

Quorum-sensing system of *Staphylococcus aureus* isolates from diabetic foot ulcers

Matias, C. ^{(1), (2)}, Oliveira, M. ⁽¹⁾

⁽¹⁾ Interdisciplinary Centre of Research in Animal Health (CIISA) / Faculdade de Medicina Veterinária da Universidade de Lisboa, Avenida da Universidade Técnica, 1300-477, Lisboa, Portugal.

⁽²⁾ Instituto Superior Técnico, Avenida Rovisco Pais 1, 1049-001 Lisboa, Portugal.

November 2015

Foot ulceration is a major complication of diabetes. Several microorganisms can colonize these wounds, being *Staphylococcus aureus* frequently isolated. It produces numerous virulence factors controlled by Quorum-sensing system, encoded by *agr* (accessory gene regulator). Currently four distinct genetic *agr* groups have been established.

Quorum-sensing system of a collection of 23 *S. aureus* DFU isolates was characterized. *agr* type was evaluated by PCR, and gene copy number by absolute quantification with qPCR. The occurrence of alterations in *agr* copy numbers in *S. aureus* from polymicrobial infections was evaluated. Presence of *mecA* and *mecC* genes, responsible for methicillin resistance, was studied.

It was possible to detect *agrI* and *agrII* in 52.2% and 39.1% of the isolates, respectively. In two isolates it was not possible to identify any *agr* type, and types III and IV were not detected. Copy number obtained ranged from 7.1 to 94279 copies of total gDNA and from 72.9 to 1487.9 copies of total gDNA. Variation in *agr* copy number of *S. aureus* from polymicrobial infections was observed. *mecA* occurred in 35% of the isolates, yet none tested positive for *mecC*.

In conclusion, results show that *agr* type varies among DFU isolates and that *agrI* has a higher variance between copy numbers, in comparison with *agrII*, which may be related with a higher production of virulence factors. However, more studies targeting the characterization and variations of *S. aureus* DFU isolates *agr* system are still required in order to assess their impact in wounds prognostic and establishment of therapeutic protocols.

Keywords: diabetic foot ulcers, *Staphylococcus aureus*, Quorum-sensing, qPCR

1. Introduction

Diabetic foot ulcers (DFU) consists in a major complication of Diabetes *mellitus* [1], representing one of the main causes of hospitalization among diabetic patients [2].

DFU can be colonized by a variety of microorganisms given rise to complex polymicrobial communities, being *Staphylococcus aureus* (*S. aureus*) one of the most common isolated bacteria, frequently co-isolated with *Pseudomonas aeruginosa* (*P. aeruginosa*) [3], [4].

S. aureus is able to produce biofilms and to express antimicrobial resistance traits and a variety of virulence factors such as surface proteins, exoenzymes and endotoxins, which enhances its virulence [5]. Expression of these traits is controlled by several factors in a mechanism called Quorum-sensing (QS) that controls the up and down

regulation of adhesion and expression of genes associated with growth-phase dependent virulence factors [6].

QS is defined as a communication system, activated by increased cell density allowing the bacteria to share information and synchronize gene expression and is encoded by the accessory gene regulator (*agr*), a classical autoactivation system (Figure 1) [5].

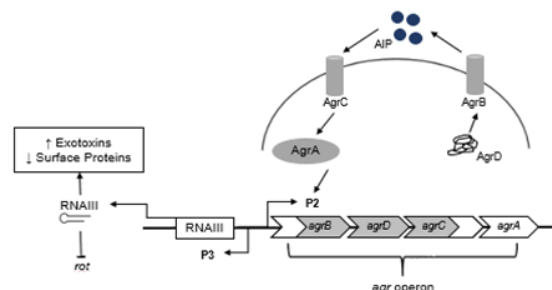


Figure 1 - *S. aureus* *agr* circuit autoactivation (Original).

The *agr* comprises two divergent transcription units, RNAII and RNAIII, expressed by P2 and P3 promoters, respectively. RNAII consists in a four gene operon, *agrBDCA*, responsible for encoding the AgrB, AgrD, AgrC and AgrA factors involved in the AIP synthesis and in the autoactivation of the regulatory system [5], [7]. RNAIII acts as a regulatory effector, responsible for activating the production of alpha-hemolysin and for inhibiting the repressor of toxins (Rot) and the production of surface proteins [8], [9].

Concerning the RNAII operon, genes *agrB* and *agrD* combine to produce and secrete the auto-inducible peptides (AIP). AgrD consists in the signaling peptide, also being the precursor peptide and AgrB acts as an integral membrane endopeptidase vital for the export and processing of AgrD [7], [10]. AgrA and AgrC constitute a two-component signaling module, being AgrC a histidine kinase present in the membrane that acts as a receptor and AgrA a response regulator [8].

The divergence of the *agr* locus has been observed among *staphylococci* strains, due to allelic variations in the *agrBDC* region, resulting into polymorphisms [7] and currently, four distinct genetic *agr* groups have been established, classified as *agr-I*, *agr-II*, *agr-III* and *agr-IV* [11].

Other adaptive mechanisms are known. Recent studies suggest that microorganisms such as yeasts and cyanobacteria are capable to adapt to environmental changes in order to persist. These mechanisms occur by alterations in gene copy number and are shown to be beneficial, since they increase survival under selective pressure conditions [12], [13]. In bacteria less is known about this type of mechanisms and its consequences in bacteria fitness. However, it has been shown that bacteria displaying several copies of rRNA respond faster to resource and nutrients availability [14]. Also, gene copy number alterations in bacteria, caused by environmental stress, can be responsible for genetic variability and it may explain the generation of antimicrobial resistant populations [15].

