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Characterization of methicillin-resistant *Staphylococcus aureus* in commercial and wild rabbits (*Oryctolagus cuniculus*) and immunological evaluation of a paternal line of commercial rabbits. Study of the pathogen-host interaction

TESIS DOCTORAL

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En Alfara del Patriarca, a_____de_____de_____.

Los Directores de la Tesis.

Fdo. Dr. D. Juan Manuel Corpa Arenas Fdo. Dra. Dña. Laura Selva Martínez

A mi madre

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“No hay nada más maravilloso que ser un científico, en ninguna parte preferiría estar más que en mi laboratorio, manchando mi ropa y cobrando por jugar.”

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SUMMARY

SUMMARY

Staphylococcus aureus is an important pathogen that is well able to develop resistance to antibiotics. One of the most remarkable clones to have spread worldwide and caused pandemic outbreaks is methicillin-resistant *Staphylococcus aureus* (MRSA), a dangerous strain that is frequently multiresistant to antibiotics. In this work, we studied the pathogen *S. aureus* by focusing on MRSA strains. We evaluated the presence of *S. aureus* on rabbit farms and in wild rabbits before carrying out an in-depth study of the isolated clones through their sequencing. Then we studied a frequent host of this pathogen, the rabbit (*Oryctolagus cuniculus*), to which this bacterium is highly adapted and is, therefore, a very useful model of infections by this pathogen. We evaluated the immune system of commercial rabbits at remarkable time points of the productive cycle and then performed experimental infection to evaluate the immune system when it faces an infectious challenge. First, an unexpected large number of MRSA strains from infrequent lesions were found on rabbit farms. This is the first time that *mec*C-MRSA strains isolated from rabbit farms are described. The MRSA strains isolated from rabbit farms showed limited genetic diversity, with ST2855 being the most prevalent clone. The majority of the tested MRSA isolates were multidrug-resistant. Second, a high percentage of *S. aureus* carriers was detected in wild rabbits and hares hunted in high-density areas in east Spain, and the ear was the main ecological niche where *S. aureus* was isolated. Another finding was the marked presence of ST1945 MRSA of the CC130 lineage containing the *mec*C gene in these animals. These strains harboured mobile genetic element SCC*mec* type XI. Therefore, farm rabbits and wild rabbits can act as a reservoir for MRSA strains being transmitted in nature. As a result of sequencing mobile genetic element SCC*mec*, we describe two new SCC*mec* cassette types: one containing the *mec*C gene and another simultaneously containing *mec*A and *mec*C genes. Two of these *mec*A-*mec*C-MRSA strains had the SCC*mec* excised from the genome. We also observed that each SCC*mec* type was associated with certain STs. The phenotypic resistance profile revealed that strains had an

antibiotic resistance profile depending on whether they had methicillin-resistance gene *mec*A or *mec*C. Nevertheless, simultaneously carrying the two genes offered no more advantages than having only the *mec*A gene in the resistance profile to the tested antibiotics. Finally, after evaluating the immune system by flow cytometry at main time points of the productive cycle of commercial rabbits the genetic selection by average daily gain did not affect rabbit females' ability to mount immune response. After evaluating lesions and immune system evolution during an experimental infection by *S. aureus* in skin, the results evidenced that this breeding programme favoured the immune system's capability to undergo an infectious challenge with *S. aureus*. To evaluate vitrification effects, a group of animals obtained from vitrified embryos was compared to another group obtained by routine artificial insemination. A long-term vitrification effect on the immune response to infection with *S. aureus* was observed insofar as the animals from the restored vitrificated population presented better performance during infection.

RESUMEN

RESUMEN

Staphylococcus aureus es un patógeno importante con gran capacidad de desarrollar resistencia a los antibióticos. Uno de los clones más notables que se ha extendido por todo el mundo y ha provocado brotes pandémicos es el *Staphylococcus aureus* resistente a la meticilina (MRSA), una cepa peligrosa que con frecuencia es multirresistente a los antibióticos. En este trabajo, estudiamos el patógeno *S. aureus* centrándonos en las cepas MRSA. Evaluamos la presencia de *S. aureus* en granjas de conejos y en conejos silvestres antes de realizar un estudio en profundidad mediante su secuenciación de los clones aislados. Luego se estudió un hospedador frecuente de este patógeno, el conejo (*Oryctolagus cuniculus*), al que esta bacteria está muy adaptada y es, por tanto, un modelo muy útil de infecciones por este patógeno. Evaluamos el sistema inmunológico de conejos comerciales en momentos notables del ciclo productivo y luego realizamos una infección experimental para evaluar el sistema inmunológico cuando se enfrenta a un desafío infeccioso. En primer lugar, se encontró una cantidad inesperada de cepas de MRSA de lesiones poco frecuentes en granjas de conejos. Además, es la primera vez que se describen cepas *mec*C-MRSA aisladas de granjas de conejos. Las cepas de MRSA aisladas de granjas de conejos mostraron una diversidad genética limitada, siendo ST2855 el clon más prevalente. La mayoría de los aislados de MRSA probados fueron multirresistentes. En segundo lugar, se detectó un alto porcentaje de portadores de *S. aureus* en conejos y liebres silvestres cazados en zonas de alta densidad del este de España, siendo la oreja el principal nicho ecológico donde se aisló *S. aureus*. Otro hallazgo fue la marcada presencia de clones ST1945 MRSA del linaje CC130 que contienen el gen *mec*C en estos animales. Estas cepas albergaron el elemento genético móvil SCC*mec* tipo XI. Por lo tanto, los conejos de granja y los conejos silvestres pueden actuar como reservorios de cepas MRSA que se transmiten en la naturaleza. Como resultado de la secuenciación del elemento genético móvil SCC*mec*, describimos dos nuevos tipos de casetes de SCC*mec*: uno que contiene el gen *mec*C y otro que contiene

simultáneamente los genes *mec*A y *mec*C. Dos de estas cepas *mec*A-*mec*C-MRSA tenían además el SCC*mec* escindido del genoma. También observamos que cada tipo de SCC*mec* estaba asociado con ciertos ST. El perfil de resistencia fenotípica reveló que las cepas tenían un perfil de resistencia a antibióticos dependiendo de si tenían el gen *mec*A o *mec*C de resistencia a la meticilina. Sin embargo, llevar simultáneamente los dos genes no evidenció más ventajas que tener solo el gen *mec*A en el perfil de resistencia a los antibióticos probados. Finalmente, tras evaluar el sistema inmunológico por citometría de flujo en los puntos principales del ciclo productivo de los conejos comerciales, la selección genética por ganancia diaria promedio no afectó la capacidad de las hembras de conejos para generar una respuesta inmune. Después de evaluar las lesiones y la evolución del sistema inmunológico durante una infección experimental por *S. aureus* en piel, los resultados evidenciaron que este programa de mejora genética favoreció la capacidad del sistema inmunológico al sufrir un desafío infeccioso con *S. aureus*. Para evaluar los efectos de la vitrificación, se comparó un grupo de animales obtenidos de embriones vitrificados con otro grupo obtenido mediante inseminación artificial rutinaria. Se observó un efecto a largo plazo de la vitrificación sobre la respuesta inmune a la infección por *S. aureus* en la medida en que los animales de la población vitrificada restaurada presentaron un mejor comportamiento durante la infección.

