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

Carina Sofia Silva Matias

Dissertation to obtain the Master of Science Degree in

Microbiology

Supervisors: Prof. Doutora Isabel Maria de Sá Correia Leite de Almeida
Prof. Doutora Maria Manuela Castilho Monteiro de Oliveira

Examination Committee

Chairperson: Prof. Doutor Jorge Humberto Gomes Leitão
Supervisor: Prof. Doutora Maria Manuela Castilho Monteiro de Oliveira
Member of the Committee: Prof. Doutor João João Duarte Alves Mendes

November 2015

“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

Albert Einstein

Acknowledgments

Firstly, I would like to express my sincere gratitude to my advisor Professor Manuela Oliveira, for the continuous support, constant motivation, patience and friendship. Her knowledge and guidance helped me in all the time of research, laboratory work and writing of this dissertation.

I could not have asked for a better advisor and mentor.

Besides my advisor, I would like to thank to Professor Sofia van-Harten, not only for her knowledge and help with the entire laboratory work, but also for her friendship and support. And also to Carla Mottola, for her friendship and constant motivation.

To Professor Isabel Sá Correia, for consenting me to develop my work in the Faculty of Veterinary Medicine. And also, for her dedication to this course.

To Professor Luis Tavares, for welcoming me at the Faculty of Veterinary Medicine.

I thank my fellow colleagues and friends, Zé, João C., Ana, Margarida, João M., Inês and Tiago, for their friendship and company.

To my beloved friends Mónica, Ricardo and Bruno, for being truly good friends. Thank you all for always cheering me up and for all the moments of distraction.

Last but not least, I would like to express gratitude to my family: my parents, my cousin Cheila, my sister and grandparents, especially to avó Teresa and to avô Gonçalves, for always supporting me and always encouraged me to want more. Without them I would not be where I am today.

This work was part of the project “Biofilms in diabetic foot: microbial virulence characterization and cross-talk of major isolates” and financially supported by “Fundação para a Ciência e Tecnologia (FCT)” (Contract PTDC/SAU-MIC/122816/2010).

Abstract

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

Resumo

O desenvolvimento de úlceras nos pés consiste numa importante complicação da diabetes, podendo ser colonizadas por vários microrganismos. *Staphylococcus aureus* é frequentemente isolado a partir destas úlceras, podendo produzir factores de virulência controlados pelo sistema Quorum-sensing, codificado pelo *agr*, com quatro grupos genéticos distintos.

O sistema Quorum-sensing de 23 isolados de *S. aureus* de úlceras de pé diabético (UPD) foi caracterizado. O tipo de *agr* foi determinado por PCR e o número de cópias do gene por qPCR, de modo a avaliar a ocorrência de alterações no número de cópias em isolados de infeções polimicrobianas. A presença de *mecA* e *mecC*, responsáveis pela resistência a meticilina, foi determinada por PCR.

Detectou-se *agrI* e *agrII* em 52.2% e 39.1% dos isolados. O número de cópias variou entre 7.1 a 94279 cópias e 72.9 a 1487.9 cópias de gDNA total, respetivamente. Em dois isolados não foi possível identificar o tipo de *agr*, e os tipos III e IV não foram detetados. Foi observada variação do número de cópias em isolados de infeções mistas. *mecA* foi observado em 35% dos isolados, e nenhum apresentou *mecC*.

O tipo de *agr* varia entre isolados de UPD e *agrI* apresenta uma maior variância entre o número de cópias obtidas comparativamente a *agrII*, o que pode estar relacionado com maior produção de fatores de virulência. Sugerem-se novos estudos para caracterização e avaliação de variações no *agr* em *S. aureus* isolados de UPD, para determinar o seu impacto no prognóstico destas feridas e no estabelecimento da terapêutica.

Palavras-Chave: Úlceras de pé diabético, *Staphylococcus aureus*, Quorum-sensing, qPCR

Table of Contents

List of Figures

List of Tables

List of Abbreviations

agr	Acessory gene regulator
AAp	Accumulation associated protein
AIPs	Autoinducing peptides
Ais	Autoinducers
bp	Base pairs
C.D.	Cross-dimers
CDC	Center for Disease Control and Prevention
CFU	Colony forming units
Cq	Quantification cycles
DFI	Diabetic foot infections
DFU	Diabetic foot ulcers
DM	Diabetes *mellitus*
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
F	Forward
GC	Guanine-cytosine content
HIV	Human Immunodeficiency Virus
M	Stability measure
MRSA	Methicillin-resistant *Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
PBP	Penicillin binding protein
PBP2a	Penicillin binding protein 2a
PBP2c	Penicillin binding protein 2c
PCR	Polymerase chain reaction
PFGE	Pulse Field Gel Electrophoresis

PIA	Polysaccharide intercellular adhesin
PVD	Peripheral Vascular disease
PVL	Panton-Valentine Leukocidin
qPCR	Quantitative real-time polymerase chain reaction
QS	Quorum-sensing
R	Reverse
RNA	Ribonucleic acid
Rot	Repressor of toxins
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
S.D.	Self-dimers
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCV	Small colony variants
sd	Standard deviation
SSSS	Staphylococcal scalded skin syndrome
T_m	Melting Temperature
TSB	Tryptic Soy Broth
TSST-1	Toxic shock syndrome toxin-1
UPD	Úlceras de Pé Diabético
WHO	World Health Organization

CHAPTER 1

Introduction

1.1. Diabetes *mellitus*

Diabetes *mellitus* (DM) is defined by World Health Organization (WHO) as a chronic disease that occurs when the pancreas produces insufficient insulin or when the body is not able to use the insulin produced [1]; in both cases, it leads to chronic hyperglycemia, a condition characterized by an increase of the concentration of glucose in the blood [2]. The term also describes a multiplicity of metabolic disorders, related with the endocrine system, including disturbances of carbohydrate, fat and protein metabolisms [3]. The prevalence of Diabetes for all age groups is increasing and there are an estimated 171 million diabetic patients worldwide and this number is expected to rise to 366 million by the year 2030 [4].

Diabetes can be manifested in several forms, still the most common are types 1 and 2. The type 1 or insulin dependent is characterized by an insulin deficit [3] related with the autoimmune mediated destruction of the pancreatic β-cells, responsible for insulin production [2]. Type 1 Diabetes is also characterized by a tendency to develop ketoacidosis, a metabolic condition that leads to fatty acid burning and production of ketones by the liver, which combined with the insulin deficiency can lead to coma and ultimately death [3], [5]. Epidemiologically, it's more common to be diagnosed in children and young adults, nevertheless it can develop in individuals from any age group [6].

The exact risk factors that can lead to the development of type 1 Diabetes are still unknown, though this disease is believed to be related with an autoimmune and genetic predisposition, since a record of family members increases the risk of developing the disease [7], [8]. Some studies also suggest the exposure to viral infections, like enteroviruses, as a major environmental factor that could trigger Diabetes development [9].

Type 2 or non-insulin dependent Diabetes, is mainly characterized by a group of heterogeneous disorders that can be related with various levels of insulin resistance demonstrated by insulin dependent cells, like adipocytes, myocytes or hepatocytes, insulin secretion and excessive production of glucose by the hepatic cells, all leading to high concentrations of glucose in the blood [2], [3]. It is also known as "adult onset" Diabetes, as it typically develops in individuals with more than 35 years old. Risk factors include genetic, immunological and environmental factors like obesity, diet, physical inactivity, high blood pressure, impaired glucose tolerance, family history of Diabetes, history of gestational Diabetes, increasing age and ethnicity [7], [8].

Concerning clinical manifestations, primary signs and symptoms of DM include polyuria, polydipsia, extreme hunger, weight lost, lack of energy, blurred vision, tingling sensation or numbness in the extremities, dry skin caused by anhidrosis, frequent infections, chronic wounds [3], [10], nauseas, vomiting and stomach aching, especially in type 1 Diabetes [7]. It is important to state that clinical manifestations do not appear all at once and may vary between individuals. Symptoms are often not severe, but clinical manifestations in type 1 Diabetes are usually sudden, while in type 2 symptoms may be moderate or absent, hindering its diagnosis [10].

Uncontrolled Diabetes can lead to a multiplicity of short and long-term complications, being related with the occurrence of high concentrations of glucose in the blood over a long period of time. Short-term or acute complications are more common among type 1 Diabetes patients and include ketoacidosis and

hypoglycemia [11], [12]. In contrast, long-term or chronic complications may include microvascular alterations like ophthalmologic, renal, neurological, gastrointestinal and genitourinary disorders, and macrovascular complications including cardiovascular and lower limb disorders [3], [13]. Chronic complications are described as developing equally in both type 1 and 2 Diabetes [11].

1.2. Diabetic foot

Foot disorders, such as diabetic foot ulcers (DFU), infection and gangrene, are very common amongst diabetic patients and may occur in both type 1 and 2 Diabetes [14]. DFU represent one of the main causes of hospitalization of diabetic patients as they cause high morbidity and often precede amputation of the affected limb [15]. The lifetime risk of developing DFU is approximately 15%, however the risk increases among DM patients with peripheral neuropathy [16].

DFU are also described to be more common among caucasian men over 60 years old, being also related to social and economic aspects [17].

1.2.1. Pathophysiology of Diabetic foot ulcer

DFU are the outcome of multifactorial phenomena induced by sustained hyperglycemia [18]. Including peripheral neuropathy, peripheral vascular disease (PVD) and arterial insufficiency, foot deformities, trauma and diminished resistance to infection [19], [20].

Diabetic neuropathy is a complication that results from degenerative changes in the axons, caused by high glucose concentrations, affecting all nerve fibers and causing impairment in the autonomic, sensory and motor functions, depending upon the affected nerve [13], [21].

The non-myelinated autonomic nerves are the first to be affected, leading to microvascular thermoregulatory dysfunction, which translates in anhidrosis and decreased function of the sebaceous glands, ensuing in dry skin and fissures. Also, it may cause artery calcification and arteriovenous shunting [18], [19].

Alterations in the autonomic functions are followed by sensory neuropathy, that affects the sensory nerves present in body extremities like the feet, causing gradual loss of sensitivity and increasing numbing sensation in the extremities [15].

Finally, motor neuropathy is described as the damage in motor nerves which affects the ability of the body to coordinate movements, leading to the development of foot deformities, Charcot's foot, a condition that causes weakening with consequent fracture of the feet bones in individuals who have significant nerve damage, hammerhead and claws toes (Figure 1) [19], [22]. It also, leads to muscle atrophy and weakness with consequent alteration in foot anatomy and osteomyelitis [23].

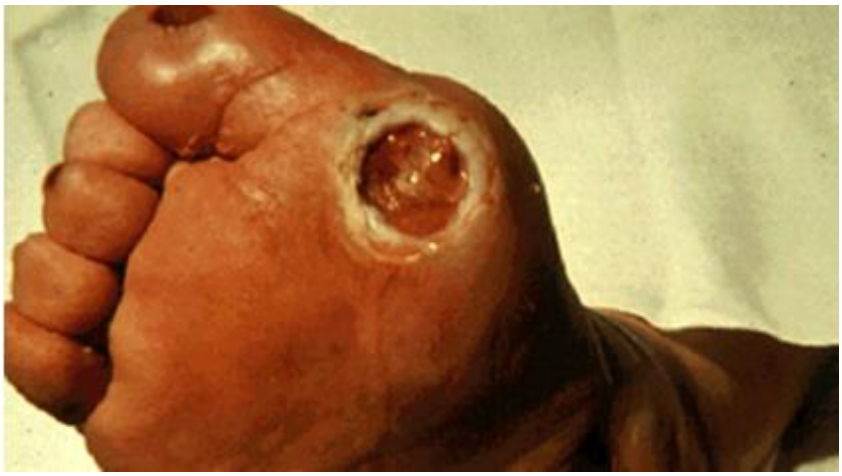


Figure 1 - Full thickness diabetic foot ulcer and claw toe [24].
Reproduced with permission from: McCulloch DK. Patient information: Foot care in Diabetes *mellitus* (Beyond the Basics)

As the disease progresses these conditions seem to contribute to the insensitiveness of the foot and to its deformity [25].

Sustainable hyperglycemia is also responsible for the development of peripheral vascular disease (PVD), a group of disorders characterized by the narrowing and consequent occlusion of the arteries, leading to gradual ischemia, i.e., inadequate arterial perfusion to organs and extremities [26].

Asymptomatic PVD is very common among DM patients, however it may be responsible for symptoms such as sporadic claudication or critical limb ischemia, which is characterized by discomfort and soreness in the extremities when at rest. Other observed consequences include the development of ischemic ulceration and gangrene [26], [27].

PVD also represents one of the major leading causes of lower extremity amputations among DM patients, being accompanied by a higher probability in developing cardiovascular and cerebrovascular disease [19].

As a result of insensitivity and altered perception, ischemic or neuropathic DFU, occur as a consequence of trauma most of the times unperceived by the individual [15]. Also, DFU affects approximately 30 to 50% of DM patients [28].

Trauma can be extrinsic or intrinsic. Extrinsic trauma can be caused by thermal events such as hot water, chemical events like foot treatment solutions or localized mechanical events, being the most common the continuous low-pressure trauma caused by uncomfortable ill-fitting shoes, repetitive trauma from walking or the appearance of blisters or other wounds [18], [20]. In contrast, intrinsic traumas result from foot deformities and biomechanical abnormalities such as callus formation and limited joint mobility [18], [29].

Once neuropathy and PVD are established, ischemia and infection are the most important factors in the prognosis of DFU [30]. It is also possible to observe a biological deficit in tissue healing and regeneration, caused by reduced neutrophil function which contribute to the development of DFU and to their slow healing and regression [29].

1.2.2. Infection and Bacteriology of DFU

Even though infection is hardly implicated in the development of DFU, once the protective layer of the skin is broken and deep tissues are exposed, the ulcers are susceptible to infection and bacterial colonization's can begin [18].

Diabetic foot infections (DFI), can range from local fungal infections in the nails to necrotizing limb and life-threatening infections [31], being the leading cause of nontraumatic amputations [32].

The infected neuropathic or ischemic ulcer is one of the most common presentations of DFI, which can include infections like paronychia, cellulitis, myositis, abscesses, necrotizing fasciitis, septic arthritis, tendonitis, and osteomyelitis [33]. Although most of infections are originated with an ulcer, infections like localized cellulitis and necrotizing fasciitis can develop in the absence of an ulcer or other traumatic injury [28].

As the term DFI comprises a multiplicity of different clinical presentations and/or manifestations, was proposed a classification by the International Working Group on the Diabetic Foot Classifications, named PEDIS. According to this classification, the DFI are divided in four grades concerning the level of perfusion, sensation and infection. Consequently they are graded as uninfected, mild, moderate and severe (Table 1) [30].

Table 1 - PEDIS classification proposed by the International Working Group on the Diabetic Foot Classifications of Diabetic Foot Infections [34].

Grade	Infection	Clinical presentations and/or manifestations
1	Uninfected	Wound without signs of inflammation or purulence
2	Mild	Signs of purulence and erythema, pain, cellulitis around the ulcer, skin/subcutaneous infection
3	Moderate	Deep tissue abscess, gangrene, involvement of muscle, tendon, joint or bone
4	Severe	Infection with systemic or metabolic instability, fever, chills, tachycardia, severe hyperglycemia, acidosis

Nevertheless, the causative microbial agents varies in the different levels of infections, being mild infections generally considered monomicrobial while moderate and severe infections considered polymicrobial [31].

The first microorganisms to colonize the wound are aerobic Gram-positive bacteria like *Staphylococcus aureus* (*S. aureus*) and β-hemolytic *Streptococcus* (groups A, B and C) [35], which are also the most common pathogens found in acute and previously untreated superficial wounds in DM patients [30].

On the other hand, DFI in patients that had recently received antibiotic treatment, deep limb-threatening infections or recurrent chronic wounds, are usually caused by a combination of multiple microorganisms, including aerobic Gram-positive bacteria like *S. aureus*, *Staphylococcus epidermidis, Corynebacterium* and β-hemolytic *Streptococcus,* aerobic Gram-negative bacteria like *Escherichia coli, Klebsiella* and *Proteus* and, finally anaerobic Gram-negative bacteria like *Fusobacterium* and *Clostridium*, especially found in deep tissue infections with ischemia or gangrene [28], [35]. *Pseudomonas aeruginosa* (*P. aeruginosa*) and other nonfermentative Gram-negative rods can also be found in chronic wounds [33].

DM patients with recent history of hospitalization, surgical procedures and prolonged antibiotic therapy are more predisposed to colonization and consequent infection by antibiotic resistant microorganisms, like Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant enterococci [28], [33].

1.2.2.1. Polymicrobial interactions between *S. aureus* and *P. aeruginosa*

DFU are usually colonized by several microorganisms that interact with each other constituting complex polymicrobial communities. Two of the microorganisms that are frequently co-isolated from this type of wounds, although sharing a competitive relationship, are *S. aureus* and *P. aeruginosa* [36], [37].

In vitro co-culture studies suggest that *P. aeruginosa* prospers better than *S. aureus*, by multiplying faster and acting as its antagonist [38].

P. aeruginosa persistence is related with the microorganism ability to produce toxins, such as pyocyanin, hydrogen cyanide and a mixture of quinoline N-oxides, that are capable of blocking the electron transport pathway inhibiting the growth of *S. aureus* [37]. *S. aureus* respond by forming electron transport-deficient small colony variants (SCV) in order to counter the effect of the toxins produced by *P. aeruginosa* [36]. Compared with *S. aureus* wild strains, SCVs are smaller, show a slower growth and metabolism rate and, are non-hemolytic. These strains are also describe to have a higher resistance to antibiotics [39]. This might be related to the fact that *P. aeruginosa* seems to induce *S. aureus* biofilm production [40].

Korgaonkar et al shown that, when in coinfection, *P. aeruginosa* uses the peptidoglycan shed by *S. aureus* as a signal to produce extracellular factors with cytolytic activity [41].

Nevertheless, *S. aureus* remains one of the bacterial strains frequently isolated from DFI, since it has the ability to modulate wound healing and avoid the host immune defenses enabling other microorganisms to colonize the wound, ultimately exacerbating the infection [42].

1.3. *Staphylococcus aureus*

The genus *Staphylococcus*, was first described in 1881 by Ogsten as a way to identify cocci arranged in a cluster, but the species *S. aureus* was only isolated and named by Anton J. Rosenbach in 1884 [43].

Taxonomically the genus belongs to the *Staphylococcaceae* family and, to date, forty-nine species have been recognized [44], among which *S. aureus* is one of the most virulent species [45].