2. Materials and Methods

The main objective of this study was the characterization of the Quorum-sensing system in *S. aureus* isolates from DFU.

2.1. Bacterial strains

A collection of twenty-three (n=23) isolates obtained from a previous epidemiological survey of diabetic foot ulcers as described by Mendes et al [16] was studied. Isolates were also, previously characterized by the research team regarding their phenotypic virulence traits including the presence of exoenzymes and biofilm production [17], [18].

2.2. DNA extraction

Selected isolates, plus a reference strain, were plated onto Columbia agar medium with 5% of sheep blood (BioMérieux®, ref. 43401) and incubated at 37°C for 24h. Genomic DNA was extracted using two different methods, the Guanidium Thiocyanate Method as described by Pitcher et al [19] and the Boiling Method as described by Millar et al [20], in order to compare their applicability to qPCR. Concentration of the extracted gDNA, was measured by spectrometry recurring to Nanodrop® (Thermo Scientific NanoDrop 2000C Spectrophotometer).

2.3. *agr* type screening

The presence and type of *agr* was determined in all isolates, including the reference strain, by polymerase chain reaction (PCR). Four PCR reactions were performed, one for each *agr* type [21] and primers were selected based on published sequences by François et al [22]. Each PCR mixture, with a final volume of 25 µl, contained 12.5 µl of Supreme NZYtaq 2x Green Master Mix (Nzytech®), 1 µl of each primer (10 µM stock solution) and 9.5 µl of sterile water, plus 1 µl of gDNA. A PCR mix without DNA template was used as no-template control. PCR amplification was performed in a thermal cycler (MyCycler Thermal Cycler, BioRad®), using the following conditions: an initial denaturation at 94°C for 4m, followed by 30 cycles consisting in denaturation at 95°C for

1m, annealing at 60°C for 1m and elongation at 72°C for 1m, and a final extension at 72°C for 5m.

Amplified products were resolved by conventional electrophoresis gel, in a 1.8% agarose gel at 70V during 1h. Also, NZYDNA ladder VIII (NZYTech®) was included as a molecular weight marker. Results were visualized by transillumination under UV (Pharmacia Biotech, Image Master®VDS).

2.4. qPCR absolute quantification of *agrI* and *agrII*

Absolute quantification for the analysis of the *agr* locus was performed recurring to quantitative real-time PCR (qPCR). Also, a reference gene was included to guarantee technique stability.

2.4.1. Primers design

Recurring to GenBank sequences, primers for the target genes *agrI* and *agrII* and reference genes *coA*, *fabD*, *glyA*, *gmk*, *gyrA*, *hla*, *nuc*, *rrsC* and *spa* [23]–[25] were designed (Table 1). Based on results obtained by PCR, no primers for *agrIII* and *agrIV* were designed.

Table 1 - Target and reference genes tested and respective function/products.

Gene	Product
<i>agrI</i>	Accessory gene regulator type I
<i>agrII</i>	Accessory gene regulator type II
<i>coA</i>	Coagulase
<i>fabD</i>	Malonyl CoA-acyl carrier protein transacylase
<i>glyA</i>	Serine hydroxymethyltransferase
<i>gmk</i>	Guanylatekinase
<i>gyrA</i>	Glycine hydroxymethyltransferase A
<i>Hla</i>	alfa-hemolysin
<i>Nuc</i>	Thermonuclease
<i>rrsC</i>	16S ribosomal RNA subunit
<i>Spa</i>	Protein A

Online software Primer3® (Biotools®) was used for primers design. This software retrieved a list of possible primers that were submitted to Primer Express Software Real Time PCR® (Applied Biosystems®), a specific software to evaluate and validate primers for qPCR

techniques, *in silico*. Primers were then chosen considering the following parameters: melting temperature (T_m), guanine-cytosine content, product size, primer length and absence of hairpins formation and lower number of self and cross dimers produced. Gene specificity for *S. aureus* of all primers was confirmed using BLAST [26].

2.4.2. Reference genes selection

First, the presence of all genes selected for this study in *S. aureus* DFU isolates was evaluated by conventional PCR (as in 2.3).

A qPCR reaction was then developed for each reference gene, in order to evaluate their stability. Based on quantification cycles (C_q) values, a set of final candidates was selected and submitted to genEx v.6 Software®, used to compare the stability of each gene by recurring to two different algorithms, NormFinder and geNorm.

2.4.3. Optimization and construction of calibration curves

Two calibration curves were constructed (one for *agrI* and other for *agrII*) and all calculations performed as described in Applied Biosystems® guidelines for creating standard curves with genomic DNA [27].

The number of copies tested, for each *agr* type, were 1×10^1 to 1×10^6 , 1.4×10^1 to 1.4×10^6 , 3×10^1 to 3×10^6 and 5×10^1 to 5×10^6 copies/ μ l, obtained by tenfold serial dilutions.

A qPCR absolute protocol was performed and the calibration curves and respective C_q values of each point in duplicate were obtained. The medium of C_q values obtained was plotted against the logarithm of their initial copy numbers and each calibration curve was generated by a correlation coefficient (R^2) of the plotted points. From the slope of each curve, calibration curves amplification efficiency was calculated according to equation 1 [28].

$$E (\%) = (10^{-1/slope} - 1) \times 100\% \quad (1)$$

2.4.4. Absolute quantification protocol for *agrI* and *agrII*

Two 96-micro well plates (Thermo Scientific®) were prepared: one with *S. aureus* isolates harboring the *agrI* gene and another with isolates harboring the *agrII* gene; in both plates the selected reference gene was included.