INDEX OF ABBREVIATIONS

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AAC	Aminoglycoside acetyltransferases
AAD	Aminoglycoside adenyltransferases
ACME	Arginine catabolic mobile element
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APH	Aminoglycoside phosphotransferases
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CAT	Chloramphenicol acetyltransferase
CC	Clonal complex
CFU	Colony forming units
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
ClfA	Clumping factor A
CoPS	*Staphylococcus* coagulase positive
CoNS	*Staphylococcus* coagulase negative
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DLV	Double locus variant
DNA	Deoxyribonucleic acid
FnbpA	Fibronectin binding protein A
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
IEC	Immune evasion cluster
kb	Kilobases
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LDM	Leukocyte differentiation molecules
mAb	Monoclonal antibody
mg	Milligrams

MGE	Mobile genetic element
MHC	Major histocompatibility complex
ml	Milliliters
MLST	Multilocus sequence typing
mm	Millimeters
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NADPH	Nicotinamide adenine dinucleotide phosphate
NLR	NOD-like receptors
ºC	Degrees Celsius
PAMP	Pathogen-associated molecular pattern
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PGRP	Peptidoglycan recognition protein
PIA	Polysaccharide intercellular adhesin
PMN	Polymorphonuclear leukocyte
PSM	Phenol-soluble modulin
PRR	Pattern recognition receptor
PVL	Panton-Valentine leucocidin
RFLP	Restriction fragment length polymorphism
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SaPI	*Staphylococcus aureus* pathogenicity island
SAG	Superantigenic toxin
SAK	Staphylokinase
SCC	Staphylococcal cassette chromosome
SCIN	Staphylococcal complement inhibitor

Sdr	Serine-Aspartate Repeat Protein
SE	Staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEC	Staphylococcal enterotoxin C
SLV	Single locus variant
SNP	Single nucleotide polymorphism
SpA	Staphylococcal protein A
ST	Sequence type
TCR	T cell receptor
TCS	Two-component system
Th	Helper T lymphocytes
TLR	Toll-like receptor
TMP-SMX	Trimethoprim-sulfamethoxazole
TNF	Tumor necrosis factor
TSB	Tryptic soy broth
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin 1
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant *Enterococcus faecalis*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

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INTRODUCTION

INTRODUCTION

In the development of diseases, the interaction established between pathogens and hosts is decisive in determining their course. In the case of *Staphylococcus aureus*, the rabbit, in addition to humans, is one of the hosts to which this bacterium is very well adapted and therefore the interaction of *S. aureus* in infections in rabbits is a very representative model of the pathogenesis of this bacterium. Within this bacterium-host binomial, we are going to start talking about the pathogen, to continue with the interaction between both and finally describe the host that has been used as a model in this study.

1. The pathogen: *Staphylococcus aureus*

The *S. aureus* bacteria was discovered in 1880 by the doctor Alexander Ogston when he observed spherical bacterial forms in clustered clusters, although Pasteur and Koch had previously observed it. Since then, it has been considered one of the most important pathogens due to its potential to produce multiple infections in humans and animals. *S. aureus* is a member of the Staphylococcaceae family (***Table 1***), it is considered the type species within its group and the most virulent due to the diversity and severity of diseases it produces and its geographical distribution is worldwide.

Rank	**Specific name**
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Baciallales
Family	Staphylococcaceae
Genus	Staphylococcus
Species	*Staphylococcus aureus*

Table 1. Taxonomy of *Staphylococcus aureus*

1.1. The Staphylococcus genus

Staphylococci are gram-positive bacteria usually living as commensals on the skin of mammals and birds. Forty-one species and several subspecies have been catalogued into the *Staphylococcus* genus. *S. aureus* and *S. epidermidis* are the most important from a human and animal health perspective.

The first division that is made between the species that make up this genus is based on the presence of the enzyme coagulase that is responsible for the passage of fibrinogen to fibrin, resulting in blood clotting (Devriese et al., 2005). As a result of this classification, we have the *Staphylococcus* coagulase positive (CoPS) and the coagulase negative (CoNS). The CoNS group is broader and more heterogeneous, however in the case of CoPS only seven species have been identified: *Staphylococcus aureus, S. intermedius, S. schleiferi subsp. coagulans, S. hyicus, S. lutrae, S. delphini*, and *S. pseudintermedius*.

1.2. General characteristics of *Staphylococcus aureus*

Staphylococcus aureus is gram-possitive cocci, which can appear in pairs, in chains or in clusters. Clustering of cocci is promoted by growth on solid medium; on occasion, the clusters may be asymmetrical. *S. aureus* is non motile, do not form spores or resting states, its size ranges from 0.5 to 1.5 microns of diameter, and some strains produce a mucoid outer capsule that increases its ability to cause infection, however, most strains produce only a microcapsule and the colonies appear non-mucoid (O'Riordan and Lee, 2004). This organism is distinguished from other staphylococcal species because of the gold pigmentation of colonies composed of carotenoids. *S. aureus* is capable of growing in a wide range of pH and temperatures and in saline solutions with a proportion up to 15% sodium chloride. *S. aureus* is part of the bacterial microbiota existing in the skin and mucous membranes of man and animals (primates, ungulates, carnivores, rodents, lagomorphs, marsupials and birds) (Williams, 1963; Kloos, 1980). Besides, it survives for weeks in carcasses, in the tissues and organs of animals

(meat) and, for days, on the skin, in the ground and on the surface of metal and glass objects. Colonies of *S. aureus* are β-hemolytic due to the production of several hemolysins: α-toxin, β-toxin, γ-toxin, and δ-toxin (Tegmark et al., 1998) (***Figure 1***).