S. aureus, although considered an ubiquitous microorganism that can be found in the environment and also colonizing the skin and mucous membranes of humans and other warm-blooded animals, is an extremely versatile human and veterinary pathogen responsible for causing not only a wide spectrum of systemic diseases, but also a diversity of acute and chronic infections [46], [47].

According to Center for Disease Prevention and Control (CDC), approximately 30% of the healthy human population is persistently colonized by this microorganism, being the rest of the population intermittently colonized [48]. Since *S. aureus* has a commensalism relationship with the host, most of the times disease only develops in cases of decreased immunity or of disruption of skin barrier. So a higher rate of colonization can be observed among individuals with skin lesions, insulin-dependent diabetic patients, intravenous drug users and Human Immunodeficiency Virus (HIV) carriers [49].

Also, *S. aureus* is one of the most frequent agents associated with health care and community acquired infections [50], representing a major public health and epidemiological problem with elevated health costs due to its increased morbidity and mortality [51].

1.3.1. General characteristics

S. aureus are Gram-positive cluster-forming coccus, with 0.5 – 1.0 μm in diameter, nonmotile and nonsporulated [45] (Figure 2).

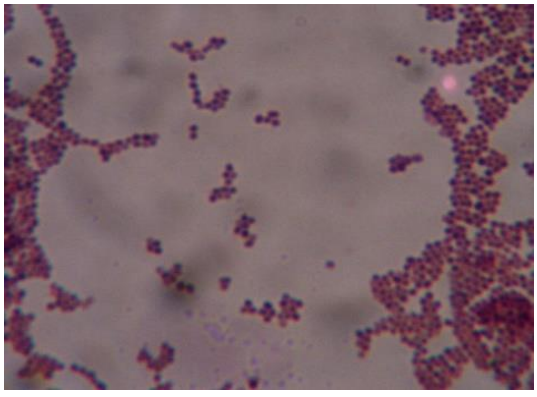


Figure 2 – Gram-positive *S. aureus* isolate, 100x (Original).

The microorganism is classified as being a facultative anaerobic, which means they grow by aerobic respiration or by fermentation with production of lactic acid [45]. *S. aureus* is also catalase positive and an important producer of coagulase. Moreover, the microorganism has the ability to grow in adverse environments, like sodium chloride rich mediums, and survives at a temperature range from 18ºC to 40ºC, characteristics that assist its dissemination [52].

1.3.2. Pathogenic determinants

S. aureus virulence results from the combined effect of a multiplicity of pathogenic determinants (i.e. virulence factors) expressed by the microorganism at different stages of infection, promoting bacterial growth, colonization, avoidance of host defense and tissue damage [53]. Their production is controlled by several factors, including cell density, energy availability and environmental signals, in a mechanism called Quorum-sensing (QS) [54].

As a pathogen, *S. aureus* also expresses antimicrobial resistance traits and a variety of proteins including surface proteins, exoenzymes and endotoxins, which may enhance its virulence. The microorganism also has the ability of producing biofilms [55].

Virulence factors are not essential to bacteria development being only produced at certain phases of the bacterial growth or under specific environmental conditions [56].

Other adaptive mechanisms are known. Recent studies suggest that microorganisms such as yeasts and cyanobacteria are capable to adapt to environmental changes, such as nutrient availability, 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 [57], [58]. In bacteria less is known about this type of mechanisms and its consequences in bacteria fitness, since studies are more focused on ecology and in the variation of ribosomal RNA gene copy numbers among different species [59], [60]. However, it has been shown that bacteria displaying several copies of rRNA respond faster to resource and nutrients availability [59].

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 [61].

1.3.2.1. Antimicrobial resistance

Infections by antibiotic-resistant *S. aureus* became problematic in the 1950's when strains acquired a plasmid-encoded β-lactamase allowing resistance to penicillin. Shortly after, methicillin was introduced to treat these infections; however in 1961 the first cases of resistance to methicillin were reported, given rise to a new strain named Methicillin-resistant *Staphylococcus aureus* (MRSA) [45], [62].

Acquired resistance in *S. aureus* can occur by different pathways. In the case of β-lactam antibiotics, resistance emerged with the production of β-lactamases, such as penicillinase, that promotes the hydrolysis of the β-lactam ring, leading to the deactivation of the molecule's antimicrobial characteristics [63].

In the case of methicillin resistance a different mechanism can be observed, in which the resistance is mediated by the acquisition of a mobile genetic element named staphylococcal cassette chromosome *mec* (SCC*mec*), which carries the *mecA* gene. This gene encodes an altered penicillin binding protein (PBP), named penicillin binding protein 2a (PBP2a) [64], with low affinity to β-lactam antibiotics [65].

SCC*mec* can be spread among strains by horizontal transfer [66] and five major types can be distinguished, related with nosocomial and community acquired strains: types I, II and III are related with nosocomial strains, while types IV and occasionally V, are related with community acquired strains [67].

Recently, a novel *mecA* homologue was described and named as *mecC*, which shares 70% DNA homology with *mecA* [68]. The gene was firstly associated with livestock [69], but it has already been identified in humans samples across Europe [68], [70].

mecC encodes for the penicillin binding protein 2c (PBP2c), that differs from PBP2a in its binding characteristics to β-lactam antibiotics, showing an higher affinity to oxacillin [65], [71]. This gene is located in a novel staphylococcal cassette chromosome *mec* element, nominated type-XI SSC*mec* [68].

1.3.2.2. Cell-wall associated components

The cell-wall represents an important structure since is responsible not only for the bacteria morphology but also acts like a mechanical protective factor against cell rupture [45]. Cell-wall associated components include the capsule, peptidoglycan, teichoic acids and protein A [52].

The capsule, only sporadically found in *S. aureus* strains, consists in a polysaccharide layer that surrounds the cell-wall protecting the microorganism against chemotaxis and phagocytosis [72]. There are eleven capsular serotypes identified, being types five and seven associated with the majority of diseases caused by this microorganism. The capsule also contributes to bacterial adhesion by being capable of binding specifically to epithelial and endothelial cells and to monocytes of the host. Also, it has been shown to protect the microorganism against dehydration by accumulating large amounts of water [73].

Peptidoglycan and teichoic acids are the main cell-wall components of *S. aureus*. Both have been shown to stimulate the release of several cytokines, like tumor necrosis factor, responsible for promoting apoptosis of the human host cells, also the peptidoglycan has a variety of toxic properties such as the ability to activate the production of monocytes and macrophages and initiate a cytokine response, which is associated with several diseases [74].

Concerning protein A, it consists in a surface protein encoded by the *spa* gene, found in the cell-wall covalently binded to the peptidoglycan layer. This protein has an unique affinity for binding to the Fc receptor of immunoglobulins (Ig)G_1, (Ig)G_2 and (Ig)G_4, and as a result, the IgG molecules are bound in the wrong orientation leading to opsonization and phagocytosis [75].

1.3.2.3. Exoenzymes

S. aureus produces a variety of exoenzymes including coagulase, deoxyribonuclease (DNase), lipase, proteases, gelatinase and hyalurodinases [73].

Coagulase is a protein located in the surface of the outer membrane and consists in a clumping factor. This protein binds to prothrombin in the host and forms a complex called staphylotrombin. This complex is responsible for the complex activation, resulting in the conversion of fibrinogen in insoluble fibrin, causing the clumping or aggregation of blood plasma [76]. Its detection consists in one of the *S. aureus* identification tests, since it the main coagulase producer specie among the genus [52].

DNase consists in a catalytic enzyme, responsible to catalyze the hydrolytic cleavage of phosphodiester linkages in the DNA, leading to is degradation, while lipases are responsible for the hydrolysis of lipids, converting them into fatty acids and glycerol, aiding the invasion of cutaneous and subcutaneous tissues [73].

On the other hand, proteases act by hydrolysing the peptide bonds in aminoacids, promoting tissue invasion and contributing to the dispersal of the microorganism into host tissues. Gelatinase is included in their group, being responsible for the degradation a range of substrates like gelatin, collagen and hemoglobin [77].

Finally, hyalurodinase is an enzyme in charge of the degradation of hyaluronic acid present in the extracellular matrix of human tissues. The enzyme acts by facilitating bacterial invasion and dispersal in the host [78].

1.3.2.4. Toxin Production

Toxins produced by *S. aureus* include staphylococcal enterotoxins, exfoliative toxins and cytolytic membrane damaging toxins, such as hemolysins and leukocidins.

Staphylococcal enterotoxins are pyrogenic exotoxins divided in eight serological types (A, B, C, D, E, G, H and I). Enterotoxins are thermostable and commonly associated with food poisoning, since they are able to resist the action of gastric enzymes [79].

The toxic shock syndrome toxin-1 (TSST-1) is an exotoxin produced by some strains during their growth, being responsible for the toxic shock syndrome that leads to multiorganic systemic failure [73]. Concerning to exfoliative toxins, they are responsible to recognize and cleave desmossomal cadherins present in the superficial layers of the skin, being the direct cause of staphylococcal scalded skin syndrome (SSSS) [80].

Cytolytic toxins produced by *S. aureus* include alpha (α), beta (β), gamma (γ) and delta (δ) toxins, presenting hemolytic and cytolitic actions or membrane-damaging functions, respectively [73].

This microorganism also produces the Panton-Valentine leukocidin (PVL) [81].

Alpha-hemolysin main function is to mediate necrotic tissue injury by binding to host cells and inducing pores formation, while beta-hemolysin is a sphingomyelinase capable of inducing cellular damage in the membranes [73]. On the other hand, gama-hemolysin and PVL promote the lysis of

leukocytes and the lipid layer of the membranes by inducing the formation of pores in the cell membrane. PVL is also associated with the higher virulence demonstrated by community acquired MRSA strains, has it may cause tissue necrosis [81].

Finally, delta-hemolysin is produced by almost all *S. aureus* strains and acts like a surfactant disrupting cellular membranes [73], [82].

1.3.2.5. Biofilms

Microbial biofilms can be defined as a structured community of microbial cells, attached to biotic (i.e. living tissues) or abiotic surfaces surrounded by a self-produced extracellular polymeric matrix [83]. They can be mono or polymicrobial and display different phenotypes depending on gene expression and protein production [84], [85].

Development and production of staphylococcal biofilms occurs in sequential steps (Figure 3) involving primary colonization and attachment followed by accumulation and maturation [83].

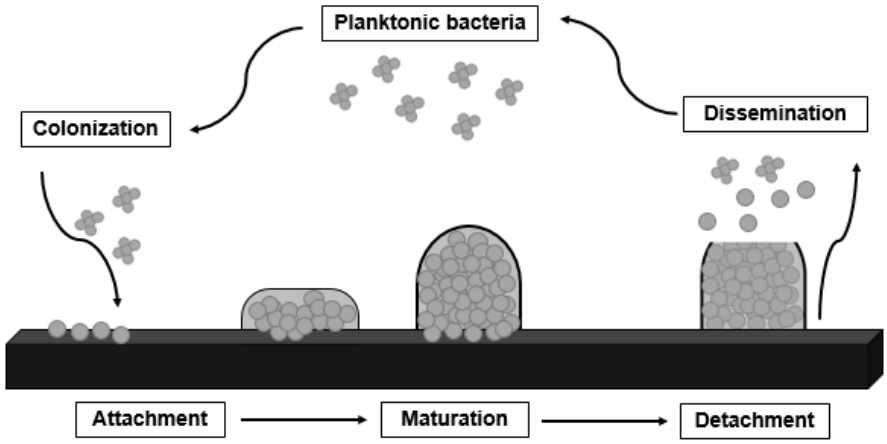


Figure 3 - Biofilms production by staphylococci (Original).

The initial attachment follows different mechanisms. While in tissues the attachment consists in a specific interaction between proteins, in abiotic surfaces this step is non-specific.

After colonization and attachment, occurs the production of the extracellular polymeric matrix that provides an essential frame for the development of the biofilm and exhibits an important role in the promotion of microbial adhesion to surfaces. It also occurs the accumulation of bacterial cells leading to multilayers, followed by growth and maturation of the biofilm [84], [86].

During the maturation step, molecules responsible for the microbial cells connection are produced, mainly proteins like polysaccharide intracellular adhesin (PIA) and accumulation-associated protein, exopolysaccharides and teichoic acids [87]; these molecules are also the main components of the extracellular polymeric matrix, often called “slyme” [83].

The final step consists in the dispersion or detachment of single or clustered cells from the biofilm structure [86], which is believed to be vital for the dissemination of the microorganism and the establishment of new colonization or infection sites [83].

The detachment of microbial cells can be promoted by several factors, including mechanical forces, interruption in the production of biofilm elements and production of detachment factors such as enzymes or surfactants like phenol soluble modulins, whose expression is controlled by the QS system [83], [88].

Biofilms are reported to display an important role in DFI since they are shown to be involved in persistent infections and related with impaired wound healing [89]. Also, bacteria residing in this structures are more resistant to antimicrobial agents when compared to planktonic bacteria [90].

1.4. Bacterial Quorum-sensing

The great majority of clinically relevant bacteria uses regulatory systems to control and regulate the collective production of virulence factors [91].

Regulation of this systems vary among bacteria species, nevertheless they all have in common the secretion of low-molecular-weight signaling molecules called autoinducers (Ais), which concentration increases with bacterial growth. In Gram-positive bacteria, like *S. aureus*, the Ais consist in small peptides referred to as autoinducing peptides (AIP). AIs are detected by receptors located in the membrane or in the cytoplasm of the bacteria, activating the expression of genes that ultimately lead to a cell to cell communication system named QS [92], [93].

QS is defined as a communication system, activated by an increase in population density, allowing the bacteria to share information and synchronize gene expression within this community and responding collectively to environmental changes [94]. QS regulates the transition between individual to collective behaviors, including processes like bioluminescence, sporulation, competence, antibiotic production and virulence factors regulation [93].

1.4.1. Quorum-sensing in *S. aureus*

In *S. aureus*, QS is encoded by the staphylococcal accessory gene regulator (*agr*), a classical autoactivation system located in the *S. aureus* chromosome and considered to be a part of the core genome [55].

This system was first described in 1988 by Peng et al [95] and it improves the ability of the microorganism to cause disease and to colonize various niches, by controlling the up and down regulation of adhesion and expression of genes associated with growth-phase-dependent virulence factors [93].

1.4.1.1. Molecular arrangement of the *agr* locus

The *agr* locus 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 *Agr*B, *Agr*D, *Agr*C and *Agr*A factors involved in the AIP synthesis and in the autoactivation of the regulatory system [55], [93].

RNAIII acts as a post-transcriptionally downstream regulatory effector, accountable for activating the production of alpha-hemolysin and inhibiting the repressor of toxins (Rot) and the production of virulence factors, like coagulase and other surface proteins [92], [96].

Concerning the RNAII operon, genes *agrB* and *agrD* combine to produce and secrete the auto-inducible peptides, i.e. the AIP. *Agr*D consists in the signalling peptide, also being the precursor peptide and *Agr*B acts as an integral membrane endopeptidase vital for the export and processing of *Agr*D [93], [97]. *Agr*A and *Agr*C are encoded by genes *agrA* and *agrC*, respectively, and constitute a two-component signaling module, being *Agr*C a histidine kinase present in the membrane that acts as a receptor and *Agr*A a response regulator [92].

Briefly, the production of AIP begins during the exponential growth phase and when its extracellular concentration reaches a threshold (i.e. near stationary growth phase) the *agr* system is autoactivated [55], [98] (Figure 4).

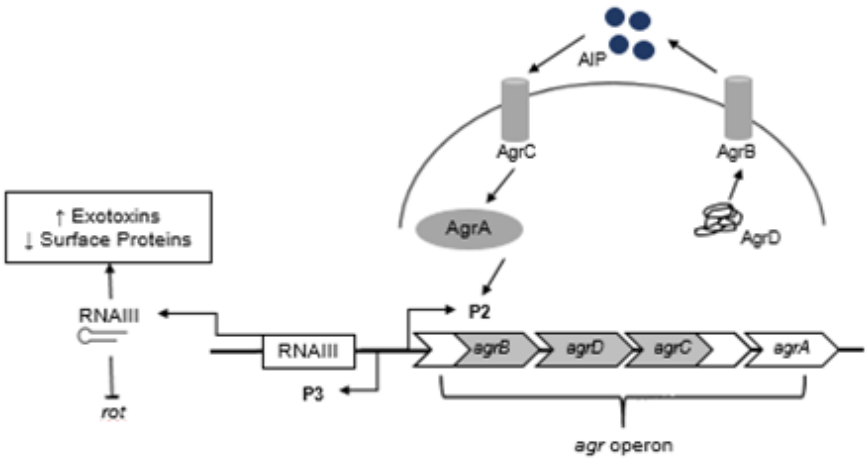


Figure 4 - *S. aureus agr* circuit autoactivation (Original).

The *Agr*D peptide is processed N-terminally by a signal peptidase named SpsB and C-terminally by *Agr*B, leading to its secretion in the form of an eight amino-acidthiolactone ring, consisting in the AIP which will be exported trough the membrane. When the threshold concentration is reached, the AIP binds to the *Agr*C receptor, activating it and resulting in the phosphorylation of the *Agr*A response regulator. Once phosphorylated, *Agr*A leads to the upregulation of its own promoter, the P2 promoter, and to the activation of the P3 promoter, leading to the expression of RNAIII, completing the autoactivation circuit [92], [93].

1.4.1.2. *agr* groups variability

The divergence of the *agr* locus has been observed among *staphylococci* strains, due to allelic variations in the *agrBDC* region, resulting into polymorphisms [93].

The variable operon region consists of the 3' end of *agrB*, plus the *agrD* and the 5' end of *agrC* (Figure 5), involved in the generation of the specific signals. The region where the main variation can be seen is in the AIP precursor, *agrD*. *agrA* which codes for the response regulator, is more conserved and does not belong to the variable region [55], [99].

Figure 5 - *agrBDC* variable region (shaded in gray) (Original).

Currently, four distinct genetic *agr* groups have been established, classified as *agr*-I, *agr*-II, *agr*-III and *agr*-IV, allowing to divide strains accordingly [100]. Within each *agr* group occurs the production of peptides capable of activating an *agr* response in other strains, however AIPs produced by different groups are usually mutually inhibitory [93]. This cross inhibition in the *agr* signaling pathway, caused by different AIP signals, constitutes a form of bacterial interference [101] and seems to be associated with the capacity of the strain to compete for infection sites with other strains [102].

This variations in the *agrBDC* region, in addition to determine *agr* groups specificity, may also explain the wide range of virulence factors which regulation is controlled by the *agr* system [96], [103].