A mixture with a final volume of 40 µl, was pipetted into individual wells of a 96-micro well plates (20 µl per well). Each well contained 1 µl of gDNA from each isolate with at final concentration of 8 ng/µl, 10 µl of SYBR GREEN I (PerfeCTa®, SYBR® Green FastMix®ROX), 4.2 µl of sterile water (water for molecular biology, Nzytech®) and 12.8 µl of a primers mix.

qPCR amplifications were performed in a thermal cycler (StepOne™ Software V2.3) and thermal cycling conditions divided in three stages as follows: holding stage at 95°C for 20s, cycling stage at 95°C for 15s and 60°C for 1m; followed by a melting curve stage, consisting of 30 cycles of 95°C for 15s, 60°C for 1m and 95°C for 15s.

To validate the methodology calibration curve and isolates were run in duplicate in parallel with no-template controls.

Amplified products were confirmed by conventional electrophoresis gel, in a 2% agarose gel at 65V during 2h. Also, NZYDNA ladder VIII (NZYTech®) was used as a molecular weight marker. Results were visualized as in 2.3.

Copy number determination for each isolate was calculated according to equation 2, where N_n stands for quantity and n means Cq [29].

$$N_n = 10^{\left(\frac{n-b}{m}\right)} \quad (2)$$

2.5. Co-culture assay

A co-culture assay was performed, using two DFU clinical isolates identified as *S. aureus* and *P. aeruginosa*, obtained from the same wound. Isolates were previously characterized, being observed that the *S. aureus* isolate expressed *agrII*. Dual bacterial suspensions with different concentrations of *S. aureus* and *P. aeruginosa* (1:9; 2:8; 5:5; 8:2; 9:1) were prepared in Tryptic Soy Broth and incubated for 24h/37°C. The *agrII* copy number in all suspensions was determined by PCR (as in 2.4.4.). As

control, the copy number of *agrII* present in a *S. aureus* suspension was also determined.

2.6. Screening of *mecA* gene and *mecA* homologous gene, *mecC*

Detection of *mecA* gene and its homologous *mecC* were performed by multiplex PCR. Primers were selected based on published sequences of *mecA* and *mecC* [30]. PCR amplification was carried out and amplified products resolved as already described in 2.3.

2.7. Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics™ V21 Software for Windows.

To evaluate the relation between *agr* type and virulence factors production, Friedman test was applied and a P-value ≤ 0.05 was considered statistically significant. Relation between *agr* type and presence of *mecA* gene was also determined.

3. Results

3.1. DNA extraction

The chosen gDNA extraction method was the Boiling Method, since it shown a lower variance between obtained gDNA concentrations, with an average concentration of 68.8 ± 27.8 ng/µl.

3.2. *agr* type screening

It was possible to detect the *agrI* gene in 52.2% (n=12) of the isolates, and the *agrII* gene in 39.1% (n=9) of the isolates. In 8.7% (n=2) of the isolates it was not possible to identify any *agr* type. Also, *agr* types III and IV were not detected. These were not included in the qPCR assays.

3.3. qPCR absolute quantification of *agrI* and *agrII*

3.3.1. Primers selection

Primers sequences selected are presented in Table 2.

Table 2 - Nucleotide sequences of the designed primers for the qPCR assay.

Gene	Sequence (5'-3')		Product size (bp)
	Forward	Reverse	
<i>agrI</i>	GCAAGTTCCGTCACGATTATGTC	ATCACGTAGGCCAGGCATGT	83
<i>agrII</i>	TTCAAACGGTGATAGCTTAATTCCA	CTCTTTAAGGGTGAAAAGCGACATTA	103
<i>coA</i>	CACAACCAGTTGCACAACCATTA	GGGACCTTGAACGATTTCCACC	118
<i>fabD</i>	TTTGAATGGCGTGATGCTAAGTT	TCAGTTTCACCTTGCGCATT	115
<i>glyA</i>	AGCGGCAGGTTTACATCCAA	CACGTGGTCCGCGTAATGT	83
<i>gmk</i>	GCGTGAAGGTGAAGTTGATGG	GCTTCAAACGCATCCCTAGTTTT	74
<i>gyrA</i>	TGCGATGAGTGTATCGTTGCT	ATCCGGTGTACATACCTTGTTTCATT	126
<i>hla</i>	TTGGCGGCCTTATTGGTG	GTTGGGCTCTCTAAAATTGTTTTGAA	78
<i>nuc</i>	CGAAAGGGCAATACGCAAAG	CTGTTTGTGATGCATTTGCTGAG	107
<i>rrsC</i>	CTTGACATCCTTTGACCGCTCT	ACACGAGCTGACGACAACCA	102
<i>spa</i>	AGCACCGAAAGCGGATAACA	TGGATGAAACCATTGCGTTG	105

3.3.2. Reference genes selection

Based on conventional PCR results reference genes *coA*, *fabD* and *spa* were excluded. qPCR quantification cycles (Cq) values of the remaining six reference candidate genes, *hla*, *glyA*, *gmk*, *gyrA*, *nuc* and *rrsC*, were compared, allowing to exclude genes *hla*, *gmk* and *glyA* (explained in the discussion). Genes *gyrA*, *nuc* and *rrsC*, were then evaluated using the genEx v.6 Software[®]. *gyrA* and *nuc* were found to be the most stable genes presenting a M value of 1.2 by geNorm and *gyrA* was found to be the ideal gene by NormFinder with a sd value of 0.8. Based on these results *gyrA* was chosen as the most suitable reference gene for this study.