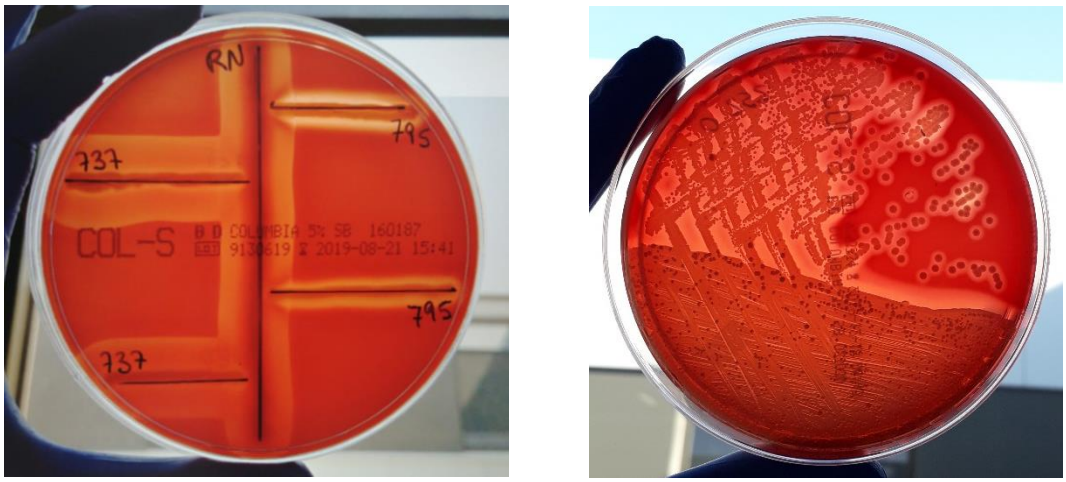

Figure 1. Hemolysis produced on blood agar by different strains of *S. aureus.*

In relation to its metabolism *S. aureus* is facultative anaerobic, coagulase positive (it produces a positive coagulase reaction in rabbit plasma), catalase positive (a feature that distinguishes them from the streptococci and enterococci, although occasional reports of catalase-negative strains have been described (Millar et al., 1986; Del'Alamo et al., 2007; Grüner et al., 2007), oxidase negative and it uses carbohydrates for their metabolism both by oxidation and by fermentation (Branson, 1968; Lowy, 1998). When grown on mannitol salt agar (media containing a pH-based indicator), the fermentation of mannitol that *S. aureus* produces generate a yellow zone around the colony in the red agar. In addition to mannitol, *S. aureus* can metabolize glucose, xylose, lactose, sucrose, maltose, and glycerol (Somerville and Proctor, 2009).

1.3. *Staphylococcus aureus* identification techniques

Due to the pathogenic potential of *S. aureus*, the comprehension of the population structure and evolutionary dynamics of the pathogenic strains and the identification of particularly successful clonal lineages it is a very important tool for the control and prevention of infections produced by this

pathogen. The first step is to discriminate the *S. aureus* isolates from other species of the genus; for this, various phenotypic and molecular techniques have been developed over the years to differentiate the species *S. aureus*, and within it the different bacterial strains and their phylogenetic relationships.

The previously mentioned mannitol salt agar does not discriminate all species of the genus *Staphylococcus*, but a negative reaction allows us to rule out species such as *S. epidermidis*, which produces white colonies on this agar with no color change. Recently, various types of commercial media have been described, some of which can also differentiate methicillin-resistant *S. aureus* (MRSA) from methicillin-sensitive *S. aureus* (MSSA) (Perry et al., 2004) (***Figure 2***); they are more expensive but highly specific for presumptive identification.

Figure 2. Medium Thermo Scientific™ Brilliance™ MRSA 2/Brilliance™ Staph 24 that discriminates methicillin resistant *S. aureus* (MRSA) and coagulase positive staphylococci.

Since the development of molecular diagnosis, there are several genotypic identification methods, such as those based on 16s rRNA sequencing and real-time polymerase chain reaction (RT-PCR)-based diagnostic platforms.

One of the most outstanding discoveries was the development of Pulse Field Gel Electrophoresis (PFGE) method during the mid to late 1980s. This method employs restriction enzymes that recognize an informative number of sites that are used to generate large fragments that are resolved by electrophoresis (Schwartz and Cantor, 1984; Goering, 1993), thereby generating a strain-specific restriction fragment length polymorphism (RFLP) banding pattern. This method can detect variation between closely related strains due to point mutations, insertions, and deletions resulting in loss or gain of individual restriction sites and alteration of fragment sizes. Therefore is still the method of choice for comparing closely related strains in which is highly discriminative. The enzyme of choice in the case of *S. aureus* is *Sma*I because it recognizes GC-rich sequences, and staphylococcal species have low GC content (Poddar and McClelland, 1991; Linhardt et al., 1992; Pantůček et al., 1996). Despite its usefulness the difficulties associated with interlaboratory comparisons, the difficulty to perform PFGE on large number of samples, have led to search for alternative methods for large-scale genotyping.

Since 1998, the multilocus sequence typing (MLST) has become the gold standard for describing the population structure of prokaryotes (Maiden et al., 1998; Enright et al., 2000). It is a DNA sequence-based method that employ seven conserved housekeeping genes that in the case of *S. aureus* are *arc* (carbamase kinase), *aro*E (shikimate 5-dehydrogenase), *glp*F (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triose phosphate isomerase) and *yqi*L (acetyl-Coa acetyltransferase). Each unique sequence recovered for a particular gene is denoted as a specific allele and the concatenation of a given allelic profile is assigned a particular sequence type (designed ST followed by a number). The variation in the sequence types is a result of point mutations within these genes (Feil et al., 2003) and allows the determination of macro or long-term genetic variation and evolution. This method allows to assess the highly clonal population structure of *S. aureus* (Robinson and Enright, 2004). The *S. aureus* MLST database is one of the largest of bacteria

database. The single nucleotide polymorphisms (SNPs) are described as single-locus variants (SLVs) or double-locus variants (DLVs). The sequence type with the greatest number of SLVs is assumed to represent the 'founder' of a given cluster, with large clusters termed 'clonal complexes' (designated CC followed by a number).

Taken together, PFGE and MLST represent give information about different aspects of the genotypic diversity: MLST can capture the large-scale population structure of an entire species but is unable to discriminate between closely related isolates, whereas PFGE is the gold standard for indexing microvariation yet is unable to assess the overall population structure. To find a middle point between the two methods the *spa*-typing emerged (Shopsin et al., 1999; Harmsen et al., 2003).

The *spa*-typing is a single-locus sequence typing method based on sequencing of a hypervariable repeat region within the coding sequence of protein A. This technique is useful for indexing microvariation and macrovariation of the strains and is being used for the analysis of the global epidemiology of *S. aureus* as well as for strain comparison in infection control and localized geographic studies.

Other method consisting of double-locus sequence typing includes two families of surface proteins in the variable genome of *S. aureus*, which are *spa* (Koreen et al., 2004), as in the *spa*-typing, and *clf*B (clumping factor B) (Koreen et al., 2005). This method combines sequencing of repeats in both genes based on sequence variation of variable repeat regions extending from the cell surface.