1.4.1.3. Role of *agr* in the regulation of virulence factors and pathogenesis

Regulation of virulence factors by the *agr* locus results in pleiotropic phenotypes, in which can be observed an increased production of secreted toxins and exoenzymes and a decreased production of adhesion factors and other surface proteins [93].

However, some virulence factors, like enterotoxins A and E, hyalurodinase and some nucleases, were shown to be unaffected by the *agr* locus [104] (Table 2).

Table 2 - Important virulence proteins of *S. aureus* and its regulation by the *agr* system, adapted from Thoendel et al and Lyon et al, [55], [104].

Virulence Factors	Response to *agr* regulation	Type of regulation
Surface Proteins		
Protein A	+	↓
Cell Wall Proteins	+	↓
Exoenzymes		
Proteases	+	↑
Nucleases	-	-------
Lipases	+	↑
Hyalurodinase	-	-------
Coagulase	+	↓
Toxins		
Enterotoxin A	-	-------
Enterotoxin B	+	↑
Enterotoxin C	+	↑
Enterotoxin D	+	↑
Enterotoxin E	-	-------
TSST-1	+	↑
Exfoliative Toxins	+	↑
Alpha-hemolysin	+	↑
Beta-hemolysin	+	↑
Gama-hemolysin	+	↑
Delta-hemolysin	+	↑
Leukocidin	+	↑

+: synthesis is altered in response to activation of the *agr* system; -: no *agr* effect.
↓: down-regulation; ↑: up-regulation.

The *agr* locus is also involved in the mechanism responsible for biofilm detachment by the up-regulation of extracellular proteases, suggesting that QS also displays an important role in the adjustment between planktonic and biofilm stages, contributing to bacterial dispersal and further colonization of new locations [55], [105].

Concerning the *agr* role in disease development many of the acute disease signals have been attributed to the variety of virulence factors induced by the *agr* system, and several authors attempted to show a correlation between *agr* type present and the type of disease caused by *S. aureus*. *agr*-I is shown to be the most common type present, linked with a variety of diseases like invasive infections as bacteremia, followed by *agr*-II, both types being linked with endocarditis [55], [102]. *agr*-I is the main type to be identified in MRSA isolates and its believed to play a major role in the increased virulence shown by these strains [46], [106].

Types *agr*-III and *agr*-IV are more rare and usually associated with exfoliative syndromes. Therefore, *agr*-III is described to be linked to toxic shock syndrome, caused by TSST-1, and to staphylococcal scarlet fever and, *agr*-IV linked to SSSS [46], [55].

The majority of the recovered *S. aureus* clinical isolates are *agr* positive, though recent studies revealed that *agr*-defective strains can also be recovered from patients in nosocomial settings, being found during colonization and infection, suggesting that this strains participate in the transmission of the microorganism [107], [108].

Studies also suggest that when the *agr* is activated the microorganism switches from a colonizing commensal to an invasive pathogen [109].

1.5. Quorum-sensing as a therapeutic target

The prevalence of infections caused by *S. aureus* coupled with the microorganism ability to quickly adapt and acquire resistance to antimicrobial compounds, leads to the constant need of investigating new therapeutic approaches [62]. Due to QS involvement in the production of a wide range of virulence factors, it constitutes a desirable target for new therapeutic protocols for infections caused by numerous microorganisms, including *S. aureus* [110].

QS has been suggested as a therapeutic target, since *agr*-defective mutants display attenuated virulence in animal models of acute infection [107], [111]. Several mechanisms of interrupting the QS circuit targeting the *agr* system have been described including the design of competitive AIPs [112] and the use of specific antibodies to inactivate AIPs or RNAIII [113].

The use of competitive AIPs consist in design of analog molecules targeting the *Agr*C receptor, which has an extracellular exposure being located in the outer membrane [112]. Analog AIPs can be constructed by varying the length, amino acid sequence or by combined substitutions and truncations. Ultimately, analog AIPs bind to *Agr*C but do not activate it, preventing AIPs binding and leading to the interruption of the QS cascade [110].

On the other hand, AIPs inhibition by inactivation through interaction with specific antibodies, consist in the use of AP4-24H11, a monoclonal antibody that was demonstrated to efficiently inhibit QS *in vitro*, through AIPs capture. Also, *in vivo* studies using a mouse model, have shown that these monoclonal antibodies can also inhibit the production of toxins by inhibiting RNAIII [92], [113].

The major concern regarding these approaches is that the inactivation of the QS system can lead to an increased biofilm production [55]. Nevertheless, they are likely to become an element of combined therapies against *S. aureus* infections [92].

CHAPTER 2

Materials and Methods

The main objective of this study was the characterization of the Quorum-sensing in *S. aureus* isolates from diabetic foot ulcers. This characterization included:

- Screening of the *agr* type present;
- Copy number determination of *agr* types present by absolute quantification recurring to quantitative real time PCR (qPCR);
- Evaluation of the influence of polymicrobial infections with *P. aeruginosa* in the *agr* copy number;
- Determination and occurrence of *mecA* and *mecA* homologous gene *mecC*, responsible for the methicillin resistance in *S. aureus*.
- Relate the results obtained with biofilms production and the presence of produced virulence factors;

2.1. Bacterial strains

Isolates under study were obtained during a previous epidemiological survey of diabetic foot ulcers (DFU), as described by Mendes et al 2012 [35].

For the present study, a collection of twenty-three (n=23) representative *Staphylococcus aureus* isolates was selected, based on Pulse Field Gel Electrophoresis (PFGE) analysis, previously performed by the research team [114].

Isolates were kept at -80ºC, in BPW (buffered peptone water) plus 20% of glycerol, until further processing.

Isolates were also previously characterized regarding their phenotypic virulence profile, including the presence of exoenzymes such as coagulase, hemolysins, gelatinase, DNase, lipase [114] and biofilm production [115] (Table 3).

A reference strain, *S. aureus* ATCC®29213™, was also included as a positive control.

Table 3 - Phenotypic characterization of the clinical isolates under study considering the production of virulence factors and biofilm production [114], [115].

Isolates	Coagulase	Hemolysis	Lipase	DNase	Gelatinase	Biofilm Production
A 1.1	+	β	+	+	-	+
A 5.2	+	β	-	+	-	+
A 6.3	+	β	+	+	-	+
B 3.2	+	β	+	+	-	+
B 3.3	+	β	-	+	-	+
B 7.3	+	β	+	+	-	+
B 13.1	+	β	-	+	-	+
B 14.2	+	β	+	+	-	+
Z 1.1	+	β	+	+	-	+
Z 2.2	+	β	+	+	-	+
Z 3.1	+	β	-	+	-	+
Z 5.2	+	β	+	+	-	+
Z 14.1	+	α	-	+	-	+
Z 16.1	+	β	+	+	-	+
Z 16.2	+	β	+	-	-	+
Z 17.2	+	β	+	+	-	+
Z 21.1	+	β	+	+	-	+
Z 21.3	+	β	+	+	-	+
Z 23.2	+	β	+	+	-	+
Z 25.2	+	β	+	+	-	+
Z 27.2	+	α	+	+	-	+
Z 27.3	+	β	-	+	-	+
Z 32.2	+	α	+	-	-	+

A: aspirate; B: biopsy; Z: swab; +: positive; -: negative; α: alfa; β: beta.

2.2. DNA extraction

Selected isolates plus the reference strain were plated onto Columbia agar medium with 5% of sheep blood (BioMérieux®, ref. 43401) and incubated at 37ºC for 24h. Genomic DNA was extracted using two different methods, the Guanidium Thiocyanate Method [116] and the Boiling Method [117], in order to compare their applicability to qPCR.

For the Guanidium Thiocyanate Method a suspension with approximately $6x10^8$cfu/ml in TE (1x) (Tris EDTA) buffer, corresponding to 2 in McFarland scale was prepared and centrifuged at 8000 rpm for 10 minutes (Hermle® Z233 MK-2). Cell pellets were resuspended in 250 µl TE with lysozyme (10 mg/ml) (Merk®) and lysostaphin (1.3 ng/ml) (Merk®) and incubated at 37ºC for 30 minutes. Afterwards, 500 µl of guanidium thiocyanate (5mM/l) (Sigma-Aldrich®) were added and suspensions were held on

ice for 10 minutes followed by incubation at 50ºC for 1h (Rotilabo® Block heater H250). Subsequently, 250 µl ammonium acetate (10 mMol/L) (Merk®) were added at 4ºC and incubated on ice for 10 minutes, after which 1 ml of chloroform:isoamilic acid (24:1) (Sigma-Aldrich®) mixture was added. Suspensions were then centrifuged at 8000 rpm for 10 minutes and the supernatant fluids were transferred to new eppendorf tubes, mixed by inversion with the same volume of cold isopropanol (Sigma-Aldrich®), followed by centrifugation at 8000 rpm for 10 minutes. DNA pellets were then resuspended in 1 ml ethanol 70% (Roth®), and centrifuged using the same conditions as described before. Supernatants were discarded and the pellets were dried at room temperature. Finally, pellets were resuspended in 100 µl of RNase (Merk®) and incubated at 37ºC during 30 minutes, followed by storage at -20ºC until further use.

For the Boiling Method, 3 to 5 colonies were picked from pure cultures grown overnight in Columbia agar medium with 5% of sheep blood (BioMérieux®, ref. 4340) and suspended in 100 µl of TE complemented with 0.1% of Tween 20 (Merk®), corresponding to 0.5 in McFarland scale, followed by homogenization. Subsequently suspensions were incubated at 100ºC for 10 minutes followed by 5 minutes on ice. Suspensions were then centrifuged at 14 000 rpm for 10 minutes and the supernatant collected to new eppendorf tubes and storaged at -20ºC until further use.

The concentration of the gDNA extracted, by both methods, 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 [118]. Primers were selected based on published sequences [119], and synthesized by STABVIDA® (Table 4).

Table 4 - Nucleotide sequences of the primers used for the amplification of *agrI, agrII, agrIII* and *agrIV* [119].

Gene	Sequence (5' – 3')	Product size (bp)
agrI	F_CCAGCTATAATTAGTGGTATTAAGTACAGTAAACT R_AGGACGCGCTATCAAACATTTT	441
agrII	F_CAATAGTAACAATTTTAGTGACCATGATCA R_GCAGGATCAGTAGTGTATTTTCTTAAAGTT	575
agrIII	F_CATTATAACAATTTCACACAGCGTGTT R_GCAAGTGCATAAGAAATTGATACATACA	323
agrIV	F_GAGTTCTCAAAAAGATTAGCTCATCATATC R_TAGCTTCATCCGAGTTTATTTGAGAAT	659

F.: forward; R.: reverse; bp: base pairs.

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 94ºC for 4m, followed by 30 cycles consisting in denaturation at 95ºC for 1m, annealing at 60ºC for 1m and elongation at 72ºC for 1m, and a final extension at 72ºC for 5m.

Amplified products were resolved by conventional electrophoresis gel, in a 1.8% agarose gel (NZYTech®) with 0.5% Tris/Boric Acid EDTA (Biorad®) buffer stained with GreenSafe (NZYTech®) at 70V during 1h. Also, NZYDNA ladder VIII (NZYTech®) was included as a molecular weight marker. The results were visualized by transillumination under UV (Pharmacia Biotech, Image Master®VDS).

2.4. qPCR SYBR GREEN I assay: absolute quantification of *agrI* and *agrII*

An absolute quantification for the analysis of the *agr* locus was performed recurring to quantitative real-time PCR (qPCR). This absolute quantification requires the construction of an absolute calibration curve for each gene in study, in order to calculate the precise number of copies of the gene per cell in a given condition [120], [121].

Also, a reference gene was included as a stability control.

Nomenclature and provided qPCR data is based in the MIQE guidelines [122].

2.4.1. Primers design

Reference genes chosen were described by Theis et al [123], Goerke et al [124] and Eleaume et al [125], as they showed better results in the literature. 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* were designed (Table 5).

Table 5 - Target and reference genes tested and respective function/products.

Gene	Product	Accession number
agrI	Accessory gene regulator type I	AF210055
agrII	Accessory gene regulator type II	AF001782
coA	Coagulase	AB489883.1
fabD	Malonyl CoA-acyl carrier protein transacylase	AF275318.1
glyA	Serine hydroxymethyltransferase	GU358685.2
gmk	Guanylate kinase	AF528947.1
gyrA	Glycine hydroxymethyltransferase A	AF044070.1
hla	alfa-hemolysin	X01645
nuc	Thermonuclease	DQ399678.1
rrsC	16S ribossomal RNA subunit	AB987928.1
spa	Protein A	J01786.1

Based on results obtained by PCR, no primers for *agrIII* and *agrIV* were designed.

Online software Primer3® (Biotools®) was used for primers design, based on the following conditions: product melting temperature (T_m) between 62.8ºC and 63.0ºC and product size 50/100/150.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 (amplicon), primer length, absence of hairpins formation and lower number of self and cross dimers produced. All primers were synthesized by STABVIDA®.

Gene specificity for *S. aureus* of all primers was confirmed using BLAST [126].

2.4.2. Reference genes selection

First, the presence of all genes selected for this study in *S. aureus* DFU isolates was evaluated. A conventional PCR was performed using the same conditions and parameters as in 2.3.

A qPCR reaction was then developed for each reference gene, in order to evaluate their stability.

First, reactions with each reference gene and two isolates was performed to check if the primers were adequate for qPCR. These two isolates were chosen randomly using an online software (Random Number Generator, Intermondino®) and each harboring a different *agr* type.

Concerning the remaining reference genes candidates, qPCR reactions were performed afterwards, using all twenty-three isolates in study plus the reference strain. Based on quantification cycles (Cq) 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.

NormFinder calculates the gene expression stability based on the combined inter and intragroup Cq values, determining the optimal reference gene among a set of candidates [127]. On the other hand, geNorm calculates the gene expression stability measure (M) for each reference gene in analysis as the average pairwise variation, excluding the ones with the highest M value and retrieving a ranking list of the genes according to their stability [128].

2.4.3. Optimization and construction of calibration curves

Two calibration curves were constructed following Applied Biosystems® guidelines for creating standard curves with genomic DNA [129]. *agrI* curve was constructed based on a *pool* of isolates DNA; i.e. 10 µl of DNA of each isolate harboring the *agrI* gene were mixed and used as template. On the other hand, construction of the *agrII* curve was made using the available type strain which harbors the *agrII* gene. Both DNA concentrations were measured by spectrometry recurring to Nanodrop® (Thermo Scientific NanoDrop 2000C Spectrophotometer).

The mass of genome was calculated using equation 1, were **n** means genome size and **m** stands for mass [129].

$$m = (n)(1.096e^{-21}\ \frac{g}{bp}) \quad (1)$$

Genome size used belongs to MRSA252, a representative strain of *S. aureus* composed of a single circular chromosome of 2.902.619 bp [130].

After obtaining the genome mass and according to equation 2 [129], the mass of gDNA that would contain the number of copies of the target gene was calculated.

$$Copy\ number\ of\ interest\ x\ mass\ of\ haploid\ genome = mass\ of\ gDNA\ needed \quad (2)$$

The number of copies of interest for *agrI* and *agrII* tested were 1 x 10^1 to 1 x 10^6, 1.4 x 10^1 to 1.4 x 10^6, 3 x 10^1 to 3 x 10^6 and 5 x 10^1 to 5 x 10^6 copies/µl.

Finally, the concentration of gDNA needed to achieve the number of copies of interest was calculated by dividing the mass needed (calculated previously) by the volume to be pipetted into each reaction. After that ten-fold serial dilutions of the *agrI* pool and the type strain, containing the number of copies of interest, were used to construct the calibration curves (supplementary data: 6.3).

A qPCR absolute protocol was performed and the calibration curves and respective Cq values of each point in duplicate were obtained, in order to check which copy number were more suitable for the study. The medium of Cq 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 3 [131].

$$E\ (\%) = (10^{-1/slope}\ - 1)\ x\ 100\% \quad (3)$$

2.4.4. Absolute quantification protocol

Once the calibration curve was constructed, the absolute quantification of *agrI* and *agrII* was carried out. 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) and cap strips were used to cover the wells (Optically clear flat 8 cap strips, Thermo Scientific®). Each well contained 1 µl of diluted 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 previous prepared mix of the forward and reverse primers with a final concentration of 10 nM.

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 95ºC for 20s, cycling stage at 95ºC for 15s and 60ºC for 1m; followed by a melting curve stage, consisting of 30 cycles of 95ºC for 15s, 60ºC for 1m and 95ºC 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 (NZYTech®) with 0.5% Tris/Boric Acid EDTA (Biorad®) at 65V during 2h. Also, NZYDNA ladder VIII (NZYTech®) was used as a molecular weight marker. Results were visualized by transillumination under UV (Pharmacia Biotech, Image Master® VDS).

Copy number determination for each isolate was calculated according to equation 4, where $\mathbf{N_n}$ stands for quantity and **n** means Cq [132].

$$N_n = 10^{\left(\frac{n-b}{m}\right)} \quad (4)$$

2.5. Co-culture assay

To evaluate if there were any changes in the copy numbers of *agr* in *S. aureus* present in polymicrobial infections, a co-culture assay was performed. Isolates B 3.2 (*S. aureus*) and B 3.1 (*P. aeruginosa*), were grown apart in Tryptic Soy Broth (TSB) (PROLABO®) for 24h at 37ºC. Afterwards, dual suspensions with different concentrations of *S. aureus* and *P. aeruginosa* (1:9; 2:8; 5:5; 8:2; 9:1) were prepared in TSB, using 0.5 McFarland cultures, and incubated for 24h at 37ºC (Table 6).

Table 6 - Suspensions used in co-cultures.

Suspensions		Volumes	≅ Number of Bacteria (CFU/ml)
A1	1:9	100 µl *S. aureus* + 900 µl *P. aeruginosa*	$1.5 \times 10^7 + 1.35 \times 10^8$
A2	2:8	200 µl *S. aureus* + 800 µl *P. aeruginosa*	$3 \times 10^7 + 1.2 \times 10^8$
A3	5:5	500 µl *S. aureus* + 500 µl *P. aeruginosa*	$7.5 \times 10^7 + 7.5 \times 10^7$
A4	8:2	800 µl *S. aureus* + 200 µl *P. aeruginosa*	$1.2 \times 10^8 + 3 \times 10^7$
A5	9:1	900 µl *S. aureus* + 100 µl *P. aeruginosa*	$1.35 \times 10^8 + 1.5 \times 10^7$
	Control +	1000 µl *S. aureus*	1.5×10^8

Following incubation, gDNA was extracted using the Boiling Method and its concentration was measured using Nanodrop® (Thermo Scientific NanoDrop 2000C Spectrophotometer). To confirm the presence of *S. aureus*, a conventional PCR was performed, screening for the presence of *agrII*, under the same conditions as in 2.3. The *agrII* copy number in all suspensions was determined by absolute quantification using qPCR.