3.3.3. Calibration curves optimization and selection

For both *agr* types calibrations curves were obtained for the copies of interest described in 2.4.3, were compared based on their amplification efficiency (Table 3).

Table 3 - Amplification efficiency for each calibration curve.

Number of copies (copies/μl)	<i>agrI</i>	<i>agrII</i>
	Efficiency (%)	
1 x 10 ¹ to 1 x 10 ⁶	86.9	81.1
1.4 x 10 ¹ to 1.4 x 10 ⁶	105.3	102.8
3 x 10 ¹ to 3 x 10 ⁶	94.8	90.9
5 x 10 ¹ to 5 x 10 ⁶	116.1	113.7

Calibration curves for both *agr* types chosen were the ones with copies ranging from 3 x 10¹ to 3 x 10⁶ copies/μl.

3.3.4. Absolute quantification of *agrI* and *agrII*

For copy number determination of both *agrI* and *agrII*, calibration curves were generated (Figure 2 (A) and (B)). Dilutions with 3 x 10¹ copies/μl in (A) and 3 x 10¹ copies/μl and 3 x 10⁶ copies/μl in (B) were discarded to compensate for pipetting errors, for normalization purposes.

Averaged Cq values for the calibration curves obtained ranged from 19.4 ± 0.1 to 33.1 ± 0.4 for *agrI* and from 18.9 ± 0.9 to 29.7 ± 0.7 for *agrII*. For isolates which Cq values were not included within this range was not possible to calculate the number of the gene copies.

Calibration curves were generated by plotting the DNA (pg) log against the Cq values determined for *agrI* and *agrII* by qPCR. For *agrI*, a high correlation coefficient was obtained (R² = 0.998), indicating a low interassay variability. The slope of the calibration curve was -3.454, which corresponds to an efficiency of 94.8%. For *agrII*, a lower value of correlation was obtained (R² = 0.916), which may affect the interassay variability. The slope of the calibration curve was -3.558, which corresponds to an efficiency of 90.9%.

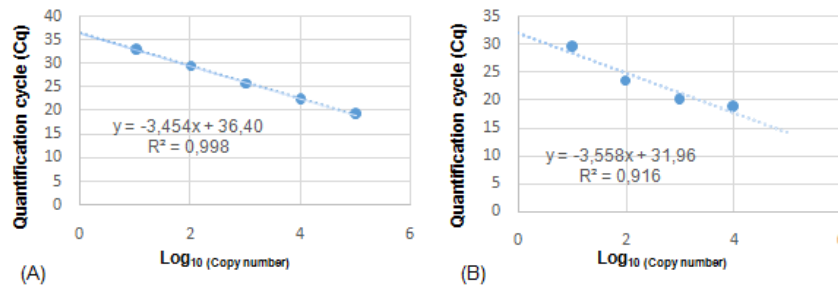


Figure 2 - Calibration curves generated from the amplification of a tenfold dilutions of target gDNA for *agrI* (A) and *agrII* (B).

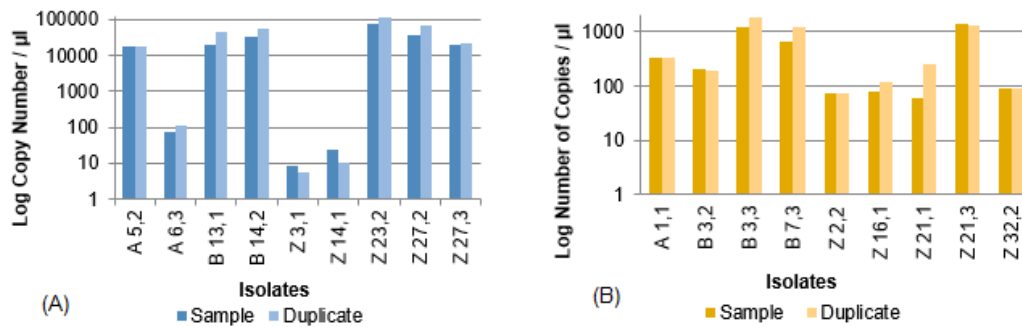


Figure 3 - Determination of the absolute copy number of *agrI* (A) and *agrII* (B) using the absolute quantification method. A: aspirate; B: biopsy; Z: swab.

Concerning *agrI*, copy number obtained ranged from 7.1 ± 2.4 to 94279 ± 28507 copies of total gDNA, determined for each isolate (Figure 3 (A) and (B)). For isolates Z 1.1, Z 5.2 and Z 25.2 it was not possible to calculate the *agrI* copy numbers.

All assays were validated by analyzing the reference gene, *gyrA*, used as a stability control.

3.4. Co-culture assay

For copy number determination of *agrII* in the dual suspensions, a calibration curve, with copies ranging from 3×10^1 to 3×10^6 copies/ μ l was generated (Figure 4).

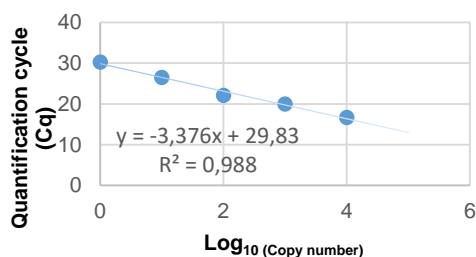


Figure 4 - Calibration curve generated from the amplification of a tenfold dilutions of target gDNA for and *agrII* when in co-culture.