1.4. Cell wall, cell membrane and surface proteins

The cell wall is made up of 50 percent peptidoglycan, approximately 30 percent theicoic acid and polysaccharides, and contains the high osmotic pressure of the cytoplasm of staphylococci. The peptidoglycan may have endotoxin-like activity, stimulating the release of cytokines by macrophages, activation of complement, and aggregation of platelets. Differences in the

peptidoglycan structure of staphylococcal strains may contribute to variations in their capacity to cause disseminated intravascular coagulation (Kessler et al., 1991). Theicoic acids are approximately 30% of the dry cell wall weight. They take part in cell division, autolytic activity, and biofilm formation (Jorasch et al., 2000).The polysaccharide intercellular adhesin (PIA) is required for the maturation of biofilms (Heilmann et al., 1996) and is important for evasion of the host innate immune system (Vuong et al., 2004a and 2004b).

Penicillin-binding proteins (PBPs) are involved in the biosynthesis of cell wall peptidoglycan; they catalyze the last step of peptidoglycan biosynthesis (Frère et al., 1992). PBPs are of particular importance because MRSA strains have acquired resistance to all β-lactam antibiotics due to a modified PBP2 called PBP2a, which is capable of producing cell wall biosynthesis in the presence of high concentrations of antibiotics due to its low affinity for β-lactam antibiotics (Hartman and Tomasz, 1984; Reynolds and Brown, 1985).

Most staphylococci produce microcapsules made of polysaccharide that increases its adherence capacity, prevents it from being recognized, as well as reinforces the anti-phagocytic effect (Nilsson et al., 1997).

S. aureus has a large array of surface proteins and carbohydrates that enable binding to a broad range of host tissues, including platelets, epithelial cells, endothelial cells and intercellular matrix proteins (Harraghy et al., 2006). Some of the most important *S. aureus* surface proteins are protein A, coagulase (a prothrombin activator that converts fibrinogen to fibrin), and the clumping factor. The synthesis of many of the surface proteins is dependent on the growth phase and is controlled by regulatory genes such as *agr*. These proteins are usually synthesized during the exponential-growth phase. However, many of the secreted proteins are synthesized in the stationary phase, such as Enterotoxin B, TSST-1 (toxic shock syndrome toxin 1) and α-Toxin. The *agr* gene is a global virulence regulator that induces the expression of exoprotein (extracellular protein) while

suppressing the expression of surface protein. This sequential expression of genes may have clinical importance. In the early stages of infection, activation of surface proteins allows host tissues to be successfully colonized while exoprotein synthesis allows the spread to adjacent tissues. This hypothesis is supported by studies in animals showing that the inactivation of regulatory genes reduces bacterial virulence (Cheung et al., 1994). Nearly all strains secrete a group of enzymes and cytotoxins that have the main function of converting local host tissues into nutrients required for bacterial growth, destroy tissue and they have very diverse mechanisms of action. Some of these enzymes and cytotoxins are four hemolisins (alpha, beta, gamma and delta), nucleases, lipases, proteases, hyaluronidase and collagenase. Some strains produce other additional exoproteins, some of which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI), and leucocidin. Their primary function in vivo is to inhibit host immune responses to *S. aureus*, thus these toxins have potent effects on cells of the immune system, but many of them have other biological effects as well.

1.5. Genomes

Genome sequencing has enabled investigators to explore questions of virulence, resistance, physiology, and host interactions; and has provided insights into their success as significant bacterial pathogens. The first *S. aureus* genome sequences were published in 2001 comparing the genomes of two methicillin-resistant strains, N315 and Mu50 (Kuroda et al., 2001). Subsequently many other genomes of *S. aureus* were published as MW2 (Baba et al., 2002), MRSA252 and MSSA476 (Holden et al., 2004), COL (Gill et al., 2005), USA300 (Diep et al., 2006), ET3-1 (Herron-Olson et al., 2007), JH1 and JH9 (Mwangi et al., 2007), and Newman (Baba et al., 2008).

The *S. aureus* genome consists of a circular haploid chromosome of approximately 2.8 to 2.9 Mb encoding 2563-2721 proteins (Kuroda et al., 2001; Baba et al., 2002; Diep et al., 2006; Herron-Olson et al., 2007) which

contains two distinct regions. The first region called core genome, represent approximately 80% of the bacterial genome and contains conserved genes that encode essential metabolic and regulatory functions, surface proteins with roles in tissue adhesion and surface architecture as well as virulence-associated factors that are expressed by nearly all strains, such as protein A (*spa*), clumping factor (*clfAB*), coagulase (*coa*), fibrinogen-binding protein A (*fnbA*), α-toxin (*hla*), lipase (*lip*), and superoxide dismutase (*sodM*). A region called "*ori*C environ" situated downstream from the origin of replication includes important *S. aureus* genes such as *spa* (encoding protein A) and *coa* (encoding coagulase) and is thought to be related with the evolution and differentiation of the staphylococcal species. Phylogenetic classification indicated that over 50% of predicted proteins encoded by the *S. aureus* genome are most similar to those in *Bacilllus subtilis* and *Bacillus halodurans* (Kuroda et al., 2001). Blast homology search for the most closely related orthologues indicates that the genetic background of *S. aureus* has been vertically transmitted from a common ancestor that subsequently diverged to *Bacillus* and *Staphylococcus* species (Ito et al., 2003). The other region called accessory genome (dispensable genetic material), performs about 20% of the genome and is composed of other variable genes that often show variation associated with lineage. These variable regions may be more prone to diversifying events such as single nucleotide polymorphisms (SNPs), partial or complete gene deletions likely by recombination events, and repeat variation. This is the region with the greatest interest for the study and is mainly composed of MGEs that are integrated throughout the genome and carry about 50% of *S. aureus* virulence factors.

The MGEs of *S. aureus* are pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids and transposons. These MGEs play significant roles in the evolution of *S. aureus* virulence and emergence of new strains with clinical implications because they are regions of the genome that undergo continuous mutations (Lowy, 1998; Baba et al., 2008; Chen et al., 2018). The MGEs can transfer horizontally

between strains differentially, from high frequency to low frequency, while some do not transfer at all. In general, there are three mechanisms of lateral gene transfer in bacteria: conjugation, transformation, and transduction (Thomas and Nielsen, 2005). The horizontal gene transfer (HGT) is of considerable importance in medicine because is the major route by which bacteria acquire virulence factors and antibiotic resistance. MGE-mediated movement of toxin genes is associated with particular lineages that may dominate in carriage and disease (Moore and Lindsay, 2001; Peacock et al., 2002). Comparative analysis of the S. *aureus* genome sequences has given evidence of substantial homologous recombination within MGEs, such that each MGE is composed of multiple short mosaic fragments that are randomly spread through other MGEs of the same type (phage or SaPI). MGEs strongly associated with a particular lineage are presumable stable and transmitted by vertical transmission to next generation bacterial cells. In contrast, MGEs that are randomly distributed among multiple lineages are likely to be transferred horizontally. Combinations of virulence factor carried by the mobile genetic elements (MGEs) and their exchange among strains results in evolution of clones able to infect selected hosts and cause specific diseases (Herron-Olson et al., 2007).