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

qPCR was performed and amplified products were confirmed as described in 2.4.4.

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* described by Stegger et al [133] (Table 7).

Table 7 - Nucleotide sequences of the *mecA* and *mecC* primers.

Gene	Sequence (5' – 3')	Product size (bp)
mecA	F_TCCAGATTACAACTTCACCAGG R_CCACTTCATATCTTGTAACG	162
mecC	F_GAAAAAAAGGCTTAGAACGCCTC R_GAAGATCTTTTCCGTTTTCAGC	138

F.: forward; R.: reverse; bp: base pairs

For each isolate, a mixture containing 12.5 µl of Supreme NZYTaq 2x Green Master Mix (Nzytech®), 1 µl of each primer (forward and reverse) for the *mecA* gene (STABVIDA®), 1 µl each primer (forward and reverse) for the *mecC* gene (STABVIDA®) and 5.5 µl of sterile water (water for molecular biology, Nzytech®), was prepared. To this mixture 1 µl of the previous extracted gDNA was added, resulting in a total reaction volume of 25 µl.

A PCR mix without DNA template was used as no-template control. As a positive control for *mecC*, a strain of *S. aureus* LGA251 was used, gently provided by Dr. Mark Holmes, Senior Lecturer of the University of Cambridge [133].

PCR amplification was carried out and the amplified products were 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, including coagulase, hemolysins, gelatinase, DNase, lipase and biofilm production, previously determined by the research team [114], [115], 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.

CHAPTER 3

Results

For the Quorum-sensing characterization a collection of twenty-three clinical DFU isolates was analysed. Identification of the *agr* type present was performed by PCR and, afterwards the quantification of *agr* genes copy number in each isolate was performed recurring to qPCR. It was also possible to evaluate the influence of polymicrobial infections with *P. aeruginosa* in the *agr* copy number. The occurrence of *mecA* and *mecA* homologous gene *mecC* was also determined. Finally, results obtained were related with biofilms and virulence factors production, previously described.

3.1. DNA extraction

The Guanidium Thiocyanate Method and the Boiling Method for gDNA extraction were compared. Average gDNA concentrations obtained were 157.1 ± 84.8 ng/µl and 68.8 ± 27.8 ng/µl, respectively. Based on the results, the chosen extraction method for the subsequent analysis was the Boiling Method, since it shown a lower variation between obtained gDNA concentrations.

3.2. *agr* type screening

S. aureus agr type characterization was performed by PCR (Figure 6). Isolates were distributed in two groups, based on their *agr* type, *agr*-I or *agr*-II. For illustration purposes only two isolates of each type are presented in Figure 6 A and B.

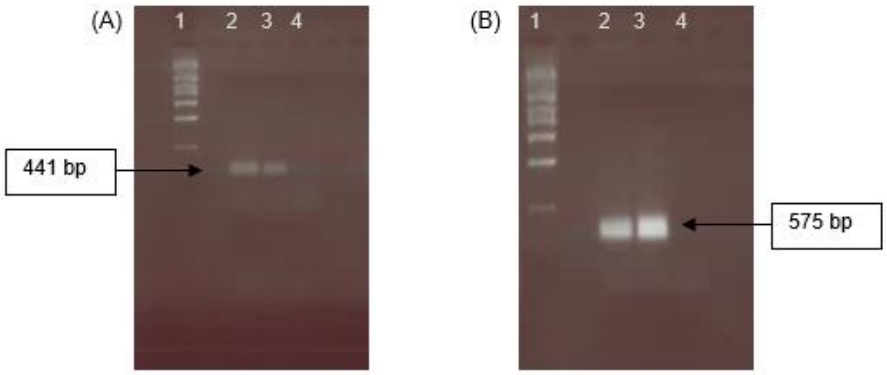


Figure 6 - Amplification of *agrI* (441 bp) (A) and *agrII* (575 bp) (B).
(A) Lane 1 – NZYDNA ladder VIII; Lane 2, 3 - *agr*-I isolates; Lane 4 - no-template control;
(B) Lane 1 – NZYDNA ladder VIII; 2 – *agr*-II isolates; Lane 3 - no-template control.

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.

Detailed characterization of each isolate may be found in supplementary data (6.1).

3.3. qPCR SYBR GREEN I assays: absolute quantification of *agrI* and *agrII*

3.3.1. Primers selection

Sequences and characteristics of the primers designed for target and reference genes are presented in Table 8.

Table 8 - Nucleotide sequences and characteristics of the primers designed for the qPCR assay.

Gene	Sequence (5'-3')	Product size (bp)	Length (bp)	GC (%)	Characteristics
agrI	F_GCAAGTTCCGTCACGATTATGTC R_ATCACGTAGGCCAGGCATGT	83	23 20	47.8 55	Hairpins: no S.D.: no and C.D.: 5 Tm (ºC): 59.5 and 59
agrII	F_TTCAAACGGTGATAGCTTAATTCCA R_CTCTTTAAGGGTGAAAAGCGACATTA	103	25 26	36 38.4	Hairpins: no S.D: 4 and C.D.: 3 Tm (ºC): 59.1 and 59.7
coA	F_CACAACCAGTTGCACAACCATTA R_GGGACCTTGAACGATTTCACC	118	23 21	43.7 52.4	Hairpins: no S.D.: 4 and C.D.: 4 Tm (ºC): 59.4 and 59.3
fabD	F_TTTGAATGGCGTGATGCTAAGTT R_TCAGTTTCACCTTGCGCATTT	115	23 21	39.1 42.9	Hairpins: no S.D.: no and C.D.: 4 Tm (ºC):59.6 and 59.3
glyA	F_AGCGGCAGGTTTACATCCAA R_CACGTGGTCCGCGTAATGT	83	20 19	50 57.9	Hairpins: no S.D.: no and C.D.: 4 Tm (ºC): 59.2 and 59
gmk	F_GCGTGAAGGTGAAGTTGATGG R_GCTTCAAACGCATCCCTAGTTTT	74	21 23	52.4 43.5	Hairpins: no S.D.: no and C.D.: 4 Tm (ºC):59.2 and 59.6
gyrA	F_TGCGATGAGTGTTATCGTTGCT R_ATCCGGTGTCATACCTTGTTCATT	126	22 24	45.5 41.7	Hairpins: 4 S.D.: 4 and C.D.: no Tm (ºC): 59.4 and 59.5
hla	F_TTGGCGGCCTTATTGGTG R_GTTGGGCTCTCTAAAATTGTTTTGAA	78	18 26	55.6 34.6	Hairpins: no S.D.: 4 and C.D.: no Tm (ºC): 59.1 and 59.9
nuc	F_CGAAAGGGCAATACGCAAAG R_CTGTTTGTGATGCATTTGCTGAG	107	25 23	50 43.5	Hairpins: no S.D.: no and C.D.:4 Tm (ºC): 59.3 and 59.4
rrsC	F_CTTGACATCCTTTGACCGCTCT R_ACACGAGCTGACGACAACCA	102	22 20	50 55	Hairpins: no S.D.: no and C.D.: 4 Tm (ºC): 59.4 and 58.9
spa	F_AGCACCGAAAGCGGATAACA R_TGGATGAAACCATTGCGTTG	105	20 20	50 45	Hairpins: no S.D.: no and C.D.: 3 Tm (ºC): 59.3 and 59

T_m: melting temperature; GC: guanine- cytosine content; S.D.: self-dimer; C.D.: cross-dimer; F: forward; R: reverse; bp: base pairs.

3.3.2. Reference genes selection

Based on conventional PCR results reference genes *coA*, *fabD* and *spa* were excluded. Since for *coA* and *spa* the amplification of nonspecific bands was observed and for *fabD* there was no amplification in the isolates harboring *agrII*.

qPCR quantification cycles (Cq) values results of the remaining six reference candidate genes, *hla*, *glyA, gmk*, *gyrA*, *nuc* and *rrsC*, were compared, allowing to exclude genes *hla*, *gmk* and *glyA* since they presented a high variability of Cq values between isolates.

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 (supplementary data: 6.2).

3.3.3. Optimization and construction of calibration curves for *agrI* and *agrII*

For both *agr* types, calibration curves, with the following copies of interest 1 x 10^1 to 1 x 10^6, 1.4 x 10^1 to 1.4 x 10^6, 3 x 10^1 to 3 x 10^6 and 5 x 10^1 to 5 x 10^6 copies/µl of *agrI* and *agrII*, were compared based on their amplification efficiency (Table 9).

Table 9 - Amplification efficiency for each calibration curve.

Number of copies (copies/µl)	***agrI***	***agrII***
	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

The chosen calibration curves, for both *agrI* and *agrII*, were the ones with copies ranging from 3 x 10^1 to 3 x 10^6 copies/µl.

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

Concerning the qPCR analysis of isolates harboring *agrI* and *agrII*, Cq values obtained ranged from an averaged 16.6 ± 0.2 to 36 ± 1.6 and 20.7 ± 0.4 to 25.3 ± 0.0, respectively.

Dissociation curves obtained for *agrI* and *agrII* reveal a single dissociation peak, which indicates primers specificity (Figure 7, (A) and (B)). Also, *agrI* no-template control dissociation curve suggests the

formation of primer dimers (in red), since an amplification peak can be observed (Figure 7, (A)). In fact, primers contamination can be discarded since amplification occurred at a lower T_m, compared with the remaining reactions.

For *agrII,* no-template control dissociation curve presented a flat outline (in red), suggesting that there were no primer dimers formation and no contamination of the primers used (Figure 7, (B)).

The averaged T_m was found to be 76.7 ºC ± 0.3 ºC for *agrI* and 72.6 ºC ± 0.1 ºC for *agrII*.

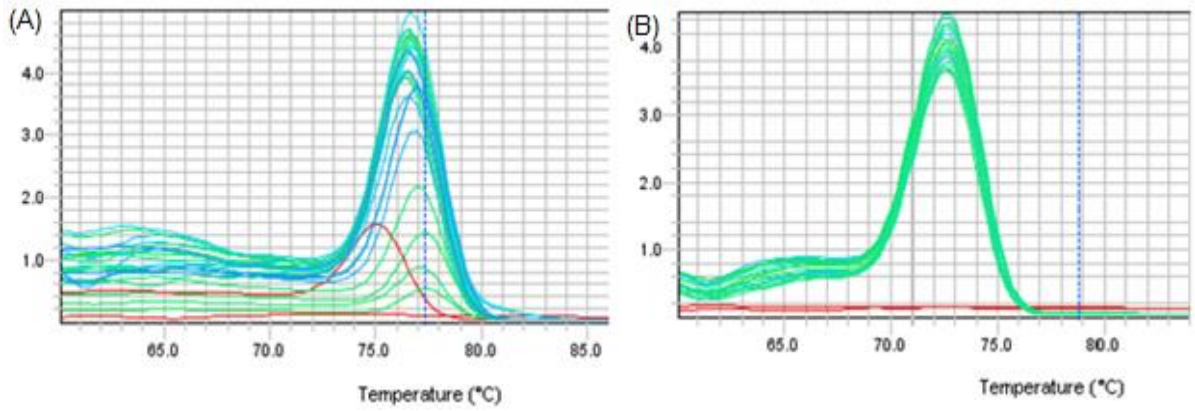


Figure 7 - Dissociation curves obtained in *agrI* (A) and *agrII* (B) assays.

Amplified products of qPCR, for both *agrI* and *agrII*, were observed in a 2% agarose gel electrophoresis, to allow amplification confirmation.

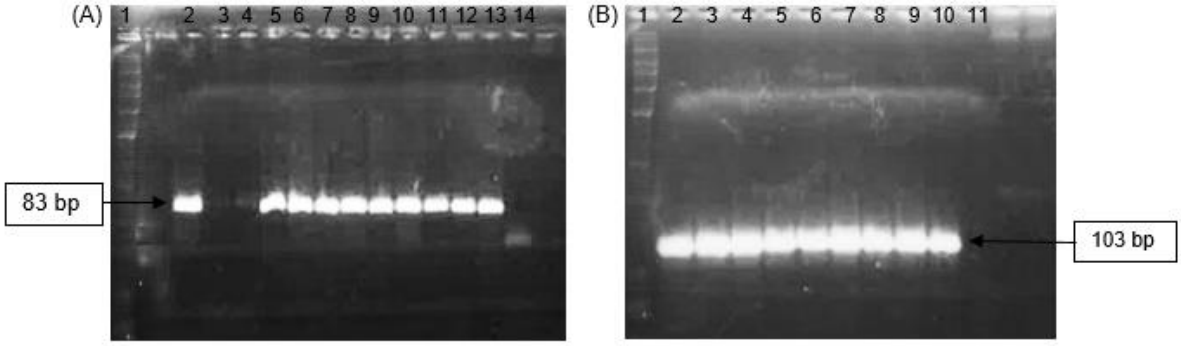


Figure 8 - qPCR amplification products confirmation for *agrI* (83 bp) (A) and *agrII* (103 bp) (B) assays.
(A) Lane 1- NZYDNA ladder VIII, Lanes 2 - 13 - *agrI* isolates; Lane 14 - no-template control.
(B) Lane 1 - NZYDNA ladder VIII; Lane 2 -10 - *agrII* isolates; Lane 11 - no-template control.

It was possible to confirm qPCR amplification products in *agrI* and *agrII* assays, validating previous conventional PCR results, since the obtained results in gel electrophoresis showed clear single bands with the expected size of 83 bp and 103 bp, respectively (Figure 8, (A) and (B)). Isolates Z 3.1 and Z 5.2, which amplified products revealed less intensity (lanes 4 and 3 of Figure 8 (A), respectively), presented higher Cq values (>30) in the qPCR analysis, suggesting a lower quantity of gDNA, when

compared with the remaining isolates. Also, the observed band in lane 14 (Figure 8, (A)), corresponding to the no-template control, confirms the formation of primer dimers, shown in the dissociation curve.

For copy number determination of both *agrI* and *agrII*, calibration curves, ranging from 3×10^1 to 3×10^6 copies/µl were generated (Figure 9, (A) and (B)). Nevertheless, dilution with 3×10^1 copies/µl in (A) and dilutions with 3×10^1 copies/µl and 3×10^6 copies/µl in (B) were discarded for normalization purposes.

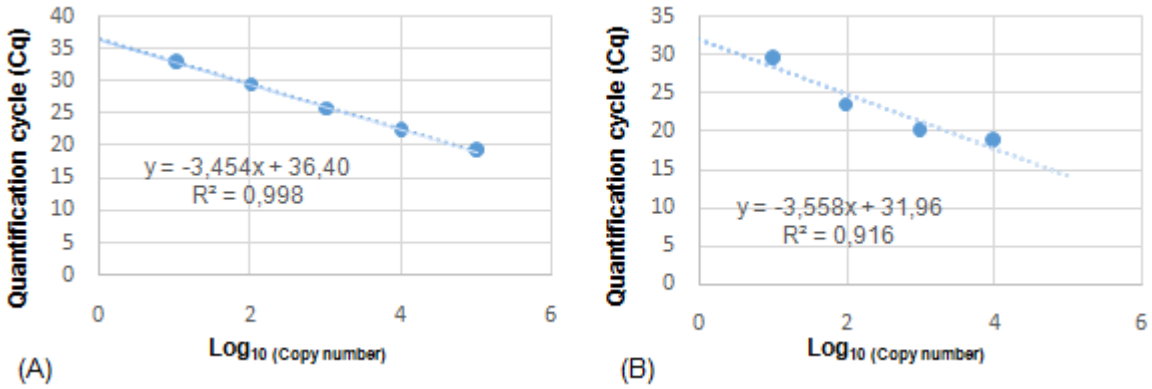


Figure 9 - Calibration curves generated from the amplification of a ten-fold dilutions of target gDNA for *agrI* (A) and *agrII* (B).

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* (supplementary data: 6.3.). For isolates which Cq values were not included within this range was not possible to calculate the number of copies of *agrI* and *agrII*.

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 ($R^2 = 0.998$), 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 ($R^2 = 0.916$), which may affect the interassay variability. The slope of the calibration curve was -3.558, which corresponds to an efficiency of 90.9%.

Concerning *agrI,* copy number obtained ranged from 7.1 ± 2.4 to 94279 ± 28507 copies of total gDNA, determined for each isolate (Figure 10). For isolates Z 1.1, Z 5.2 and Z 25.2 it was not possible to calculate the *agrI* copy numbers.

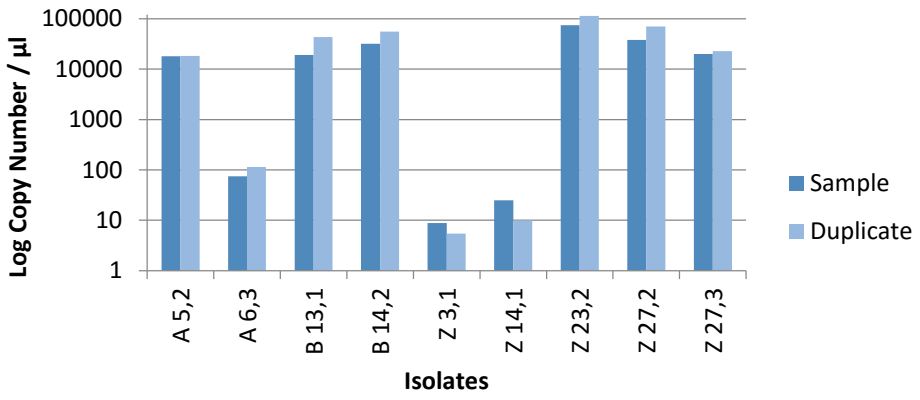


Figure 10 - Determination of the absolute copy numbers of *agrI* using the absolute quantification method. A: aspirate; B: biopsy; Z: swab.

In relation to *agrII*, copy numbers obtained ranged from 72.9 ± 0.2 to 1487.8 ± 405.4 copies of total gDNA, determined for each isolate (Figure 11).

No isolates were excluded.