Dilution with 3×10^1 copies/ μ l was discarded to compensate for pipetting errors.

agrII copy number was then calculated. The copy number obtained in the control suspension was 13.6 ± 12.2 copies of total gDNA. For the dual suspensions, copy numbers obtained ranged from 20.3 ± 5.5 to 99.2 ± 25 copies of total gDNA (Figure 5).

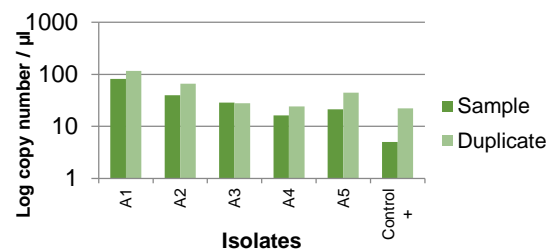


Figure 5 - Determination of the absolute copy number of *agrII* of *S. aureus* when in co-culture using the absolute quantification method. A1: 1:9; A2: 2:9; A3: 5:5; A4: 8:2; A5: 9:1; Control +: isolate B 3.2.

It was possible to observe that higher copy numbers were obtained in the 1:9 suspensions (A1). This number decreased inversely with the increase of *S. aureus* concentration in the dual suspensions.

3.5. Screening of *mecA* gene and *mecA* homologous gene, *mecC*

DFU staphylococcal isolates were also screened for the presence of *mecA* and *mecC* by multiplex PCR. PCR results showed that 35% of the isolates tested positive for *mecA*, however none tested positive for *mecC*.

3.6. Virulence factors relation with *agr* type

agr type and virulence factors coagulase ($P = 0.035$), type of hemolysis ($P = 0.002$), lipase ($P = 0.003$) and DNase ($P = 0.011$) are considered statistically significant, according to Friedman test. Also, *agr* type was related with the presence of *mecA* gene ($P = 0.000$) and considered significant. Relation between *agr* type present and biofilm production ($P = 0.035$) was also considered significant.

4. Discussion

In this study, the QS system in *S. aureus* isolates from DFU, obtained in a previous epidemiological survey as described by Mendes et al [16], was characterized, namely at the level of the *agr* system. Recurring to conventional PCR, a collection of twenty-three DFU *S. aureus* isolates was first screened for the presence of the genes related with the four *agr* types described, *agr*-I, *agr*-II, *agr*-III and *agr*-IV. This analysis allowed the distribution of the DFU isolates in two main groups, according to *agr* type present. Occurrence of *agr*-I was observed in approximately half the isolates and the occurrence of *agr*-II in almost 40% of the isolates. This is in accordance with previous studies, where *agr*-I was the most prevalent type to be identified followed by *agr*-II [31], [32]. *agr* types III and IV, associated with the production of TSST-1 and exfoliative syndromes, were not identified in our collection. *agr*-III was described as having a lower prevalence among *S. aureus* clinical isolates [31], [33] and *agr*-IV was also reported as absent in previous studies [34], [35], [36].

Finally, it was not possible to detect any of the *agr* genes in two isolates, suggesting that they consist in *agr*-defective strains. Grundmeier et al, already suggested that *agr* deficiency may represent an adaptive approach that allows microorganism avoid the host immune system [37].

For the analysis and determination of gene copy number in all isolates an absolute qPCR protocol was developed. This type of quantification depends on the use of a calibration curve constructed with known concentrations, to measure the copy numbers of a given target gene [38]. The use of a reference gene is not compulsory but it may be included, as a control, to guaranty technique stability [39]. Ideal reference genes should have an essential function, preferably not being related with the target genes in study [23].

However, since for *S. aureus* the available number of reference genes described in the literature is limited, in this study some of the reference genes tested are related with the target genes. This was the case of genes *spa* and *coA* that are down-regulated and of *hla* that is up-regulated by RNAIII [5]. Remaining reference genes were analysed by qPCR, for genes *glyA*, *gmk* and *hla*, Cq values obtained showed a high variability between isolates and therefore were excluded. Cq values obtained for the remaining reference genes, *gyrA*, *nuc* and *rscC*, were similar between isolates. Therefore, they were submitted to GenEx v.6 Software[®] that uses two different algorithms, NormFinder and geNorm, to choose the most adequate reference gene for the isolates in study. The internal control gene stability measure (M) given by the algorithm geNorm retrieved *gyrA* and *nuc* as the most stable genes ($M = 1.2$), while NormFinder retrieved *gyrA* as the ideal gene ($sd = 0.7$). Based in these results *gyrA* was chosen as the most suitable reference gene for the assay, having an average Cq value of 20.5 ± 2.9 .

For absolute quantification, the choice of the calibration curve is based on an ideal efficiency range, between 90 – 100% [40].

In this study, four different calibration curves, were tested for the absolute quantification of *agrI* and *agrII*, with copies ranging from 1×10^1 to 1×10^6 , 1.4×10^1 to 1.4×10^6 , 3×10^1 to 3×10^6 and 5×10^1 to 5×10^6 copies/ μ l. Obtained efficiencies for each calibration curve allowed choosing the curve with ranging from 3×10^1 to 3×10^6 copies/ μ l as the most suitable for the assay, since an efficiency of 94.8%

and 90.9% was obtained for *agrI* and *agrII*, respectively. Efficiencies calculated for the remaining curves were not within the ideal range. Also, chosen calibration curves were the ones that included the Cq values of the target genes to be quantified, allowing gene copy number determination.