The former virulence and resistance genes tend to be found in *S. aureus* pathogenicity islands (SaPI) whereas the latter rely on SCC, plasmids and transposons for transfer.

1.5.1 Bacteriophages

Staphylococcus aureus harbor temperate and lytic bacteriophages (Kwan et al., 2005) that play key roles in mobilization of virulence factors between strains and genome evolution of virulent isolates. Bacteriophages are the most abundant gene-transfer particles, and phage transduction is generally regarded as the most important mechanism of horizontal gene transfer between bacterial cells. A recent study reports that *S. aureus* temperate bacteriophages engage in a form of transduction different from HGT termed

lateral transduction (Chen et al., 2018) and it can be a universal mechanism of gene transfer present in other bacterial species.

There are two groups of *S. aureus* bacteriophages that are likely responsible for the widespread horizontal gene transfer between strains; they are the generalized transducing phage (Iandolo et al., 2002) and the temperate phage associated with SaPI (Novick and Subedi, 2007; Tallent et al., 2007). The SaPI element encodes proteins that remodel the phage capsid to accommodate the smaller SaPI genome (Tormo et al., 2008).

A subgroup of temperate *S. aureus* bacteriophages produces the inactivation of virulence genes by inserting into these genes, this phenomenon is called lysogenic conversion. An example is the serogroup F bacteriophages which insert into and inactivate the β-hemolisyn (*hlb*) gene. The Hlb-converting phages are most frequently associated with clinical isolates of *S. aureus*. Phages encoding immune evasion molecules (SCIN, CHIP) which integrate specifically into the *hlb* gene in *S.aureus*, are widely distributed. The dynamics of these phages during host infection lead to the splitting of phage population into two populations exhibiting different virulence potentials: phages that produce Hlb and phages that produce immune evasion molecules (SCIN and CHIP) (Goerke et al., 2006).

Another mechanism that leads to the emergence of virulent *S. aureus* clinical isolates is the mobilization of bacteriophages encoding specific virulence factors. For example, Panton-Valentine leucocidin (PVL) is encoded by bacteriophages from Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates associated with soft tissue infections and necrotizing pneumonia in children (Gillet et al., 2002).

1.5.2. Pathogenicity islands

Pathogenicity islands (SaPIs) have a general genomic organization extremely conserved and like that of temperate phages. They also depend on staphylococcal phages to carry out excision, replication, packaging into infectious small-headed phage-like particles, and finally mobilization into recipient strains (Tormo et al., 2008) resulting in extremely high transfer

frequencies. SaPIs are highly common in *S. aureus* and are composed from 15 to 17 kb. At least 16 SaPIs have been found on the *S. aureus* chromosome. They are named according to the loci of the chromosome to which the islands are integrated; SaPI1 is considered as the prototype (Novick, 2003; Subedi, 2007). Core genes include two open reading frames encoding transcriptional regulatory proteins and a region encoding integrase, Rep protein, and terminase. Some SaPIs confer pathogenicity while others confer antibiotic resistance. The two SaPI families, SaPI2 and SaPI3, encode virulence genes such as leucocidine (lukDE) and staphylococcal superantigens (enterotoxins and exotoxins), and all SaPIs encode enterotoxins or toxic shock syndrome toxin (TSST) (Thomas et al., 2007; Yarwood et al., 2002). Antibiotic treatment is thought to induce the mobilization of SaPIs (Úbeda et al., 2005; Maiques et al., 2006).

1.5.3. Genomic islands

In contrast to the SaPIs, the genomic islands are stably integrated in the same position in all *S. aureus* genomes and do not appear to be mobile. All sequences *S. aureus* genomes contain sets of three genomic islands that are variable between strains. Gene diversification in the island occurs as a result of gene loss and recombination (Fitzgerald et al., 2003). Given the composition of genomic islands, it is suspected that genomic islands were once mobile elements acquired by HGT (Dobrindt et al., 2004). Both flanking DNA segments contribute to the stability of genomic islands within the *S. aureus* chromosome.

1.5.4. Staphylococcal cassette chromosome

The staphylococcal cassette chromosome (SCC) is an important MGE because it contains multiple virulence and antimicrobial resistance genes. In contrast to other MGE, cassette chromosome *mec* (SCCmec; *mec* standing for methicillin resistance) preferentially carries antibiotic resistance genes rather than pathogenicity factors. SCC is characterized by a set of site-specific recombinase genes (*ccr*A and *ccr*B). The SCC*mec* element is the most common in *S. aureus*, which contains the *mec*A gene and the

recently discovered *mec*C gene (Paterson et al., 2014a) encoding methicillin resistance. The SCC*mec* elements are classified in different groups based on whether they are found in Hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA) or CA-MRSA isolates: types I-III are HA-MRSA and types IV-VII are CA-MRSA. Evolutionary studies suggest that SCC*mec* likely originated in coagulase-negative staphylococci (Yuki Katayama et al., 2001). This MGE, in contrast to other MGEs, have variable contents of insertion elements and plasmids suggesting that recombination events are modifying the element (Robinson and Enright, 2003) to convert it into a smaller element capable of being mobilized by transduction at a higher frequency.

1.5.5. ACME element

The arginine catabolic mobile element (ACME) is a genomic island in Staphylococci that contain the six-gene arc cluster that plays several roles in virulence and survival in an infection. Some authors have suggested that ACME is a new member of the SCC family. The ACME element is integrated downstream of the staphylococcal chromosomal cassette (SCC) harboring the methicillin resistance gene *mec*A (SCC*mec*) and use the same attachment site as SCC*mec* for integration within *orf*X. Similar to SCC*mec* elements, ACME is flanked by repeat sequences, and SCC*mec*-encoded cassette chromosome recombinase (*ccr*) genes catalyze integration and excision of ACME from the staphylococcal chromosome (Diep et al., 2006). ACME exists as a composite island with SCC*mec* IVa (ACME/SCC*mec*-CI) in ST8-MRSA-Iva (also known as USA300) and this strain is the predominant CA-MRSA strain in the United States. The extensive spread and success of USA300 has been partially attributed to the presence of the ACME element, which is thought to play an important role in its growth and survival (Shore et al., 2011b).

1.5.6. Plasmids

Plasmids are auto-replicating DNA molecules. *S. aureus* possess relatively few plasmids. Most of the sequenced *S. aureus* genomes contain one or

more extrachromosomal plasmids that use to contain genes encoding resistance to antibiotics and heavy metals or antiseptics and virulence factors including enterotoxins and exfoliative toxins. *S. aureus*, unlike other pathogens, utilizes transduction for horizontal transfer of plasmids between isolates. In some cases, extrachromosomal replicating plasmids have integrated into de *S. aureus* genome.