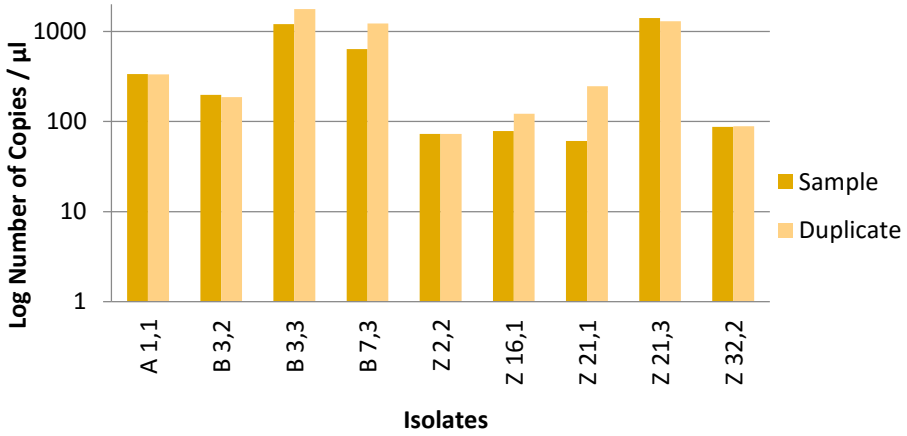


Figure 11 - Determination of the absolute copy number of *agrII* using the absolute quantification method. A: aspirate; B: biopsy; Z: swab.

Detailed results are reported as the mean ± standard deviation between the sample and duplicate (supplementary data: 6.4.)

All assays were validated by analysing the reference gene *gyrA*, used as a stability control indicating technique reproducibility. The gene as an averaged Cq values of 19.1 ± 2.6 in the *agrI* assay and 22.5 ± 2.2 in the *agrII* assay (supplementary data: 6.5.).

3.4. Co-culture assay

To evaluate changes in *agrII* copy number of *S. aureus* in polymicrobial infections, the presence of *S. aureus* in the dual suspensions was first confirmed by conventional PCR, by targeting the *agrII* gene, present in the isolate used in this assay (Figure 12).

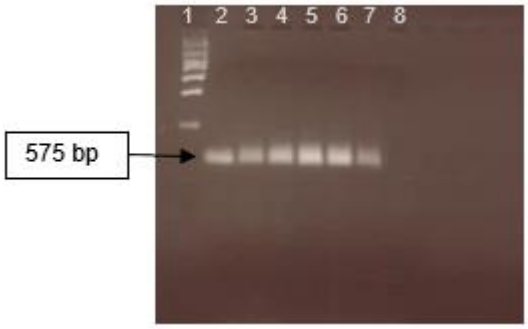


Figure 12 - Amplification products of *agrII* (575 bp).
Lane 1 – NZYDNA ladder VIII; Lane 2-6 – dual suspensions; Lane 7 – positive control (B 3.2);
Lane 8 – no-template control.

After confirming the presence of *S. aureus*, absolute quantification by qPCR was performed. Average Cq values obtained for the positive control were 26.4 ± 1.5, while for the dual suspensions Cq values obtained ranged from an average 21.9 ± 0.0 to 25.4 ± 0.4.

Dissociation curves obtained for the positive control and suspensions, presented only one peak, confirming primers specificity. The dissociation curve of the no-template control presented a flat outline (in red), suggesting that there was no primer dimers formation and no contamination of the primers used in the assay (Figure 13).

The averaged T_m was found to be 73.1 ºC ± 0.1 ºC for the positive control and 72.9 ºC ± 0.1 for the dual suspensions.

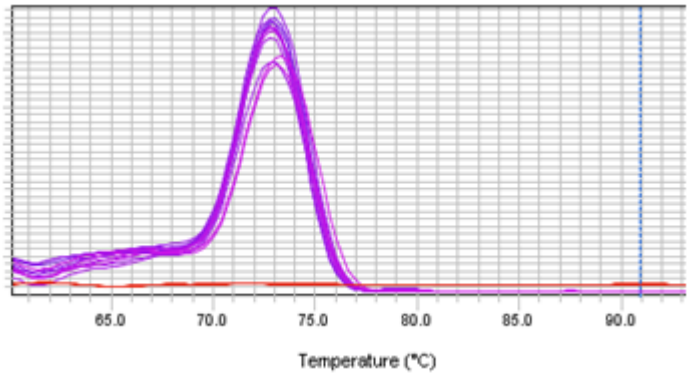


Figure 13 - Dissociation curve obtained for *agrII* when in co-culture.

Amplified products of qPCR were observed in a 2% agarose gel electrophoresis. It was possible to confirm the presence of *agrII* in all dual suspensions, corroborating conventional PCR previous results. Nevertheless, amplification shown in lane 8 (no-template control) suggests the formation of primer dimers (Figure 14), which was not observed in the dissociation curve presented above.

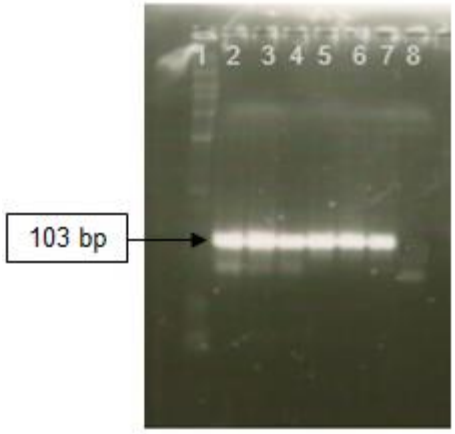


Figure 14 - qPCR amplification products of *agrII* (103 bp) when in co-culture.
Lane 1 – ladder; Lane 2 – 6 – suspensions; Lane 7 – positive control; Lane 8 – no-template control.

For copy number determination of *agrII* in the dual suspensions, a calibration curve, ranging from 3×10^1 to 3×10^6 copies/µl was generated (Figure 15). Dilution with 3×10^1 copies/µl was discarded for normalization purposes.

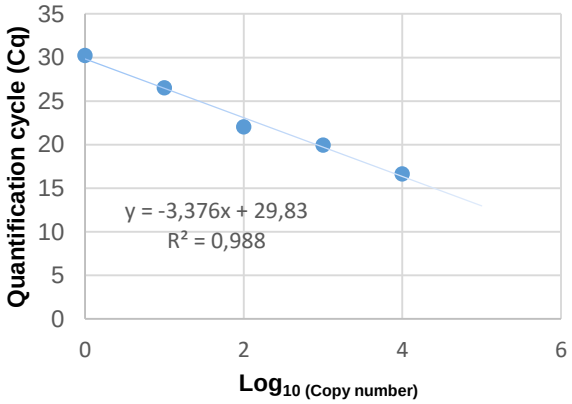


Figure 15 - Calibration curve generated from the amplification of a ten-fold dilutions of target gDNA for and *agrII* when in co-culture.

Averaged Cq values for the obtained curve ranged from 16.6 ± 0.3 to 30.2 ± 0.6, with an average T_m of 72.8 ºC ± 0.6 ºC (supplementary data: 6.3).

Calibration curve was generated by plotting the DNA (pg) log against the Cq values determined for *agr*II when in co-culture. A very high correlation value was obtained (R^2 = 0.988), indicating a low

interassay variability. The slope of the calibration curve was -3.376, which corresponds to an efficiency of 97.9%.

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 16), (supplementary data: 6.4.).

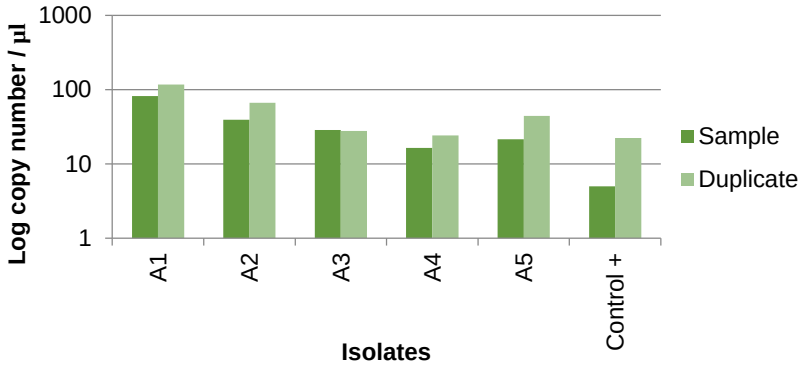


Figure 16 - Determination of the absolute copy number of *agrII* of *S. aureus* when in co-culture using the absolute quantification method.
A1: 100 µl *S. aureus* + 900 µl *P. aeruginosa* (1:9); A2: 200 µl *S. aureus* + 800 µl *P. aeruginosa* (2:9); A3: 500 µl *S. aureus* + 500 µl *P. aeruginosa* (5:5); A4: 800 µl *S. aureus* + 200 µl *P. aeruginosa* (8:2); A5: 900 µl *S. aureus* + 100 µl *P. aeruginosa* (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 (n=8) tested positive for *mecA*, however none tested positive for *mecC* (Figure 17).

For illustration purposes, only the *mecA* positive are presented in Figure 17.

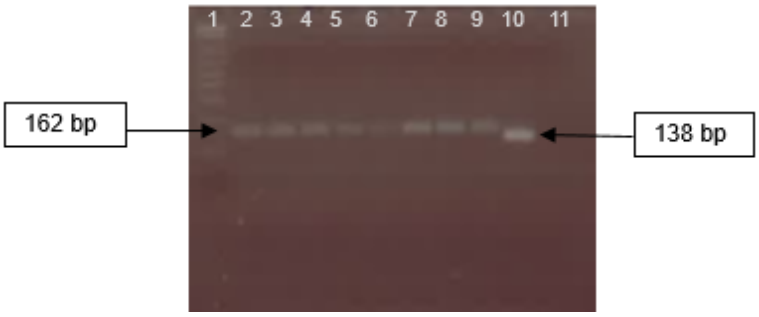


Figure 17 - Multiplex PCR amplification of *mecA* (162 bp) and *mecC* (138 bp).
Lane 1 – NZYDNA ladder VIII; Lane 2-9 – isolates; Lane10 - positive control for *mecC*; Lane 11 - no-template control

3.6. Virulence factors relation with *agr* type

The relation between *agr* type, virulence factors and biofilm production (Figure 18), was evaluated [114], [115].

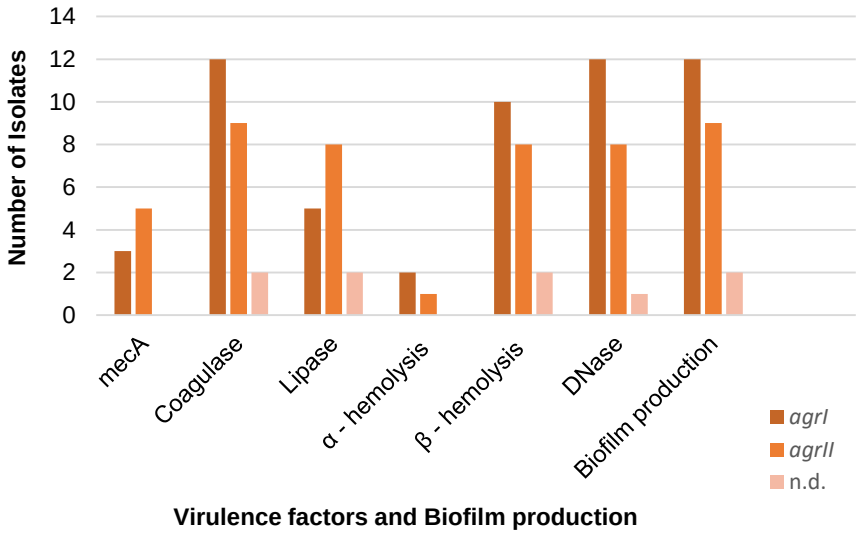


Figure 18 - Relation between *agr* type, virulence factors and biofilm production.
mecA: *mecA* gene; α: alfa; β: beta; n.d.: not determined

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.

CHAPTER 4

Discussion

S. aureus is an important human pathogen, responsible for a wide range of both community and nosocomial infections, and recognized as one of the most common bacterial species to be isolated from skin and soft tissue infections, such as DFI [39].

This microorganism uses a Quorum-sensing communication system, QS, activated by an increase in population density, which allows bacteria to share information and synchronize gene expression, responding collectively to environmental changes. QS is encoded by *agr* (accessory gene regulator), a classical autoactivation system which is responsible for controlling the expression of several genes that code for virulence factors [93], [102].

In this study, the QS system in *S. aureus* isolates from DFU, obtained in a previous epidemiological survey as described by Mendes et al 2012 [35], 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 [118]. 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, were *agr*-I was the most prevalent type to be identified followed by *agr*-II [102], [134].

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 [102], [135] and *agr*-IV was also reported as absent in previous studies [101], [136], [137].

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 the microorganism avoid the host immune system [108].

For the analysis and determination of gene copy number in all isolates an absolute qPCR protocol was developed. qPCR is a molecular biology technique highly sensitive and reproducible, based in the kinetics and signal issued from both target and reference genes amplification [138]. Opposite to conventional PCR, that only allow the amplification and further detection of nucleic acids by electrophoresis [139], qPCR allow their amplification, detection and quantification in simultaneous [140].

For nucleic acid detection in qPCR, specific probes or fluorescent dyes can be used [138]. Specific probes are fluorescent–labelled and target-specific, with a complementary oligonucleotide sequence to the target gene, being highly specific [141]. On the other hand, SYBR GREEN I, which was used in this work, is a non-specific fluorescent dye that binds to DNA double strands and can used as an alternative to specific probes. This dye binds to newly formed double stranded DNA and promotes the emission of fluorescence proportionally to the formed product, allowing quantification. However, since it is highly nonspecific, has the disadvantage of binding to any molecule or double-strand DNA, including dimers [138], [142], rendering the evaluation of primers *in silico* an important step for the technique optimization.

Quantification by qPCR can be relative or absolute [138]. For relative quantification, one of the most used methods is the comparative Cq method ($2^{\Delta\Delta Cq}$), which measures the expression of both target and reference genes, used as internal control. On the other hand, absolute quantification depends on the use of a calibration curve constructed with known concentrations, to measure the copy numbers of a

given target gene [140]. The use of a reference gene is not compulsory but it may be included, as a control, to guaranty technique stability [143]. Ideal reference genes should have an essential function, preferably not being related with the target genes in study [123].

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 [55].

Genes *coA*, *fabD* and *spa* were initially excluded, based on a conventional PCR technique. In fact, for genes *coA* and *spa* nonspecific bands were amplified, which can be related with the fact that *agr* down-regulates the expression of these genes. In the case of *fabD* there was no amplification in the *agrII* isolates.

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 to be used as reference genes for the isolates in study. Cq values obtained for the remaining reference genes, *gyrA, nuc* and *rrsC*, 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.

It is also important to refer that, when the objective is to compare different genes between isolates, an absolute quantification using gDNA as a template is considered to be more informative and consistent, since it relies on a calibration curve with known concentrations [144], while a relative quantification can miss relevant biological information, since data is reported as a ratio of the expression of the target genes divided by the expression of the selected reference genes, also is more prone to contaminations, since the template used is mRNA [140].

Regarding the selection of the gDNA extraction method, results showed that the Guanidium Thiocyanate Method had a higher recovery of gDNA as the concentrations obtained were higher. Even so, since lower concentrations of nucleic acids are more adequate for qPCR techniques [145], the chosen method was the Boiling Method, as gDNA concentrations were lower and showing a smaller variation between isolates, however it was necessary to perform dilutions of gDNA in order to obtained the ideal concentration for qPCR. Also, qPCR reproducibility using the Boiling Method was higher, when compared with the Guanidium Thiocyanate Method, which was also demonstrated by Fu et al [144].

In this work, it was necessary to design primers specific for the reference and target genes to be used in the qPCR techniques. Primers design consider the following parameters: optimal primers length of 20 bp; optimal melting temperature between 58ºC and 60ºC (though melting temperature parameters may vary with the equipment used); optimal GC content in the primers of approximately 50%, although it may fluctuate between 30% and 80%; and product size (amplicon) ideal length is considered to be between 80-120 bp [143]. Primers designed for this study shown an average length of 21.1 bp ± 4.6 bp, a melting temperature of 59.3 ºC ± 0.3 ºC and an average GC% content of 46.8% ± 6.6%. Also, the average product size (amplicon) was 99.5 bp ± 17.5 bp, values that lie within the parameters described

above. Formation of self and cross dimers was observed *in silico* in Primer Express Software Real Time PCR® (Applied Biosystems®); however, primers chosen were the ones that produced lower values of dimers formation.

For absolute quantification, other important step for assay optimization is to obtain a suitable and stable calibration curve. Besides allowing to determine the actual copy number of the target genes, by relating the Cq values obtained, calibration curves can also provide important information regarding assay efficiency, replicate consistency and also allow to detect the limit of the reaction, that consists in the lowest amount of analyte in given sample that can be detected within the linear range that can be reliably quantified [132]. The choice of the calibration curve is based on an ideal efficiency range, between 90 – 100% [146].

In this study, four different calibration curves, were tested for the absolute quantification of *agrI* and *agrII,* with copies ranging from 1 x 10^1 to 1 x 10^6, 1.4 x 10^1 to 1.4 x 10^6, 3 x 10^1 to 3 x 10^6 and 5 x 10^1 to 5 x 10^6 copies/µl. Obtained efficiencies for each calibration curve allowed choosing the curve with ranging from 3 x 10^1 to 3 x 10^6 copies/µl 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 absolute quantification, a qPCR protocol was developed that allowed the distinction between *agrI* and *agrII*. In fact, melt curve analysis of the calibration curves obtained for *agrI* and *agrII*, showed the primers to be specific for these genes.

Formation of dimers for *agrI* was observed in the analysis of the qPCR assay melt curve and confirmed by agarose gel electrophoresis, while for *agrII* no dimers formation was observed.

Amplification plot and dissociation curve obtained for *agrI* showed a higher oscillation of Cq values among isolates, while the opposite was observed for *agrII*. The same was observed for the gene copy number calculated.

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.

Although, *S. aureus* is described as one of the most common microorganisms found in DFU, those wounds are often colonized by complex polymicrobial communities, being two of the microorganisms more frequently co-isolated *S. aureus* and *P. aeruginosa* [36], [37]. *In vitro* studies demonstrated that when in co-culture these bacterial species share a competitive relationship and that *P. aeruginosa* acts as an antagonist of *S. aureus,* through the secretion of respiratory inhibitors [36]. *S. aureus* survival is related with its ability to adapt to changing selective pressures during long-term colonization by forming electron transport-deficient SCVs. SCVs occurrence has already been described in DFU by

Cervantes-Garcia et al [39] and by Windmüller et all in clinical isolates from the lungs of cystic fibrosis patients [147].

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*.