For isolates harboring *agrI* calculated gene copy number ranged from 7.1 ± 2.4 to 94279 ± 28507 copies of total gDNA. It was necessary to exclude, from the analysis, three of the twelve isolates characterized as *agrI*, since Cq values obtained were not include in the calibration curve range, which represents a limitation in this type of approach. For *agrII*, gene copy numbers were found to be between 72.9 ± 0.2 and 1487.8 ± 405.4 copies of total gDNA and no isolates were excluded. Therefore, it was possible to demonstrate not only that *agr* type varies among DFU *S. aureus* isolates but also that gene quantification can vary among isolates harboring the same *agr* type.

Taking into account the selective pressure that *S. aureus* is subjected in polymicrobial relationships, a co-culture assay was performed to evaluate if there were any changes in the copy numbers of *agr* in *S. aureus* present in polymicrobial infections. Using two DFU clinical isolates identified as *S. aureus* and *P. aeruginosa*, obtained from the same wound. Isolates were previously characterized, being observed that the *S. aureus* isolate expressed *agrII*.

For the co-culture assay, dual suspensions with different concentrations (1:9; 2:8; 5:5; 8:2; 9:1) of both *S. aureus* and *P. aeruginosa*, were prepared. Also, a *S. aureus* suspension was included, in the assay, as a control. The presence of *S. aureus* was confirmed in all suspensions by conventional PCR, by targeting the *agrII* gene, present in the isolate used in this assay. Afterwards, *agrII* copy number was determined by resorting to absolute quantification with qPCR.

For the control suspension, calculated *agrII* copy numbers was 13.6 ± 12.2 copies of total gDNA and for the dual suspensions ranged between 20.3 ± 5.5 to 99.2 ± 25 copies of total gDNA. These results show that in the *S. aureus* suspension, used as control, a lower copy

number of *agrII* was obtained in comparison with the dual suspensions. Also, a high copy number was obtained in the dual suspensions where the *S. aureus* concentration was lower and this number decreased inversely as the concentration of *S. aureus* in the suspensions increased.

Our results suggest that variations in gene copy number can also occur in bacteria regarding genes involved in QS activation. This event can be related with isolates adaptive potential, increasing their survival under selective pressure, allowing bacteria to adapt and thrive in polymicrobial communities.

In this study it was possible to confirm the use of qPCR as a reproducible technique for absolute gene quantification, since the use of *gyrA* as a reference gene showed a low variance in Cq values between assays. However, some limitations must be considered, such as pipetting errors that originate variations between samples and the respective duplicates for some isolates. Also, differences between the efficiencies obtained for the calibration curves used in the co-culture assay (97.8%) and the *agrII* assay (90.4%) showed that storage of serial dilutions of gDNA used for calibration curves determination can affect the accuracy of the assays. This can result in lower copy numbers obtained for the target genes, since assays were performed 24h apart.

To determine the presence of *mecA* and *mecC* genes a PCR multiplex assay was performed. Being possible to detect the *mecA* gene in 35% of the *S. aureus* DFU isolates (n=8), yet *mecC* was not detected in any isolates.

The presence of *mecA* positive strains among the isolates in study can be associated with the increasing prevalence of antibiotic-resistant bacteria, particularly MRSA, in DFU isolates, as described by Bowling et al [41]. Also, Djahmi et al suggested that this prevalence may be related with antimicrobial treatment and the high frequency of recurrent ulcers [32]. Correlation of MRSA isolates with specific *agr* types was evaluated. It was possible to observe a higher frequency of *agr-II* among DFU isolates classified as MRSA (n=5); nevertheless, three MRSA isolates were confirmed to be *agr-I*. Comparing with other

studies, Pérez-Vásquez et al, reported a higher prevalence of MRSA strains harboring *agr-II* [42]. However, Jarraud et al described a higher prevalence of *agr-I* among MRSA strains [43]. Besides antimicrobial resistance, *S. aureus* displays multiple mechanisms of virulence that contribute to the microorganism pathogenicity, including the production of virulence factors [3]. In this study the relation between the production of the previous characterized virulence factors and the isolates *agr* type was evaluated. It was possible to observe a higher production of these factors among isolates harboring *agrI*. Concerning to biofilm production, it was observed in all strains past 24h, which may suggest a rapid colonization by *S. aureus*.

However, it was not possible to establish a direct association between a specific virulence factor and the *agr* type present, since production of the virulence factors described was observed in both types. The same was observed considering biofilm production.

To our knowledge, this work represents the first report on the characterization of the QS system of *S. aureus* isolates from DFU in Portugal. *S. aureus* ability to control the regulation of virulence factors by *agr* can contribute to the microorganism adaptation according to type of disease and environmental conditions. Our results showed that gene copy number can vary among isolates and that the microorganism may also thrive in polymicrobial infections by variation of *agr* copy numbers.

However, more studies targeting the characterization and variations in the *agr* system of staphylococci DFU clinical isolates are still required in order to assess the impact of these differences in the prognostic of the wounds. These studies can also display a key role in the establishment of therapeutic protocols, since the possibility of using QS as therapeutic target in the future can aid to relieve the use of antibiotics, ultimately contributing for the decrease in bacterial antibiotic resistance.

References

[1] S. S. Rich, "Genetics of diabetes and its complications.," *Journal of the American Society of Nephrology*, vol. 17, no. 2, pp. 353–360, 2006

[2] A. Boulton and H. Rathur, "The diabetic foot.," *Clinics in Dermatology*, vol. 25, no. 1, pp. 109–120, 2014.