2. Interaction between the pathogen and the host: Pathogenesis of *Staphylococcus aureus*

Staphylococcus aureus is a versatile and dangerous pathogen in humans that has a wide host range, diverse cellular and environmental lifestyles, and the ability to highly develop drug-resistant forms. It is the species of the genus *Staphylococcus* that most frequently causes clinical processes in the hosts it colonizes (Witte et al., 2014). In fact, has long been recognized as a major human pathogen and remains a frequent cause of morbidity and mortality. Several studies show that *S. aureus* is one of the most common cause of nosocomial infections (Fridkin et al., 2002). It can be transmitted by direct contact or through secretions or fomites. Despite of the high pathogenicity of *S. aureus*, it is an opportunistic pathogen. This means that it is a commensal microorganism of the skin and mucous membranes that awaits favorable conditions, like wounds, immunosuppression or other concomitant diseases, to multiply and to produce an infection (Kobayashi et al., 2015).

2.1. *Staphylococcus aureus* colonization and infection

S. aureus infections in humans differ in community-acquired and hospital-acquired staphylococcal infections. Besides, *S. aureus* has two distinct lifestyles: a commensal asymptomatic state, and acute state where it invades specific tissues and pathogenicity ensures (Lowy, 1998). Humans are a natural reservoir of *S. aureus*; in healthy individuals, it can colonize the skin, nails and nostrils without causing any type of lesion. These individuals are known as carriers, which can be persistent or intermittent.

Colonization with *S. aureus* does not have a protective effect; on the contrary, it increases the risk of infection by this pathogen (Wenzel and Perl, 1995). Nevertheless, one study shows that after nosocomial infection, colonized individuals have less severe *S. aureus* disease compared with noncolonized individuals (Wertheim et al., 2004).

When immunosuppression occurs (due to stress, other diseases, etc.) or wounds on the skin and mucosa, the bacteria can multiply and invade the tissues, usually producing suppurative lesions. *S. aureus* causes a broad range of infections in humans that are varied in their severity and tissue tropism ranging from relatively mild conditions including folliculitis, superficial and deep skin abscesses, skin and soft tissue infections, to more severe disease such as wound infections, impetigo, osteomyelitis, pneumonia, suppurative arthritis, pleural emphysema, meningitis, which can be complicated by septicemia and endocarditis, toxic shock (Lowy, 1998). It is obvious that there is host susceptibility and host-pathogen dynamics at play to determine if colonization or infection occurs, although these phenomena are poorly understood.

Nasal carriage is the most frequent form of colonization of *S. aureus*, a high percentage of the population is carrier without showing symptoms. However, carriers have been found in the throat and on the skin, with negative culture in the nose (von Eiff et al., 2001; Kluytmans and Wertheim, 2005). Therefore, anterior nares cultures will detect most, but not all, people who are colonized with *S. aureus* (Shurland et all., 2009). Host immune deterrents for bacterial nasal colonization include antimicrobial peptides, lysozyme, lactoferrin, and IgA (Wertheim et al., 2005). However, little is known of the critical host defenses against *S. aureus* colonization.

A higher incidence rate of nasal carriage of community-acquired *S. aureus* (CA-MRSA) has also been associated with individuals having frequent contact with animals, suggesting that they can be vectors in the spread of CA-MRSA (Hanselman et al., 2006; Weese et al., 2006; Weese and Lefebvre, 2007).

Infection is often preceded by colonization or direct or indirect contact with the pathogen and occurs when *S. aureus* enters tissues and establish an invasive infection on breaches in the skin or mucous membranes (Lowy, 1998). *S. aureus* has a versatile tissue tropism attributed to its remarkable set of virulence factors involved in pathogenesis.

2.2. *Staphylococcus aureus* immune evasion: Virulence factors

This organism produces more than 30 virulence factors that provide the ability to colonize the host, adhere to surfaces as biofilm, invade or evade the immune system, develop resistance to multiple antibiotics and cause toxicity to the host that lead pathogenic bacteria adapt to survival in their host. One of the mechanisms of *S. aureus* to cause disease is evasion of innate host defense, which includes resistance to antimicrobial peptides (AMPs) and killing by phagocytic leukocytes. Two of the *S. aureus* two-component systems (TCSs) GraSR/VraFG, play an essential role in antimicrobial peptide resistance (Li et al., 2007; Meehl et al., 2007). Initial exposure of *S. aureus* to host tissues beyond the mucosal surface or skin is thought to trigger up-regulation of virulence genes (Novick, 2003). *S. aureus* has an astounding repertoire of immune evasion factors that try to subvert host defense mechanisms. Although the pathogen produces a proinflammatory response, it generates several molecules that block chemotaxis of polymorphonuclear leukocytes (PMNs).

Phagocyte function may be subverted at many different stages. *S aureus* may hide from recognition by producing protective coats, such as capsular polysaccharide or biofilm. Further, they produce or secrete specific molecules to block phagocyte receptor function. After ingestion, the bacteria use mechanisms to decrease the efficiency of antimicrobial mechanisms, which likely account for noted post phagocytosis survival (Voyich et al., 2005). Finally, they often produce toxins that lyse phagocytes, thus using the same kind of weapon that neutrophils use to kill bacteria.

There are numerous virulence factors and mechanisms of *S. aureus* to evade the host innate immune response that determines the success or

failure of the pathogen in an infection. The most important virulence factors in *S. aureus* are listed below.

2.2.1. The accessory gene regulator *agr*

Among the genes that control *S. aureus* colonization and virulence, the best global regulator is the accessory gene regulator *agr*. Although it is not directly a virulence factor, this gene is a quorum sensing locus which directly controls expression of a high number of virulence and colonizing factors such the coagulases, protein A and haemolysins (Wolz et al., 1996). Down-regulation of *agr* is associated with colonization and activation of *agr* with host invasion (Traber et al., 2016).

2.2.2. Polysaccharide intercellular adhesion

Biofilm formation is the main pathogenetic mechanism leading to the chronicity and irreducibility of infections. The extracellular polymeric substances of staphylococcal biofilms are the polysaccharide intercellular adhesin (PIA), extracellular-DNA, proteins, and amyloid fibrils (Arciola et al., 2015). Polysaccharide intercellular adhesion (PIA) is a biofilm-related extracellular matrix substance with a positive net charge whose synthesis is mediated produced by the *ica*ADBC locus, and its main function is protecting from neutrophil phagocytosis and AMPs (Vuong et al., 2004b). The *ica* locus was found present in biofilm producing *S. aureus* strains responsible for catheter and implant infections (Arciola et al., 2001).