In our collection, only two wounds were colonized by *S. aureus* and *P. aeruginosa*, but it was only possible to include the isolates from one wound in this assay, since the *S. aureus* isolated from the other wound was considered *agr*-defective. The fact that the assay was conducted with isolates from only one wound can be considered a limitation.

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.

Considering the qPCR assay, formation of dimers was not observed in the melt curve analysis, however it was observed by agarose gel electrophoresis. To calculate *agrII* copy numbers in the dual suspensions a calibration curve with copies ranging 3 $x10^1$ to 3 x 10^6 copies/µl was used.

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.

As selective pressure can be responsible for alterations in gene copy numbers in microorganisms such as yeasts and cyanobacteria [57], [58], 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. Dhanasekaran et al also reported that copy numbers can vary significantly over a short period of time and are highly influenced by storage conditions, showing that quantification is dependent on the use of suitable calibration curves [140]. Therefore, gDNA

dilutions for calibration curve determination should be prepared at the same day as the assay to reduce storage time and, consequently, possible errors in gene copy numbers determination.

PCR amplification of *mecA* is considered the “gold standard” technique for detection of methicillin resistance among *S. aureus* [148]. However, the discovery of a new *mecA* homologous gene, *mecC* determined the need to establish new detection protocols [133]. Although normally the screening of the homologous gene is only made in oxacillin-resistant *mecA* negative isolates [68], in this study a multiplex assay was applied for the screening of *mecA* and *mecC* in all isolates in simultaneous. 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 [149]. Also, Djahmi et al suggested that this prevalence may be related with antimicrobial treatment and the high frequency of recurrent ulcers [134].

The 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 [150]. However, Jarraud et al described a higher prevalence of *agr*-I among MRSA strains [46] and the same was observed by Azimian et al [151].

Besides antimicrobial resistance, *S. aureus* displays multiple mechanisms of virulence that contribute to the microorganism pathogenicity, including the production of virulence factors [36]. Previously to this study, the isolates were characterized phenotypically considering the production of virulence factors such as coagulase, hemolysis, gelatinase, DNase and lipase [114]. Biofilm production was also previously determined [115]. 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 that presently impairs the treatment of numerous bacterial infections diseases, including the ones promoted by MRSA strains.

REFERENCES

[1] “Health Topics - Diabetes Mellitus,” *Who - World Health Organization*. [Online]. Available: http://www.who.int/topics/diabetes_mellitus/en/. [Accessed: 17-Oct-2014].

[2] K. G. Alberti and P. Z. Zimmet, “Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation.,” *Diabetic Medicine: a Journal of the British Diabetic Association*, vol. 15, no. 7, pp. 539–53, Jul. 1998.

[3] J. L. Jameson, “Endocrionologia e Metabolismo - Diabetes melito.,” in *Harrison - Manual de Medicina*, 17th ed., M. Hill, Ed. 2011, pp. 942–947.

[4] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, “Global prevalence of Diabetes - estimates for the year 2000 and projections for 2030.,” *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[5] “Ketosis,” *Online Medical Dictionary*, 2015. .

[6] “Diabetes type 1,” *Diabetes Research Institute Foundation*. [Online]. Available: http://www.diabetesresearch.org/what-is-type-one-diabetes. [Accessed: 02-Jun-2015].

[7] J. Kelly, “What is diabetes?,” *Center for Disease Control and Prevention*. pp. 1–3, 2014.

[8] “Diabetes Risk Factors,” *International Diabetes Federation*, 2015. [Online]. Available: http://www.idf.org/about-diabetes/risk-factors. [Accessed: 02-Jun-2015].

[9] K. T. Coppieters, T. Boettler, and M. von Herrath, “Virus infections in type 1 diabetes.,” *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 1, pp. 1–14, 2012.

[10] “Signs and symptoms of Diabetes.,” *International Diabetes Federation*, 2015. [Online]. Available: http://www.idf.org/signs-and-symptoms-diabetes. [Accessed: 03-Jun-2015].

[11] M. J. Fowler, “Microvascular and macrovascular complications of Diabetes.,” *Clinical Diabetes*, vol. 26, no. 2, pp. 77–82, Apr. 2008.

[12] H. Fishbein and P. J. Palumbo, “Acute metabolic complications in Diabetes mellitus.,” *Diabetes In America*, vol. 131, no. 5, pp. 283–92, 1995.

[13] “Complications of Diabetes,” *International Diabetes Federation*, 2015. [Online]. Available: http://www.idf.org/complications-diabetes. [Accessed: 28-May-2015].

[14] N. Singh, D. G. Armstrong, and B. A. Lipsky, “Preventing foot ulcers in patients with Diabetes.,” *The Journal of the American Medical Association*, vol. 293, no. 2, pp. 217–128, 2005.

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

[16] D. K. Wukich, “Current concepts review: diabetic foot ulcers.,” *Foot & Ankle International*, vol. 31, no. 5, pp. 460–7, May 2010.

[17] K. Al-Rubeaan, M. Al Derwish, S. Ouizi, A. M. Youssef, S. N. Subhani, H. M. Ibrahim, and B. N. Alamri, “Diabetic foot complications and their risk factors from a large retrospective cohort study.,” *PloS One*, vol. 10, no. 5, p. e0124446, 2015.

[18] J. J. Mendes and J. Neves, “Diabetic foot infections: current diagnosis and treatment.,” *The Journal of Diabetic Foot Complications*, vol. 4, no. 2, pp. 26–45, 2012.

[19] S. Noor, M. Zubair, and J. Ahmad, “Diabetic foot ulcer - a review on pathophysiology, classification and microbial etiology.,” *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, 2015.

[20] P. D. Sinwar, “The diabetic foot management – recent advance.,” *International Journal of Surgery*, vol. 15, pp. 27–30, 2015.

[21] A. Boulton, R. Malik, J. Arezzo, and J. Sosenko, “Diabetic Somatic Neuropathies.,” *Diabetes Care*, vol. 27, no. 6, 2004.

[22] L. . Rogers, R. Frykberg, D. Armstrong, A. Boulton, M. Edmonds, G. Ha Van, A. Hartemann, F. Game, W. Jeffcoate, A. Jirkovska, E. Jude, S. Morbach, W. Morrison, M. Pinzur, D. Pitocco, L. Sanders, D. Wukich, and L. Uccioli, “The Charcot foot in diabetes.,” *Diabetes Care*, vol. 34, no. 9, pp. 2123–2129, 2011.

[23] S. Gardner and R. Frantz, “Wound bioburden and infection-related complications in diabetic foot ulcers.,” *Biological Research For Nursing*, vol. 10, no. 1, pp. 44–53, 2008.

[24] D. K. McCulloch, “Patient information: Foot care in diabetes mellitus (Beyond the Basics),” *In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. Copyright © 2015 UpToDate, Inc.*, 2015. [Online]. Available: http://www.uptodate.com/contents/image?imageKey=PI/64087&topicKey=PI/1739&source=outline_link. [Accessed: 16-Jun-2015].

[25] M. P. Khanolkar, S. C. Bain, and J. W. Stephens, “The diabetic foot.,” *QJM - International Journal of Medicine*, vol. 101, no. 9, pp. 685–695, 2008.

[26] E. B. Jude, I. Eleftheriadou, and N. Tentolouris, “Peripheral arterial disease in diabetes - a review,” *Diabetic medicine: a Journal of the British Diabetic Association*, vol. 27, no. 1, pp. 4–14, 2010.

[27] N. Clark, “Peripheral arterial disease in people with Diabetes.,” *Diabetes Care*, vol. 26, no. 12, pp. 3333–3341, 2003.

[28] M. S. Bader, “Diabetic Foot Infection.,” *American Academy of Family Physicians*, vol. 78, no. 1, pp. 71–82, 2008.

[29] R. G. Frykberg, “Diabetic foot ulcers: pathogenesis and management.,” *American Family Physician*, vol. 66, no. 9, pp. 1655–1662, 2002.

[30] G. Clerici and E. Faglia, “Saving the limb in diabetic patients with ischemic foot lesions complicated by acute infection.,” *The International Journal of Lower Extremity Wounds*, vol. 13, no. 4, pp. 273–93, Dec. 2014.

[31] C. Adams and E. Deitch, “Surgical Treatment: Evidence-Based and Problem-Oriented,” 2001. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK6985/. [Accessed: 17-Jun-2015].

[32] J. J. Mendes, C. Leandro, S. Corte-Real, R. Barbosa, P. Cavaco-Silva, J. Melo-Cristino, A. Górski, and M. Garcia, “Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds.,” *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*, vol. 21, no. 4, pp. 595–603, 2013.

[33] B. A. Lipsky, A. R. Berendt, H. G. Deery, J. M. Embil, W. S. Joseph, A. W. Karchmer, J. L. LeFrock, D. P. Lew, J. T. Mader, C. Norden, and J. S. Tan, “Diagnosis and treatment of diabetic foot infections.,” *Clinical Infectious Diseases*, vol. 39, no. 7, pp. 885–910, Oct. 2004.

[34] B. Lipsky, A. Berendt, P. Cornia, J. Pile, E. Peters, D. G. Armstrong, H. G. Deery, M. Embil, W. Joseph, A. Karchmer, M. Pinzur, and E. Senneville, “2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections.,” *Clinical Infectious Diseases*, vol. 54, no. 12, pp. e132–73, Jun. 2012.

[35] 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.

[36] 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.

[37] 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.

[38] L. Voggu, S. Schlag, R. Biswas, R. Rosenstein, C. Rausch, and F. Götz, “Microevolution of cytochrome bd oxidase in staphylococci and its implication in resistance to respiratory toxins released by Pseudomonas.,” *Journal of Bacteriology*, vol. 188, no. 23, pp. 8079–8086, 2006.

[39] E. Cervantes-Garcia, R. Garcia-Gonzalez, A. Reyes-Torres, A. A. Resendiz-Albor, and P. M. Salazar-Schettino, “*Staphylococcus aureus* small colony variants in diabetic foot infections.,” *Diabetic Foot and Ankle*, vol. 1, no. 4, pp. 4–8, 2015.

[40] C. F. Michelsen, A. J. Christensen, M. S. Bojer, N. Høiby, H. Ingmer, and L. Jelsbak, “*Staphylococcus aureus* alters growth activity, autolysis and antibiotic tolerance in a human host adapted Pseudomonas aeruginosa lineage.,” *Journal of bacteriology*, vol. 196, no. 22, pp. 3903–3911, Sep. 2014.

[41] A. Korgaonkar, U. Trivedi, K. P. Rumbaugh, and M. Whiteley, “Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 3, pp. 1059–64, Jan. 2013.

[42] B. Peters, M. Jabra-Rizk, G. O’May, J. Costerton, and M. Shirtliff, “Polymicrobial interactions: impact on pathogenesis and human disease.,” *Clinical Microbiology Reviews*, vol. 25, no. 1, pp. 193–213, Jan. 2012.

[43] A. Orenstein, “The discovery and naming of *Staphylococcus aureus*,” 2008. [Online]. Available: http://www.antimicrobe.org/h04c.files/history/S-aureus.asp.

[44] “Genus Staphylococcus,” *List of Prokaryotic names with standing in nomenclature (LPSN)*. [Online]. Available: http://www.bacterio.net/staphylococcus.html. [Accessed: 07-Jun-2015].

[45] K. Todar, “*Staphylococcus aureus* and Staphylococcal Disease,” *Todar’s Online Textbook of Bacteriology*, 2012. [Online]. Available: http://textbookofbacteriology.net/staph.html. [Accessed: 07-Jun-2015].

[46] 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.

[47] M. Burian, C. Wolz, and C. Goerke, “Regulatory adaptation of *Staphylococcus aureus* during nasal colonization of humans.,” *PloS One*, vol. 5, no. 4, p. e10040, Jan. 2010.

[48] “General Information about *Staphylococcus aureus*,” *Center for Disease Control and Prevention (CDC)*. [Online]. Available: http://www.cdc.gov/HAI/organisms/staph.html. [Accessed: 07-Jun-2015].

[49] A. Fauci, E. Brauland, and D. Kasper, “Doenças Infecciosas - Infeções Estafilococicas.,” in *Harrison’s Manual of Medicine*, 17th ed., McGraw Hill, 2011, pp. 489–503.

[50] J. J. Mendes, “*Staphylococcus aureus* antibiotic resistance: from basic research to clinical practice.,” *Revista Oficial da Sociedade Portuguesa de Cuidados Intensivos*, vol. 17, no. 1, pp. 11–15, 2010.

[51] V. Pereira, C. Lopes, A. Castro, J. Silva, P. Gibbs, and P. Teixeira, “Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal.,” *Food Microbiology*, vol. 26, no. 3, pp. 278–282, 2009.

[52] J. Melo Cristino, “Staphylococcus.,” in *Microbiologia vol. 2*, 2nd ed., Lisboa: Lidel, 2000, pp. 39–50.

[53] J. Bien, O. Sokolova, and P. Bozko, “Characterization of virulence factors of *Staphylococcus aureus*: novel function of known virulence factors that are implicated in activation of airway epithelial proinflammatory response.,” *Journal of Pathogens*, no. Article ID 601905, pp. 1–13, Jan. 2011.

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

[55] 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.

[56] J. Pratten, S. J. Foster, P. F. Chan, M. Wilson, and S. P. Nair, “*Staphylococcus aureus* accessory regulators: expression within biofilms and effect on adhesion.,” *Microbes and Infections*, vol. 3, pp. 3–7, 2001.

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

[58] B. E. Schirrmeister, 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.

[59] 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.

[60] T. Větrovský and P. Baldrian, “The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses.,” *PLoS ONE*, vol. 8, no. 2, pp. 1–10, 2013.

[61] 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.

[62] B. Strommenger, M. D. Bartels, K. Kurt, F. Layer, S. M. Rohde, K. Boye, H. Westh, W. Witte, H. de Lencastre, and U. Nübel, “Evolution of methicillin-resistant *Staphylococcus aureus* towards increasing resistance.,” *Journal of Antimicrobial Chemotherapy*, vol. 69, no. 3, pp. 616–622, 2014.

[63] B. Shrestha, “Study of beta lactamase activity of *Staphylococcus aureus* isolated from healthy nasal carriers and hospital isolates.,” *Nepal Medical College Journal*, vol. 9, no. 2, pp. 107–110, 2007.

[64] M. Holden, L. Hsu, K. Kurt, L. Weinert, A. Mather, S. Harris, B. Strommenger, F. Layer, W. Witte, H. de Lencastre, R. Skov, H. Westh, H. Zemlicková, G. Coombs, A. Kearns, R. Hill, J. Edgeworth, I. Gould, V. Gant, J. Cooke, G. Edwards, P. McAdam, K. Templeton, A. McCann, Z. Zhou, S. Castillo-Ramírez, E. Feil, L. Hudson, M. Enright, F. Balloux, D. Aanensen, B. Spratt, J. Fitzgerald, J. Parkhill, M. Achtman, S. Bentley, and U. Nübel, "A genomic portrait of the emergence, evolution, and global spread of a methicillin-resistant *Staphylococcus aureus* pandemic.," *Genome research*, vol. 23, no. 4, pp. 653–64, 2013.

[65] B. Ballhausen, A. Kriegeskorte, N. Schleimer, G. Peters, and K. Becker, "The mecA homolog mecC confers resistance against β-lactams in *Staphylococcus aureus* irrespective of the genetic strain background," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 7, pp. 3791–3798, 2014.

[66] A. M. Hanssen and J. E. Sollid, "SCCmec in staphylococci: Genes on the move.," *FEMS Immunology and Medical Microbiology*, vol. 46, no. 1, pp. 8–20, 2006.

[67] D. Espadinha, N. A. Faria, M. Miragaia, L. M. Lito, J. Melo-Cristino, and H. de Lencastre, "Extensive dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) between the hospital and the community in a country with high prevalence of nosocomial MRSA.," *PLoS ONE*, vol. 8, no. 4, pp. 1–8, 2013.

[68] L. García-Álvarez, M. Holden, H. Lindsay, C. Webb, D. Brown, M. Curran, E. Walpole, K. Brooks, D. Pickard, C. Teale, J. Parkhill, S. Bentley, G. Edwards, E. Girvan, A. Kearns, B. Pichon, R. Hill, A. Larsen, R. Skov, S. Peacock, D. Maskell, and M. Holmes, "Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study.," *The Lancet. Infectious diseases*, vol. 11, no. 8, pp. 595–603, Aug. 2011.

[69] A. Petersen, M. Stegger, O. Heltberg, J. Christensen, A. Zeuthen, L. Knudsen, T. Urth, M. Sorum, L. Schouls, J. Larsen, R. Skov, and A. Larsen, "Epidemiology of methicillin-resistant *Staphylococcus aureus* carrying the novel mecC gene in Denmark corroborates a zoonotic reservoir with transmission to humans.," *Clinical Microbiology and Infection*, vol. 19, no. 1, pp. e16–e22, 2013.

[70] F. García-Garrote, E. Cercenado, M. Marín, M. Bal, P. Trincado, J. Corredoira, C. Ballesteros, J. Pita, P. Alonso, and A. Vindel, *Methicillin-resistant Staphylococcus aureus carrying the mecC gene.*, vol. 69, no. 1. 2005, pp. 1–17.

[71] C. Kim, C. Milheiriço, S. Gardete, M. Holmes, M. Holden, H. de Lencastre, and A. Tomasz, "Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain LGA251 and its contribution to the β-lactam-resistant phenotype.," *Journal of Biological Chemistry*, vol. 287, no. 44, pp. 36854–36863, 2012.

[72] K. O'Riordan and J. C. Lee, "*Staphylococcus aureus* capsular polysaccharides.," *Clinical Microbiology Reviews*, vol. 17, no. 1, pp. 218–234, 2004.

[73] P. R. Murray and K. S. Rosenthal, "Staphylococcus and related organisms.," in *Medical Microbiology*, 4th ed., Mosby, 2002, pp. 202–216.

[74] R. Mercier, Y. Kawai, and J. Errington, "General principles for the formation and proliferation of a wall-free (L-form) state in bacteria," *eLife*, vol. 3, pp. 1–14, 2014.

[75] T. J. Foster, J. Geoghegan, V. Ganesh, and M. Höök, "Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*.," *Nature Reviews Microbiology*, vol. 12, no. 1, pp. 49–62, 2014.

[76] M. Dinges, P. Orwin, and P. Schlievert, "Exotoxins of *Staphylococcus aureus*.," *Clinical Microbiology Reviews*, vol. 13, no. 1, pp. 16–34, 2000.