[3] N. Nair, R. Biswas, F. Götz, and L. Biswas, "Impact of *Staphylococcus aureus* on pathogenesis in polymicrobial infections.," *Infection and Immunity*, vol. 82, no. 6, pp. 2162–2169, 2014.

[4] L. Hoffman, E. Deziel, D. D'Argenio, F. Lépine, J. Emerson, S. Mcnamara, R. L. Gibson, B. W. Ramsey, and S. I. Miller, "Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*.," *Proceedings of the National Academy of Sciences*, 2006.

[5] M. Thoendel, J. S. Kavanaugh, C. E. Flack, and A. R. Horswill, "Peptide signaling in the Staphylococci.," *Chemical Reviews*, vol. 111, no. 1, pp. 117–151, 2011.

[6] R. P. Novick, "Autoinduction and signal transduction in the regulation of staphylococcal virulence.," *Molecular Microbiology*, vol. 48, no. 6, pp. 1429–1449, 2003.

[7] R. P. Novick and E. Geisinger, "Quorum sensing in staphylococci.," *Annual Review of Genetics*, vol. 42, pp. 541–64, Jan. 2008.

[8] S. Rutherford and B. Bassler, "Bacterial Quorum sensing: its role in virulence and possibilities for its control.," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. a012427, 2012.

[9] E. Geisinger, R. Adhikari, R. Jin, H. Ross, and R. Novick, "Inhibition of rot translation by RNAIII, a key feature of *agr* function.," *Molecular Microbiology*, vol. 61, no. 4, pp. 1038–1048, 2006.

[10] R. Koenig, J. Ray, S. Maleki, M. Smeltzer, and B. Hurlburt, "Staphylococcus aureus *agrA* binding to the RNAIII- *agr* regulatory region.," *Journal of Bacteriology*, vol. 186, no. 22, pp. 7459–7555, 2004.

[11] E. Geisinger, J. Chen, and R. Novick, "Allele-dependent differences in quorum-sensing dynamics result in variant expression of virulence genes in *Staphylococcus aureus*.," *Journal of Bacteriology*, vol. 194, no. 11, pp. 2854–2864, 2012.

[12] Y. Tang and A. Amon, "Gene copy number alterations: a cost-benefit analysis.," *Cell*, vol. 152, no. 3, pp. 394–405, 2013.

[13] B. E. Schirmeister, D. a Dalquen, M. Anisimova, and H. C. Bagheri, "Gene copy number variation and its significance in cyanobacterial phylogeny.," *BMC Microbiology*, vol. 12, no. 1, p. 177, 2012.

[14] J. Klappenbach, J. Dunbar, and T. Schmidt, "rRNA operon copy number reflects ecological strategies of bacteria.," *Applied and Environmental Microbiology*, vol. 66, no. 4, pp. 1328–1333, 2000.

[15] P. Hasting, J. Lupski, S. Rosenberg, and G. Ira, "Mechanisms of change in gene copy number.," *Nature Reviews Genetics*, vol. 10, no. 8, pp. 551–564, 2010.

[16] J. J. Mendes, A. Marques-Costa, C. Vilela, J. Neves, N. Candeias, P. Cavaco-Silva, and J. Melo-Cristino, "Clinical and bacteriological survey of diabetic foot infections in Lisbon.," *Diabetes research and clinical practice*, vol. 95, no. 1, pp. 153–61, Jan. 2012.

[17] C. Mottola, T. Semedo-Lemsadeck, J. J. Mendes, J. Melo Cristino, P. Cavaco Silva, L. Tavares, and M. Oliveira, "Molecular typing, virulence traits and antimicrobial resistance of diabetic foot staphylococci.," *Submitted to publication in Medical Microbiology and Immunology*, 2015.

[18] C. Mottola, J. J. Mendes, J. Melo Cristino, P. Cavaco Silva, L. Tavares, and M. Oliveira, "Polymicrobial biofilms by diabetic foot clinical isolates.," *Folia Microbiologica*, 2015.