2.2.3. The staphylococcal immune evasion cluster

The immune evasion cluster (IEC) located on β-haemolysin-converting bacteriophages (βC-Φs), harbor *scn* and a different combination of *sak*, *chp* and *sea* (or *sep*) genes that encode the human-specific immune modulators including staphylococcal complement inhibitor (SCIN), staphylococcal enterotoxin A (SEA), staphylokinase (SAK) and chemotaxis inhibitory protein of *S. aureus* (CHIPS). The SCIN secreted *S. aureus* molecules block the complement system, thereby reducing phagocytosis by PMN after

opsonization and neutrophil chemotaxis (Wamel et al., 2006). SEA is involved in the down-regulation of chemokine receptors of monocytes (Balaban and Rasooly, 2000). SAK achieves its function primarily by forming a plasminogen activating complex together with plasmin itself, which prevents biofilm formation (Kwiecinski et al., 2016), and also initiates the fibrinolytic cascade to help the invading bacterium move deeper into tissues (Bergmann and Hammerschmidt, 2007). SAK is also able to neutralize host antimicrobial peptides (AMPs) (Rooijakkers et al., 2005). Finally, CHIPS is a molecule that blocks receptor-mediated recognition of formylated peptides, which are PAMPs secreted by bacteria and central for phagocyte detection of bacterial invaders (De Haas et al., 2004).

2.2.4. The microbial surface component recognizing adhesive matrix molecules (MSCRAMM)

The ability to adhere effectively to multiple host tissues is a critical factor for the pathogenic success of *S.aureus*. The microbial surface component recognizing adhesive matrix molecules (MSCRAMM) are adhesin proteins that mediate the initial attachment of bacteria to host tissue, providing a critical step to establish infection. Nevertheless, MSCRAMM proteins provide an excellent target for immunological attack by antibodies because the highly specific MSCRAMM antibodies prevent microbial adherence (McDevitt et al., 1995; Rennermalm et al., 2001), as well as recolonization of host tissues or biomaterials; and because the increased level of MSCRAMM protein antibodies bound to the bacterial cell wall facilitates rapid clearance of the organism through opsonophagocytosis (Rozalska and Wadstrom, 1993; Nilsson et al., 1998). Some of these proteins include protein A, clumping factor A (ClfA), fibronectin binding protein A (FnbpA) and SdrG (Foster et al., 2014). All these MSCRAMMs bind to fibrinogen except protein A, that binds IgG.

Clumping factor A (ClfA) is an MSCRAMM protein expressed by *S. aureus* that promotes binding of fibrinogen and fibrin to the bacterial cell surface (McDevitt et al., 1994). This protein mediate direct binding to human

platelets (Siboo et al., 2001) and protects bacteria from neutrophil phagocytosis through the recruitment of fibrinogen to the bacterial cell surface (Higgins et al., 2006).

The staphylococcal protein A (SpA), encoded by the *spa* gene, has several functions. The interaction of protein A with the Fc part of IgG molecules enables *S. aureus* to sequester nonspecific antibodies on its surface, which protects efficiently from attacks by the innate and acquired immune systems (Forsgren and Nordström, 1962). Protein A also has a more specific role in the pathogenesis of airway infections by interacting with the tumor necrosis factor (TNF) a receptor on airway epithelia which leads to the activation of intracellular signaling, the expression of cytokines and the recruitment of neutrophils (Gómez et al., 2004).

Staphylococcus aureus has been shown to adhere to and invade human endothelial cells, potentially the first step in the pathogenesis of disseminated infection. *S. aureus* fibronectin-binding protein (FnBPA) is central to the invasion of endothelium, fibronectin forming a bridge between bacterial fibronectin-binding proteins and host cell receptors. FnBPA is capable of conferring both adherence to fibronectin and endothelial cells, and endothelial cell invasion (Massey et al., 2001).

The Serine-Aspartate Repeat Proteins (Sdr) are encoded by the *sdr*C, *sdr*D, and *sdr*E genes located in the sdr locus. At least two *sdr* genes are present in all tested *S. aureus* strains (Josefsson et al., 1998) and always include *sdr*C (Peacock et al., 2002). The Sdr proteins are characterized by the presence of an R region containing various numbers of the Ser-Asp dipeptides and have a similar structural organization. A signal peptide is followed by an A region which is similar in size among the different members of the Sdr family. The function of Sdr proteins in *S. aureus* remains unknown; however, there have been a few studies which reported a strong correlation between *sdr* genes and certain human diseases (Peacock et al., 2002; Trad et al., 2004; Sabat et al., 2006). The presence of insertion mutation and deletion mutation in the *sdr* genes suggests that they are

variable (Xue et al., 2011). These findings lead to better understanding the emergence of traits such as increased virulence or antibiotic resistance.

2.2.5. Toxic shock syndrome toxin (TSST)

Potentiation or overstimulation of the immune response is a very effective way of interfering with the human immune system. *S. aureus* produces many superantigenic toxins (SAGs), a class of secreted toxins that activate T-cells without the need for the presence of an antigen on an antigen-presenting cell (McCormick et al., 2001). These toxins include the toxic shock syndrome toxin (TSST), a low-molecular-weight single peptide toxin secreted by some strains of *S. aureus* that is highly associated with menstrually related toxic shock syndrome (TSS) and is considered a likely causative toxin. These superantigens bypass the normal pathway for activation of T cells resulting in over-activation of cytokines and inflammatory cells what produces symptoms like fever, rash formation, and hypotension that can lead to multiple organ failure and lethal shock, as well as desquamation in patients that recover (Wiesenthal et al., 1985). Nonmenstrual-associated staphylococcal TSS is normally associated with TSST-1, staphylococcal enterotoxin (SE) serotype B (SEB), or SEC (Bohach et al., 1990).

2.2.6. Phenol-soluble modulins and α-toxin

S. aureus has a great number of immune evasion passive mechanisms to hide from recognition or blocking receptors or effectors involved in the elimination of the bacteria. But it also produces toxins that directly attack white and red blood cells. These toxins include the large family of leukocidins and α-toxin (also known as α-hemolysin), phenol-soluble modulins (PSMs), and other hemolysins.

The PSMs (PSMα1, PSMα2, PSMα3, PSMα4) are secreted peptides that interpose in the acute response of the host against *S. aureus*, diminishing the response of neutrophils. PSMs contribute significantly to increase virulence of CA-MRSA relative to HA-MRSA (Wang et al., 2007).

S. aureus α-toxin (toxin A, hemolysin A (Hla)) is a major virulence-associated factor contributing significantly to the pathogenicity of the respective *S. aureus* strains that plays an essential role in pneumonia. Hla activates defensive mechanisms of the epithelium (Räth et al., 2013) and affects activation or inhibition of different intracellular signaling pathways that triggers necrotic or apoptotic cell death (Below et al., 2021).