[77] L. Shaw, E. Golonka, J. Potempa, and S. Foster, “The role and regulation of the extracellular proteases of *Staphylococcus aureus*.,” *Microbiology*, vol. 150, no. 1, pp. 217–228, 2004.

[78] K. Zaleski, T. Kolodka, C. Cywes-Bentley, R. McLoughlin, M. Delaney, B. Charlton, W. Johnson, and A. Tzianabos, “Hyaluronic acid binding peptides prevent experimental staphylococcal wound infection.,” *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 11, pp. 3856–3860, 2006.

[79] M. Argudín, M. Mendoza, and M. Rodicio, “Food poisoning and *Staphylococcus aureus* enterotoxins.,” *Toxins*, vol. 2, no. 7, pp. 1751–1773, 2010.

[80] S. Ladhani, “Understanding the mechanism of action of the exfoliative toxins of *Staphylococcus aureus*.,” *FEMS Immunology and Medical Microbiology*, vol. 39, no. 2, pp. 181–189, 2003.

[81] R. Nazareth, J. Gonçalves-Pereira, A. Tavares, M. Miragaia, H. de Lencastre, J. Silvestre, P. Freitas, E. Gonçalves, F. Martins, V. Mendes, C. Tapadinhas, and P. Póvoa, “Infeção por *Staphylococcus aureus* meticilina-resistente da comunidade em Portugal.,” *Revista Portuguesa de Pneumologia*, vol. 18, no. 1, pp. 34–38, 2012.

[82] F. Vandenesch, G. Lina, and T. Henry, “*Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors?,” *Frontiers in Cellular and Infection Microbiology*, vol. 2, no. February, p. 12, Jan. 2012.

[83] M. Otto, “Staphylococcal biofilms.,” *Current Topics in Microbiology and Immunology*, vol. 322, pp. 207–228, 2008.

[84] N. Archer, M. Mazaitis, J. Costerton, J. Leid, M. Powers, and M. Shirtliff, “*Staphylococcus aureus* biofilms: Properties, regulation and roles in human disease.,” *Virulence*, vol. 2, no. 5, pp. 445–459, 2011.

[85] A. Malik, Z. Mohammad, and J. Ahmad, “The diabetic foot infections: biofilms and antimicrobial resistance.,” *Diabetes & Metabolic Syndrome*, vol. 7, no. 2, pp. 101–7, 2013.

[86] F. Götz, “Staphylococcus and biofilms.,” *Molecular Microbiology*, vol. 43, no. 6, pp. 1367–1378, 2002.

[87] C. Cucarella, C. Solano, and J. Valle, “Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation.,” *Journal of Bacteriology*, vol. 183, no. 9, pp. 2888–2896, 2001.

[88] S. Periasamy, H.-S. Joo, C. Duong, T.-H. Bach, V. Tan, S. Chatterjee, G. Cheung, and M. Otto, “How *Staphylococcus aureus* biofilms develop their characteristic structure.,” *Proceedings of the National Academy of Sciences*, vol. 109, no. 4, pp. 1281–1286, 2012.

[89] G. Zhao, M. L. Usui, S. I. Lippman, G. a James, P. S. Stewart, P. Fleckman, and J. E. Olerud, “Biofilms and Inflammation in Chronic Wounds.,” *Advances in wound care*, vol. 2, no. 7, pp. 389–399, 2013.

[90] D. Citron, E. Goldstein, C. Merriam, B. A. Lipsky, and M. A. Abramson, “Bacteriology of moderate-to-severe diabetic foot infections and in vitro activity of antimicrobial agents.,” *Journal of Clinical Microbiology*, vol. 45, no. 9, pp. 2819–28, Sep. 2007.

[91] A. Beceiro, M. Tomás, and G. Bou, “Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world?,” *Clinical Microbiology Reviews*, vol. 26, no. 2, pp. 185–230, 2013.

[92] 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.

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

[94] Y.-H. Dong and L.-H. Zhang, “Quorum-sensing and Quorum-quenching enzymes.,” *Journal of Microbiology*, vol. 43, no. March, pp. 101–109, 2005.

[95] H. L. Peng, R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert, “Cloning, characterization and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*.,” *Journal of Bacteriology*, vol. 170, no. 9, pp. 4365–4372, 1988.

[96] 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.

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

[98] R. Singh and P. Ray, “Quorum-sensing mediated regulation of staphylococcal virulence and antibiotic resistance.,” *Future Microbiology*, vol. 9, no. 5, pp. 669–81, Jan. 2014.

[99] P. Dufour, S. Jarraud, F. Vandenesch, R. Novick, M. Bes, J. Etienne, and G. Lina, “High genetic variability of the *agr* locus in Staphylococcus species.,” *Journal of Bacteriology*, vol. 184, no. 4, pp. 1180–1186, 2002.

[100] 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.

[101] 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.

[102] 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.

[103] D. Robinson, A. Monk, J. Cooper, E. Feil, and M. Enright, “Evolutionary Genetics of the Accessory Gene Regulator (*agr*) Locus in *Staphylococcus aureus*.,” *Journal of Bacteriology*, vol. 187, no. 24, 2005.

[104] G. J. Lyon and R. P. Novick, “Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria.,” *Peptides*, vol. 25, no. 9, pp. 1389–403, Sep. 2004.

[105] B. R. Boles and A. R. Horswill, “*Agr*-mediated dispersal of *Staphylococcus aureus* biofilms.,” *PLoS Pathogens*, vol. 4, no. 4, p. e1000052, Apr. 2008.

[106] G. Cheung, R. Wang, B. Khan, D. Sturdevant, and M. Otto, “Role of the accessory gene regulator *agr* in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis.,” *Infection and Immunity*, vol. 79, no. 5, pp. 1927–35, May 2011.

[107] B. Shopsin, A. Drlica-Wagner, B. Mathema, R. Adhikari, B. Kreiswirth, and R. Novick, “Prevalence of *agr* dysfunction among colonizing *Staphylococcus aureus* strains.,” *The Journal of Infectious Diseasess Diseases*, vol. 198, no. 8, pp. 1171–1174, 2008.

[108] M. Grundmeier, L. Tuchscherr, 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.

[109] A. Roux, S. Payne, and M. Gilmore, “Microbial telesensing: probing the environment for friends, foes and food.,” *Cell Host Microbe*, vol. 6, no. 2, pp. 115–124, 2010.

[110] T. Kievit and B. Iglewski, “Bacterial Quorum-sensing in pathogenic relationships.,” *Infection and Immunity*, vol. 68, no. 9, 2000.

[111] C. P. Gordon, P. Williams, and W. C. Chan, “Attenuating *Staphylococcus aureus* virulence gene regulation: a medicinal chemistry perspective.,” *Journal of Medicinal Chemistry*, vol. 56, no. 4, pp. 1389–1404, 2013.

[112] G. J. Lyon, P. Mayville, T. W. Muir, and R. P. Novick, “Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, *Agr*C.,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 24, pp. 13330–13335, 2000.

[113] J. Park, R. Jagasia, G. Kaufmann, J. Mathison, D. Ruiz, J. Moss, M. Meijler, R. Ulevitch, and K. Janda, “Infection control by antibody disruption of bacterial Quorum-sensing signaling.,” *Chemistry and Biology*, vol. 14, no. 10, pp. 1119–1127, 2007.

[114] 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.

[115] 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.

[116] 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.

[117] 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.

[118] 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.

[119] 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.

[120] W. Liu and D. a Saint, “A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics.,” *Analytical biochemistry*, vol. 302, no. 1, pp. 52–59, 2002.

[121] K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.,” *Methods (San Diego, Calif.)*, vol. 25, no. 4, pp. 402–408, 2001.

[122] S. A. Bustin, V. Benes, J. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer, “The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments.,” *Clinical Chemistry*, vol. 55, no. 4, pp. 611–622, 2009.

[123] 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.

[124] 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.

[125] 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.

[126] 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].

[127] B. Bonefeld, B. Elfving, and G. Wegener, “Reference genes for normalization: a study of rat brain tissue.,” *Synapse*, vol. 62, no. 9, pp. 302–309, 2008.

[128] J. Vandesompele, K. Preter, F. Pattyn, and B. Poppe, “GeNorm software manual,” *Last updated on March*, pp. 1–16, 2007.

[129] “Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR.,” *Applied Biosystems*, pp. 1–8, 2003.

[130] “*Staphylococcus aureus*.,” *Sanguer Institute*, 2015. [Online]. Available: http://www.sanger.ac.uk/resources/downloads/bacteria/staphylococcus-aureus.html. [Accessed: 10-May-2015].

[131] 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.

[132] “Real-Time PCR: applications guide.,” *Bio-Rad Laboratories*, 2006.

[133] 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.

[134] N. Djahmi, N. Messad, S. Nedjai, A. Moussaoui, D. Mazouz, J. L. Richard, A. Sotto, 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.

[135] A. Ghasemian, S. Najar Peerayeh, B. Bakhshi, and M. Mirzaee, “Acessory gene regulator specificity groups among *Staphylococcus aureus* isolated from hospitalized children.,” *Archives of Pediatric Infectious Diseases*, vol. 3, no. 2, p. e16096, 2014.

[136] 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.

[137] A. Ben Ayed, I. Boubaker, S. Ennigrou, and S. Redjeb, “Acessory gene regulator (*agr*) typing of *Staphylococcus aureus* isolated from human infections.,” *Les archives de l’Institut Pasteur Tunis*, vol. 85, pp. 3–8, 2008.

[138] V. Chini, A. Foka, G. Dimitracopoulos, and I. Spiliopoulou, “Absolute and relative real-time PCR in the quantification of tst gene expression among methicillin-resistant *Staphylococcus aureus*: evaluation by two mathematical models.,” *Letters in Applied Microbiology*, vol. 45, no. 5, pp. 479–484, Nov. 2007.

[139] M. Joshi and J. D. Deshpande, “Polymerase chain reaction: methods, principles and application,” *International Journal of Biomedical Research*, vol. 5, pp. 81–97, 2010.

[140] 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.

[141] D. T. Leong, A. Gupta, H. F. Bai, G. Wan, L. F. Yoong, H. P. Too, F. T. Chew, and D. W. Hutmacher, “Absolute quantification of gene expression in biomaterials research using real-time PCR.,” *Biomaterials*, vol. 28, no. 2, pp. 203–210, 2007.

[142] H. Fukushima, Y. Tsunomori, and R. Seki, “Duplex Real-Time SYBR Green assays for detection of 17 species of food or waterborne pathogens in stools.,” *Journal of Clinical Microbiology*, vol. 41, no. 11, pp. 5134–5146, 2003.

[143] B. D’haene, J. Vandesompele, and J. Hellemans, “Accurate and objective copy number profiling using real-time quantitative PCR.,” *Methods*, vol. 50, no. 4, pp. 262–270, 2010.

[144] J. Fu, D. Li, S. Xia, H. Song, Z. Dong, F. Chen, X. Sun, and Z. Tang, “Absolute quantification of plasmid DNA by real-time PCR with genomic DNA as external standard and its application to a biodistribution study of an HIV DNA vaccine.,” *Analytical sciences : the international journal of the Japan Society for Analytical Chemistry*, vol. 25, no. 5, pp. 675–680, 2009.

[145] J. J. Yun, L. E. Heisler, I. I. L. Hwang, O. Wilkins, S. K. Lau, M. Hyrcza, B. Jayabalasingham, J. Jin, J. McLaurin, M. S. Tsao, and S. D. Der, “Genomic DNA functions as a universal external standard in quantitative real-time PCR.,” *Nucleic Acids Research*, vol. 34, no. 12, pp. 1–10, 2006.

[146] M. T. Dorak, “Real-Time PCR: practical issues and troubleshooting.,” in *MOBGAM, Istanbul, Turkey*, 2011.

[147] N. Windmüller, A. Witten, D. Block, B. Bunk, C. Spröer, B. Kahl, and A. Mellmann, “Transcriptional adaptations during long-term persistence of *Staphylococcus aureus* in the airways of a cystic fibrosis patient.,” *International Journal of Medical Microbiology*, vol. 305, no. 1, pp. 38–46, 2015.

[148] T. Siripornmongcolchai, C. Chomvarin, K. Chaicumpar, T. Limpaiboon, and C. Wongkhum, “Evaluation of different primers for detecting mecA gene by PCR in comparison with phenotypic methods for discrimination of methicillin-resistant *Staphylococcus aureus*.,” *Southeast Asian J ournal of Tropical Medicine and Public Health*, vol. 33, no. 4, pp. 758–763, 2002.

[149] 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.

[150] 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.

[151] A. Azimian, S. Najar-pirayeh, S. Mirab-Samiee, and M. Naderi, “Occurence of methicillin-resistant *Staphylococcus aureus* (MRSA) among clinical samples in Tehran-Iran and its correlation with polymorphism of specific acessory gene regulator (*agr*) groups.,” *Brazilian Journal of Microbiology*, no. 2012, pp. 779–785, 2012.

SUPPLEMENTARY DATA

6.1. Characteristics of *S. aureus* DFU isolates

Table 10 - *agr* type characterization and methicillin - resistance (*mecA* and *mecC*) confirmation of *S. aureus* DFU isolates in study.

S. aureus	**Characteristics**		
DFU Isolates	***agr* type**	***mecA***	***mecC***
A 1.1	II	positive	negative
A 5.2	I	negative	negative
A 6.3	I	negative	negative
B 3.2	II	negative	negative
B 3.3	II	negative	negative
B 7.3	II	positive	negative
B 13.1	I	positive	negative
B 14.2	I	positive	negative
Z 1.1	I	positive	negative
Z 2.2	II	negative	negative
Z 3.1	I	negative	negative
Z 5.2	I	negative	negative
Z 14.1	I	negative	negative
Z 16.1	II	positive	negative
Z 16.2	n.d.	negative	negative
Z 17.2	n.d.	negative	negative
Z 21.1	II	positive	negative
Z 21.3	II	positive	negative
Z 23.2	I	negative	negative
Z 25.2	I	negative	negative
Z 27.2	I	negative	negative
Z 27.3	I	negative	negative
Z 32.2	II	negative	negative
ATCC®29213™	II	n.d.	n.d.

A: aspirate; B: biopsy; Z: swab; *mecA* – *mecA* gene; *mecC* – *mecC* gene; *agr:* accessory gene regulator; n.d.: not determined.

6.2. GenEx results

Table 11 - Average Cq values of *gyrA*, *nuc* and *rrsC* submitted to GenEx®.

	gyrA	*nuc*	*rrsC*	*agr* type
1	18,4395	19,4895	15,944	1
3	19,317	20,436	19,449	1
4	21,4055	22,3425	19,959	2
5	19,803	22,1405	19,7755	2
6	19,0115	22,37	21,03	1
7	22,16	21,996	22,281	2
8	21,5845	21,703	19,5315	1
10	14,6815	15,3375	13,1825	1
11	18,9775	20,6625	18,295	2
12	22,1705	22,4375	19,9885	1
13	21,749	24,6175	20,874	1
14	18,0605	19,797	17,7395	2
15	20,839	20,833	19,8395	1
16	15,958	16,688	14,609	2
17	20,0945	21,488	19,4505	2
18	23,2645	27,433	17,4045	1
19	16,824	17,928	16,0405	1
20	17,882	20,282	17,1735	1
21	23,462	23,8825	21,3555	1
22	19,594	21,944	18,804	2
23	16,643	19,0455	15,8275	2

gyrA: gyrase; *nuc*: nuclease; *rrcS:* 16s ribosomal RNA subunit; *agr:* accessory gene regulator.

6.2.1. geNorm analysis

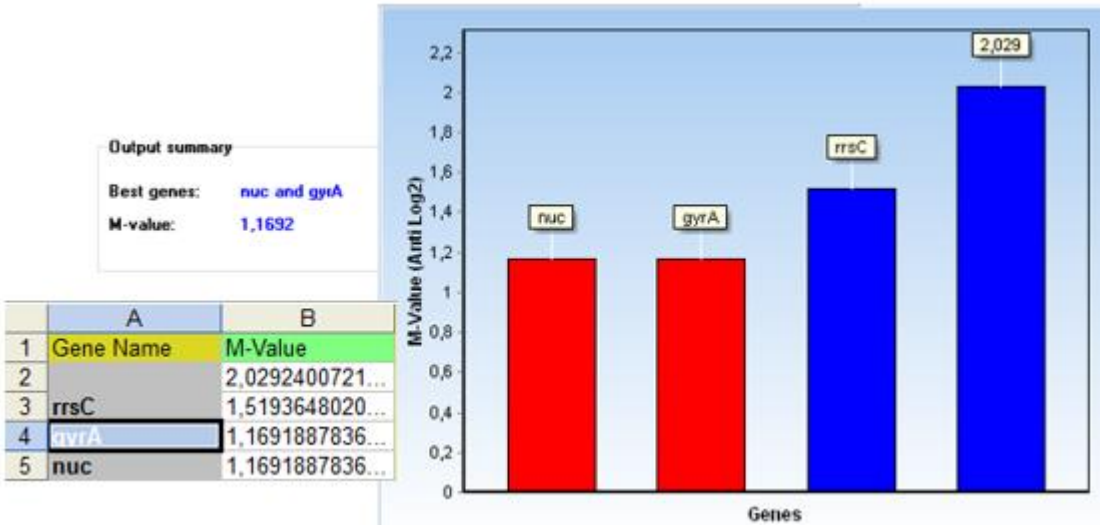
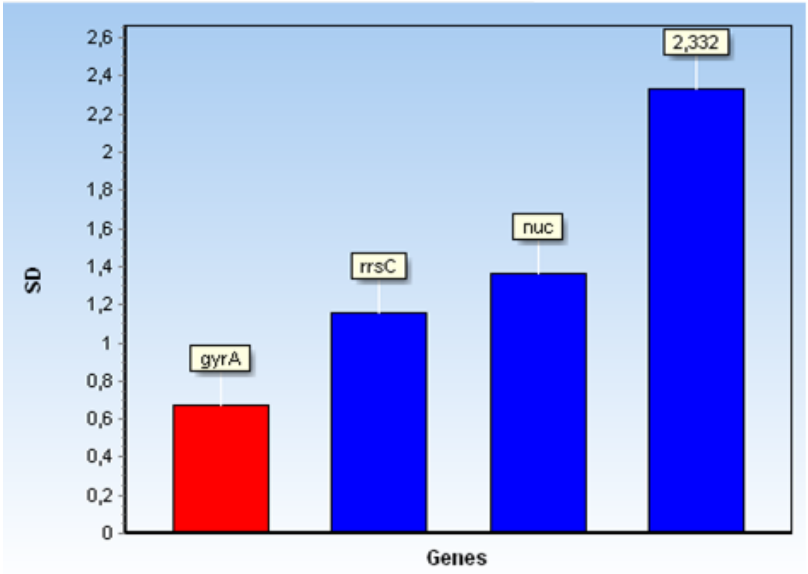


Figure 19 - geNorm algorithm analysis results.

gyrA: gyrase; *nuc*: nuclease; *rrcS:* 16s ribosomal RNA subunit.