- [19] D. G. Pitcher, N. Saunders, and R. J. Owen, "Rapid extraction of bacterial genomic DNA with guanidium thiocyanate.," *Letters in applied microbiology*, vol. 8, pp. 151–156, 1989.
- [20] B. Millar, X. Jiru, J. Moore, and J. Earle, "A simple and sensitive method to extract bacterial, yeast and fungal DNA from blood culture material.," *Journal of Microbiological Methods*, vol. 42, no. 2, pp. 139–147, 2000.
- [21] K. E. Traber, E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin, and R. P. Novick, "agr function in clinical *Staphylococcus aureus* isolates.," *Microbiology*, vol. 154, no. Pt 8, pp. 2265–74, Aug. 2008.
- [22] P. Francois, T. Koessler, A. Huyghe, S. Harbarth, M. Bento, D. Lew, D. Pittet, and J. Schrenzel, "Rapid *Staphylococcus aureus* agr type determination by a novel multiplex Real-Time quantitative PCR assay.," *Journal of Clinical Microbiology*, vol. 44, no. 5, 2006.
- [23] T. Theis, R. a Skurray, and M. H. Brown, "Identification of suitable internal controls to study expression of a *Staphylococcus aureus* multidrug resistance system by quantitative real-time PCR.," *Journal of microbiological methods*, vol. 70, no. 2, pp. 355–62, Aug. 2007.
- [24] C. Goerke, S. Campana, M. Bayer, G. Do, K. Botzenhart, and C. Wolz, "Direct quantitative transcript analysis of the agr regulon of *Staphylococcus aureus* during human infection in comparison to the expression profile in vitro.," *Infection and Immunity*, vol. 68, no. 3, pp. 1304–1311, 2000.
- [25] H. Eleaume and S. Jabbouri, "Comparison of two standardisation methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during in vitro growth.," *Journal of Microbiological Methods*, vol. 59, no. 3, pp. 363–70, Dec. 2004.
- [26] NCBI, "nucleotide BLAST." [Online]. Available: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. [Accessed: 30-Oct-2014].
- [27] "Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR.," *Applied Biosystems*, pp. 1–8, 2003.
- [28] C. Lee, J. Kim, S. Shin, and S. Hwang, "Absolute and relative qPCR quantification of plasmid copy number in *Escherichia coli*.," *Journal of Biotechnology*, vol. 123, no. 3, pp. 273–280, 2006.
- [29] "Real-Time PCR: applications guide.," *Bio-Rad Laboratories*, 2006.
- [30] M. Stegger, P. S. Andersen, A. Kearns, B. Pichon, M. a. Holmes, G. Edwards, F. Laurent, C. Teale, R. Skov, and A. R. Larsen, "Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either mecA or the new mecA homologue mecA LGA251.," *Clinical Microbiology and Infection*, vol. 18, no. 4, pp. 395–400, 2012.
- [31] S. Peerayeh, A. Azimian, Q. Nejad, and M. Kashi, "Prevalence of agr specificity groups among *Staphylococcus aureus* isolates from University Hospitals in Tehran.," *Laboratory Medicine*, vol. 40, no. 1, pp. 27–29, 2008.
- [32] N. Djahmi, N. Messad, S. Nedjai, A. Moussaoui, D. Mazouz, J. L. Richard, A. Sotio, and J. P. Lavigne, "Molecular epidemiology of *Staphylococcus aureus* strains isolated from inpatients with infected diabetic foot ulcers in an Algerian University Hospital.," *Clinical Microbiology and Infection*, vol. 19, no. 9, pp. E398–E404, 2013.
- [33] A. Ghasemian, S. Najar Peerayeh, B. Bakhshi, and M. Mirzaee, "Accessory gene regulator specificity groups among *Staphylococcus aureus* isolated from hospitalized children.," *Archives of Pediatric Infectious Diseases*, vol. 3, no. 2, p. e16096, 2014.
- [34] C. Ateba, M. Mbewe, and C. Bezuidenhout, "Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians.," *Journal of Clinical Microbiology*, vol. 41, no. 1, pp. 456–459, 2003.
- [35] N. Melake, A. Zakaria, N. Ibrahim, M. Salama, and A. Mahmoud, "Prevalence of agr specificity groups among in vitro biofilm forming Methicillin-resistant *Staphylococcus aureus* strains isolated from nasal carriers.," *International Journal of Microbiological Research*, vol. 5, no. 2, pp. 76–84, 2014.
- [36] A. Ben Ayed, I. Boubaker, S. Ennigrou, and S. Redjeb, "Accessory gene regulator (agr) typing of *Staphylococcus aureus* isolated from human infections.," *Les archives de l'Institut Pasteur Tunis*, vol. 85, pp. 3–8, 2008.
- [37] M. Grundmeier, L. Tuchscher, M. Brück, D. Viemann, J. Roth, E. Willscher, K. Becker, G. Peters, and B. Löffler, "Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells.," *The Journal of infectious diseases*, vol. 201, no. 6, pp. 871–880, 2010.
- [38] S. Dhanasekaran, T. M. Doherty, and J. Kenneth, "Comparison of different standards for real-time PCR-based absolute quantification.," *Journal of Immunological Methods*, vol. 354, no. 1–2, pp. 34–39, 2010.
- [39] B. D'haene, J. Vandesompele, and J. Hellems, "Accurate and objective copy number profiling using real-time quantitative PCR.," *Methods*, vol. 50, no. 4, pp. 262–270, 2010.
- [40] M. T. Dorak, "Real-Time PCR: practical issues and troubleshooting.," in *MOBGAM, Istanbul, Turkey*, 2011.
- [41] F. L. Bowling, E. B. Jude, and A. J. M. Boulton, "MRSA and diabetic foot wounds: contaminating or infecting organisms?," *Current Diabetes Report*, vol. 9, no. 6, pp. 440–444, 2009.
- [42] M. Pérez-Vázquez, A. Vindel, C. Marcos, J. Oteo, O. Cuevas, P. Trincado, V. Bautista, H. Grundmann, J. Campos, D. Fontanals, R. Moreno, J. Lite, P. López, G. Royo, G. Megías-Lobón, E. Ojeda, C. Miranda, M. Dolores, A. Fleites, P. Teno, P. Berdonces, T. Nebreda, Á. Campos, E. Garduño, C. Raya, B. Fernández, M. Brezmes, P. Álvarez, M. García-Campello, I. Wilhemi, A. Delgado-Iribarren, L. Marco, M. Revillo, N. Montiel, I. Cuesta, J. Calvo, and L. Martínez, "Spread of invasive Spanish *Staphylococcus aureus* spa-type t067 associated with a high prevalence of the aminoglycoside-modifying enzyme gene ant(4)-Ia and the efflux pump genes msrA/msrB.," *Journal of Antimicrobial Chemotherapy*, vol. 63, no. 1, pp. 21–31, 2009.
- [43] S. Jarraud, C. Mougel, J. Thioulouse, G. Lina, X. Nesme, and J. Etienne, "Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles) and human disease.," *Infection and Immunity*, vol. 70, no. 2, pp. 631–641, 2002.