6.2.2. NormFinder analysis



	A	B	C	D	E
1	Gene Name	SD	Acc. SD	SD	0,6755
2	gyrA	0,6755	0,6755		
3	rrsC	1,1572	0,67	Best Gene	gyrA
4	nuc	1,3628	0,6371		
5		2,3322	0,7538		

Figure 20 - NormFinder algorithm analysis results.

gyrA: gyrase; *nuc*: nuclease; *rrcS:* 16s ribosomal RNA subunit.

6.3. Calibration curves calculations

Table 12 - Calculation of the gDNA mass necessary for the construction of the calibration curves.

Copy Number (copies/µl)	Genome mass	Necessary gDNA mass (pg)
3 x 10^6	x 0.031784 pg	95352
3 x 10^5		9535.2
3 x 10^4		953.5
3 x 10^3		95.3
3 x 10^2		9.5
3 x 10^1		0.9

6.3.1. Calibration curves qPCR detailed data

6.3.1.1. qPCR results for the *agrI* calibration curve

Table 13 - *agrI* calibration curve data.

Copy Number (copies/µl)	Cq		Cq (mean ± sd)	T_m (ºC)	
	Sample	Duplicate		Sample	Duplicate
3 x 10^6	19.3	19.5	19.4 ± 0.1	76.6	76.6
3 x 10^5	22.6	22.3	22.4 ± 0.2	76.7	76.6
3 x 10^4	25.8	25.7	25.7 ± 0.1	76.6	76.4
3 x 10^3	29.5	32.6	31.1 ± 2.2	76.2	76.4
3 x 10^2	32.8	33.4	33.1 ± 0.4	76.6	76.6
3 x 10^1	n.d.	n.d.	n.d.	76.6	74.5

Cq – quantification cycles; T_m – melting temperature; sd – standard deviation; n.d.: not determined.

6.3.1.2. qPCR results for the *agrII* calibration curve

Table 14 - *agrII* calibration curve data.

Copy Number (copies/µl)	Cq		Cq (mean ± sd)	T_m (ºC)	
	Sample	Duplicate		Sample	Duplicate
3 x 10^6	21.3	23.6	22.4 ± 1.6	71.9	71.9
3 x 10^5	19.5	18.2	18.9 ± 0.8	72.6	72.6
3 x 10^4	20.3	20.2	20.2 ± 0.1	72.6	72.6
3 x 10^3	24.2	23.4	23.8 ±0.5	72.4	72.4
3 x 10^2	30.1	29.2	29.7 ±0.7	72.5	72.5
3 x 10^1	32.8	34.9	33.9 ± 1.5	72.1	72.8

Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.

6.3.1.3. qPCR results for the *agrII* co-culture assay calibration curve

Table 15 - *agrII* in co-culture calibration curve data.

Copy Number (copies/µl)	Cq		Cq (mean ± sd)	T_m (ºC)	
	Sample	Duplicate		Sample	Duplicate
3 x 10^6	17.0	16.6	17.0	72.3	72.2
3 x 10^5	20.2	19.7	20.2	72.5	72.6
3 x 10^4	22.2	21.9	22.2	72.5	72.5
3 x 10^3	27.4	25.7	27.4	72.5	72.6
3 x 10^2	30.7	29.8	30.7	72.8	72.6
3 x 10^1	36.3	36.3	36.3	73.8	74.3

Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.

6.4. qPCR absolute quantification of *agrI* and *agrII* detailed results

6.4.1. *agrI* absolute quantification

Table 16 - *agrI* absolute quantification data.

Isolates	Cq		Cq (mean ± sd)	Copy Number Sample (copies/µl)	Copy Number Duplicate (copies/µl)	Copy number (mean ± sd)	T_m (ºC)	
	Sample	Duplicate					Sample	Duplicate
A 5.2	21.7	21.7	21.7 ± 0.0	17914.0	18392.1	18153.1 ± 338.1	76.5	76.5
A 6.3	29.9	29.3	29.6 ± 0.4	74.6	113.5	94.1 ± 27.5	76.9	77.1
B 13.1	21.6	20.4	21.0 ± 0.9	18968.6	43619.4	31294 ± 17430.7	76.3	76.5
B 14.2	20.8	20.0	20.4 ± 0.6	32033.3	55556.4	43794.8 ± 16633.3	76.5	76.5
Z 1.1	16.7	17.0	16.8 ± 0.2				76.6	76.6
Z 3.1	33.1	33.9	35.0 ± 1.6	1.2	5.4	3.3 ± 3.0	77.1	77.1
Z 5.2	37.1	34.9	36 ± 1.6				77.4	77.4
Z 14.1	31.6	33.0	32.3 ± 1.0	24.8	10.0	17.4 ± 10.5	76.5	76.8
Z 23.2	18.6	17.9	18.3 ± 0.5	144358.9	222878.9	183618.9 ± 55522.0	76.5	76.6
Z 25.2	17.6	17.3	17.4± 0.2				76.5	76.5
Z 27.2	20.6	19.7	20.1 ± 0.7	37788.4	70659.3	54223.8 ± 23243.2	76.8	76.8
Z 27. 3	21.5	21.3	21.4 ± 0.1	19964.1	22785.6	21374.8 ± 1995.1	76.8	76.6
NTC							70.9	69.3

A: aspirate; B: biopsy; Z: swab; NTC – no-template control; Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.

6.4.2. *agrII* absolute quantification

Table 17 - *agrII* absolute quantification data.

Isolates	Cq		Cq (mean ± sd)	Copy Number Sample (copies/µl)	Copy Number Duplicate (copies/µl)	Copy number (mean ± sd)	T_m (ºC)	
	Sample	Duplicate					Sample	Duplicate
A 1.1	22.9	22.9	22.9 ± 0.0	336	332.6	334.3 ± 2.4	72.6	72.7
B 3.2	23.7	23.8	23.8 ± 0.1	197.8	186.1	192 ± 8.3	72.6	72.6
B 3.3	20.9	20.4	20.7 ± 0.4	1201.1	1774.5	1487.8 ± 405.4	72.4	72.4
B 7.3	21.9	20.9	21.4 ± 0.7	636.2	1223.1	929.7 ± 415	72.6	72.4
Z 2.2	25.3	25.3	25.3 ± 0.0	73.1	72.8	72.9 ± 0.2	72.6	72.6
Z 16.1	25.2	24.5	24.8 ± 0.5	78.3	122.2	100.3 ± 31.1	72.6	72.7
Z 21.1	25.6	23.4	24.5 ± 1.5	60.8	246.4	153.6 ± 131.2	72.7	72.7
Z 21.3	20.7	20.8	20.8 ± 0.1	1402.3	1295.6	1348.9 ± 75.4	72.6	72.6
Z 32.2	25	24.9	24.9 ± 0.1	87.6	88.9	88.3 ± 1	72.6	72.6
NTC							87.9	90.9

A: aspirate; B: biopsy; Z: swab; NTC – no-template control; Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.

6.4.3. *agrII* absolute quantification in co-culture

Table 18 - *agrII* in co-culture absolute quantification data.

Suspensions	Cq		Cq (mean ± sd)	Copy Number Sample (copies/µl)	Copy Number Duplicate (copies/µl)	Copy number (mean ± sd)	T_m (ºC)	
	Sample	Duplicate					Sample	Duplicate
A1	23.4	22.8	23.1 ± 0.4	81.5	116.9	99.2 ± 25.1	72.9	72.9
A2	24.4	23.7	24.1 ± 0.5	39.4	66.4	52.9 ± 19.1	72.8	72.8
A3	24.9	24.9	24.9 ± 0.0	28.6	27.8	28.2 ± 0.5	72.9	72.9
A4	25.7	25.3	25.4 ± 0.4	16.4	24.2	20.3 ± 5.5	72.8	72.9
A5	25.3	24.3	24.8 ± 0.8	21.4	44.4	32.9 ± 16.2	72.9	72.9
Control +	27.5	25.3	26.4 ± 1.5	4.9	22.3	13.6 ± 12.2	73.1	73.2
NTC							88.1	90.9

A1: 100 µl *S. aureus* + 900 µl *P. aeruginosa* (1:9); A2: 200 µl *S. aureus* + 800 µl *P. aeruginosa* (2:9); A3: 500 µl *S. aureus* + 500 µl *P. aeruginosa* (5:5); A4: 800 µl *S. aureus* + 200 µl *P. aeruginosa* (8:2); A5: 900 µl *S. aureus* + 100 µl *P. aeruginosa* (9:1); Control +: isolate B 3.2; NTC – no-template control; Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.

6.5. qPCR results for the reference gene

6.5.1. qPCR results for *gyrA* (reference gene) in the *agrI* and *agrII* assays

Table 19 - Reference gene qPCR data.

	Isolates	Cq		Cq (mean±sd)	T_m (ºC)	
		Sample	Duplicate		Sample	Duplicate
***agrI* assay**	**A 5.2**	21.9	21.6	21.7 ± 0.1	78.6	78.6
	A 6.3	15.4	14.6	14.9 ± 0.4	78.6	78.4
	B 13.1	18.6	18.3	18.5 ± 0.1	78.4	78.4
	B 14.2	21.2	21.2	21.2 ± 0.0	78.3	78.3
	Z 1.1	16.5	16.5	16.5 ± 0.0	78.4	78.4
	Z 3.1	21.7	21.4	21.6 ± 0.2	78.4	78.4
	Z 5.2	21.6	21.5	21.5 ± 0.1	78.4	78.4
	Z 14.1	15.3	15.3	15.3 ± 0.0	78.6	78.6
	Z 23.2	21.2	20.4	20.8 ± 0.5	78.4	78.4
	Z 25.2	15.8	15.7	15.8 ± 0.1	78.4	78.4
	Z 27.2	20.6	20.6	20.6 ± 0.0	78.3	78.6
	Z 27. 3	20.4	20.3	20.3 ± 0.0	78.6	78.6
	NTC				61.5	61.7
***agrII* assay**	**A 1.1**	24.1	23.4	23.7 ± 0.5	77.9	77.9
	B 3.2	20.5	20.4	20.5 ± 0.1	77.9	77.9
	B 3.3	21.8	21.4	21.6 ± 0.3	77.8	77.8
	B 7.3	25.5	24.2	24.8 ± 0.9	77.8	77.8
	Z 2.2	24.6	23.6	24.1 ± 0.7	77.9	77.9
	Z 16.1	18.4	17.9	18.1 ± 0.4	77.9	77.9
	Z 21.1	23.7	22.8	23.3 ± 0.6	78.1	78.1
	Z 21.3	21.9	21.7	21.8 ± 0.2	78.1	78.1
	Z 32.2	24.4	24.4	24.4 ± 0.0	77.9	77.9
	NTC				61.7	61.7

A: aspirate; B: biopsy; Z: swab; NTC – no-template control; Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.
Note: isolates Z 16.2 and Z 17.2 are not included, since no *agr* type was determined.

6.5.2. qPCR results for *gyrA* (reference gene) in the *agrII* co-culture assay

Table 20 - Reference gene qPCR data for the co-culture assay.

Suespensions	Cq		Cq (mean ± sd)	T_m (ºC)	
	Sample	Duplicate		Sample	Duplicate
A1	23.8	23.28	23.5 ± 0.4	77.8	77.8
A2	23.9	22.7	23.3 ± 0.9	77.8	78.0
A3	20.9	20.9	20.9 ± 0.0	77.7	77.7
A4	22.9	22.2	22.5 ± 0.5	77.7	77.7
A5	23.4	22.6	23.0 ± 0.6	77.8	77.8
Control +	24.7	24.4	24.6 ± 0.2	78.1	78.1
NTC				61.9	61.8

A1: 100 µl *S. aureus* + 900 µl *P. aeruginosa* (1:9); A2: 200 µl *S. aureus* + 800 µl *P. aeruginosa* (2:9);
A3: 500 µl *S. aureus* + 500 µl *P. aeruginosa* (5:5); A4: 800 µl *S. aureus* + 200 µl *P. aeruginosa* (8:2);
A5: 900 µl *S. aureus* + 100 µl *P. aeruginosa* (9:1);
Control +: isolate B 3.2; NTC – no-template control; Cq – quantification cycles; T_m – melting temperature; sd – standard deviation.

6.6. Accepted abstracts to MicroBiotec 2015

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

Matias, C., van-Harten, S., Mottola, C., Tavares, L., Oliveira M.

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

Diabetes *mellitus* constitutes a significant health problem. One of the most important complications of diabetes consists in foot ulceration, which can be colonized by a multiplicity of microorganisms, being *Staphylococcus aureus* (*S. aureus*) one of the most frequent ones. *S. aureus* produces a variety of virulence factors controlled in their majority by a Quorum-sensing system, encoded by the accessory gene regulator (*agr)* system. Currently four distinct genetic *agr* groups have been established (*agr*-I, *agr*-II, *agr*-III and *agr*-IV).

The Quorum-sensing system of a collection of 23 representative *S. aureus* isolates from diabetic foot ulcers was characterized. The screening of *agr* type was performed resorting to PCR and the gene copy number determined by absolute quantification with real-time quantitative PCR (qPCR).

It was possible to detect *agrI* and *agrII* in 52.2% (n=12) and 39.1% (n=9) of the isolates, respectively. In two isolates (8.7%) it wasn't possible to identify any *agr* type, and *agr* types III and IV were also not detected. Copy number obtained for *agrI* ranged from 7.1 to 94279 copies of total gDNA and for *agrII* ranged from 72.9 to 1487.9 copies of total gDNA.

In conclusion, it was possible to demonstrate that *agr* type vary among DFU isolates and that *agrI* shown a higher variance between number of copies, in comparison with *agrII*, which may be related with a higher production of virulence factors. The characterization of the *agr* system in staphylococci isolated from DFU can have a key role for the establishment of therapeutic protocols, once *agr* has been suggested as a therapeutic target.

Polymicrobial interactions result in variations of *agrII* copy numbers in *Staphylococcus aureus* from diabetic foot ulcers

Matias, C., van-Harten, S., Mottola, C., Tavares, L., Oliveira M.

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

Diabetic foot ulcers (DFU) are usually colonized by several microorganisms that interact with each other, forming complex polymicrobial communities. Bacteria present in these communities interact through Quorum-sensing (QS), defined as a communication system activated by an increase in population density, allowing bacteria to share information and synchronize gene expression and responding collectively to environmental changes.

Two of the microorganisms more frequently co-isolated from DFU are *Staphylococcus aureus* and *Pseudomonas aeruginosa.* In *S. aureus*, QS is encoded by the accessory gene regulator *agr*, a classical autoactivation system located in the bacterial chromosome. Currently, four distinct genetic *agr* groups have been established, classified as *agr*-I, *agr*-II, *agr*-III and *agr*-IV.

In vitro co-culture studies suggest that *P. aeruginosa* multiplies faster than *S. aureus,* acting as its antagonist. As selective pressure can be responsible for alterations in copy number of genes in other organisms, the aim of this study was to evaluate the occurrence of alterations in *agr* copy numbers in *S. aureus* present in polymicrobial DFU infections. 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 using 0.5 MCFarland cultures, incubated for 24h/37ºC. The *agrII* copy number in all suspensions was determined by absolute quantification using a real-time quantitative PCR protocol developed by our research team. As control, the copy number of *agrII* present in a *S. aureus* suspension was also determined. Assays were performed in triplicate.

The copy number obtained in the control suspension was 13.6 of total gDNA. For the dual suspensions, copy numbers obtained ranged from 20.3 to 99.2 copies of *agrII*/µl of total gDNA, being observed that the higher copy numbers were obtained in the 1:9 suspensions. This number decreased inversely with the increase of *S. aureus* concentration in the dual suspensions.

Results show that variations in gene copy numbers can also be observed in bacteria. This event can be related with adaptive potential, increasing their survival under selective pressure, as already described for yeasts and tumor cells.

6.7. UpToDate permission for image use

Wolters Kluwer

UpToDate
230 Third Avenue
Waltham, MA 02451

781 392 2000 tel
www.wolterskluwerhealth.com

19 August 2015

Carina Matias
University of Lisbon
Avenida Rovisco Pais 1
Lisbon, Portugal 1049-001
Phone: +351927917366
Email: carinasofiam@gmail.com

Dear Ms. Matias,

Figures: Full thickness diabetic foot ulcer and claw toe (64087)
Topic: McCulloch DK. Patient information: Foot care in diabetes *mellitus* (Beyond the Basics).

UpToDate, Inc. received your reprint request and we are pleased to give you permission to the non-exclusive right to reproduce and publish this material from its database in your master thesis entitled, "Characterization of the Quorum-sensing system of *Staphylococcus aureus* from diabetic foot ulcers" (September 2015).

The fee for this request has been waived and the rights to reproduce this content are subject to the following conditions:

- You must publish a proper copyright notice and attribution to UpToDate, Inc. as the source in your work. The UpToDate copyright notice should appear next to or on the same page as the figure and should read:

  Reproduced with permission from: McCulloch DK. Patient information: Foot care in diabetes *mellitus* (Beyond the Basics). In: UpToDate, Post TW (Ed), UpToDate, Waltham, MA. (Accessed on [DATE].) Copyright © 2015 UpToDate, Inc. For more information visit www.uptodate.com.

- The copyright notice should also include a reference to UpToDate's website http://www.uptodate.com. See above.

- Your rights are limited to **this print-based media and companion CD only** and the UpToDate material may not be reproduced in any additional print or electronic (including internet) publishing usage with the prior written consent of UpToDate, Inc.

- The permission is valid for one-time use or life of an edition only.
- Permission granted is non-exclusive, and is valid throughout the world in the English language
- If the material is being printed for distribution during a lecture or course, it should be printed directly from the UpToDate program. Cards should not be sold or included in a course packet that is for sale, instead they should be given free of charge.

Thank you again for your request to reproduce material from UpToDate. If you have any questions, please do not hesitate to contact me.

Sincerely,

Jason Davis

Jason Davis, Journals and Rights Manager

Wolters Kluwer Health | UpToDate

230 Third Ave | Waltham, MA 02451 | United States

Phone: 781.392.2005 | Fax: 781.370.4793 | Email: J.Davis0@wolterskluwer.com