2.2.7. Panton-Valentine leukocidin

Panton-Valentine leukocidin (PVL) has an epidemiologic correlation with community-associated MRSA because some CA-MRSA harbor a phage harboring PVL genes, and they are thought to be responsible at least in part for the increased virulence of CA-MRSA. This is because *pvl* has been associated with epidemic CA-MRSA strains causing skin and soft tissue infections, necrotizing pneumonia and lethal necrotizing fasciitis (Mongkolrattanothai et al., 2003; Miller et al., 2005). Some studies show that there is a high association between MRSA USA300 nasal/axillary carriage and presence of PVL in persons with abscesses (Immergluck et al., 2017). The subunits of the PVL are encoded by the genes *luk*S-PV and *luk*F-PV (*pvl*). PVL is a bicomponent, pore-forming leukotoxin with ability to lyse leukocytes or cause apoptosis (depending on the concentration). But unlike other *S. aureus* pore-forming leukocidins, PVL is not hemolytic (Boyle-Vavra and Daum, 2007). Regardless, the precise role of PVL in pathogenesis remains to be elucidated.

2.2.8. Coagulases

Coagulation is an ancient innate defence mechanism against microbial pathogens that traps and immobilizes invading bacteria in a clot (Loof et al., 2011), however coagulation is also the target of bacterial immune evasive strategies. To date, two coagulases have been described in *S. aureus*: coagulase (Coa) and von Willebrand Factor Binding Protein (Vwbp); both promote similar modifications of the coagulation cascade during host infection. The coagulase protein is encoded by the *coa* gene and is a

polypeptide that bind to and activate prothrombin, thereby converting fibrinogen to fibrin and promoting the clotting of plasma or blood; this leads to the formation of abscesses and bacterial persistence in host tissues and also enables the pathogen to cause lethal sepsis (McAdow et al., 2012). The Vwbp, that was discovered at the beginning of this century (Bjerketorp et al., 2002), binds to the von Willebrand factor and also to the prothrombin, triggering the coagulation as well. The Vwbp protein is encoded by the *vwb* gene. When both *coa* and *vwb* genes are deleted, the bacterium is unable to coagulate the plasma and, therefore, the pseudocapsule of the abscess is not formed and the lesion is less severe than those produced by the wild type. But the effect of *coa* is greater than *vwb*, as deletion of the *coa* gene makes the bacteria take longer to coagulate the plasma than deleting only the *vwb* (Cheng et al., 2010).

2.2.9. Haemolysins

Haemolysins also play an important role in the development of the infection, as they cause the lysis of blood cells. *S. aureus* can produce four different haemolysins (alpha, beta, delta, and gamma).

A high percentage of strains produce alpha-hemolysin, encoded by the *hla* gene, that is the most characterized virulence factor of *S. aureus* This haemolysin is toxic to a wide range of mammalian cells, and it is also dermonecrotic and neurotoxic. The defining characteristic of alpha-toxin is its ability to lyse erythrocytes. In particular, rabbit erythrocytes are extraordinarily susceptible to hemolysis by alpha-toxin, at least 100 times more so than other mammals and 1,000 times more than human erythrocytes (Bhakdi et al., 1984). Alpha-hemolysin produces pores on the cell surface leading to necrotic death of the target cell (Vandenesch et al., 2012).

S. aureus β-hemolysin is selectively cytotoxic to monocytes and is inactive against lymphocytes, granulocytes, and fibroblasts (Walev et al., 1996). It is highly hemolytic for sheep but not rabbit erythrocytes and it is not dermonecrotic in guinea pigs, and it is not lethal in mice. The gene that

encodes alpha-hemolysin is *hlb* gene. Beta-hemolysin is produced in large quantity by a high number of *S. aureus* strains, particularly animal isolates. The role of β-hemolysin in disease is not clearly understood, but this high level of expression in animal strains indicates that β-hemolysin producers have some selective advantage from toxin secretion (Dinges et al., 2000).

Delta-hemolysin, encoded by the *hld* gene, causes membrane damage in a variety of mammalian cells. Delta-hemolysin is capable of lysing erythrocytes and other mammalian cells, as well as subcellular structures such as membrane-bound organelles, spheroplasts, and protoplasts (Freer and Birkbeck, 1982). However, its importance in disease etiology remains unclear.

Two types of bicomponent toxins are synthetized by *S. aureus*, gamma-hemolysin and PVL. Gamma-hemolysin is produced by almost every strain of *S. aureus*, while only 2 to 3% of strains secrete PVL. These toxins affect neutrophils and macrophages, and gamma-hemolysin is additionally able to lyse many varieties of mammalian erythrocytes (Dinges et al., 2000). Gamma-hemolysin is not identifiable on blood agar plates, due to the inhibitory effect of agar on toxin activity (Prevost et al., 1995).

2.3. Host defense against *Staphylococcus aureus*

The crucial role of innate host defense is eliminating invading *S. aureus*. In humans, the primary cellular defense against *S. aureus* infections are the polymorphonuclear leukocytes (PMNs), also called neutrophils or granulocytes, which constitute the greatest number of leukocytes in people (DeLeo et al., 2009). A first step in the eradication of invading microorganisms is active recruitment of PMNs to the site of infection by chemotaxis. The host- and pathogen-derived chemotactic factors make neutrophils being mobilized from peripheral blood or bone marrow. It has been demonstrated that stimulation of CD4+ T-cells by *S. aureus* capsular polysaccharide leads to production of chemokines that recruit neutrophils to the site of infection (Tzianabos et al., 2001; McLoughlin et al., 2006) .

Many chemoattractants are priming agents (rather than activating agents) for neutrophils. Neutrophils can be primed for enhanced adhesion, phagocytosis, production of reactive oxygen species (ROS), cytokine secretion, leukotriene synthesis, degranulation, and bactericidal activity. Many neutrophil priming agents are host-derived molecules such as cytokines, chemokines, and growth factors (Kobayashi et al., 2005). Primed neutrophils produce mobilization of secretory vesicles (and thus up-regulation of specific cell surface receptors, eg, CD11b/CD18) and secretion of cytokines, but fails to trigger release of azurophilic granules or elicit production of superoxide that is produced by fully activated neutrophils (DeLeo et al., 1998). In summary, chemotactic/priming agents ultimately promote efficient clearance of invading microorganisms.

One of the most important processes made by neutrophils is phagocytosis. It consists of binding and ingesting invading microorganisms. It is a critical step in the removal of bacteria during infection. PMNs recognize many surface-bound or freely secreted molecules produced by bacteria known as pathogen-associated molecular patterns (PAMPs). They interact with pattern recognition receptors expressed on the neutrophil cell surface, including TLRs. Neutrophil TLRs activate signal transduction pathways that contribute to microbicidal activity (Hayashi et al., 2003). Peptidoglycan recognition protein (PGRP) is a secreted host protein that contributes to intracellular killing of gram-positive bacteria by neutrophils. In contrast to TLRs, which promote recognition of bacteria, PGRP-S contributes directly to bactericidal activity (Liu et al., 2000). The concerted action of pattern recognition receptors/molecules and antibody and complement receptors promotes efficient phagocytosis of microbes (***Figure 3***).

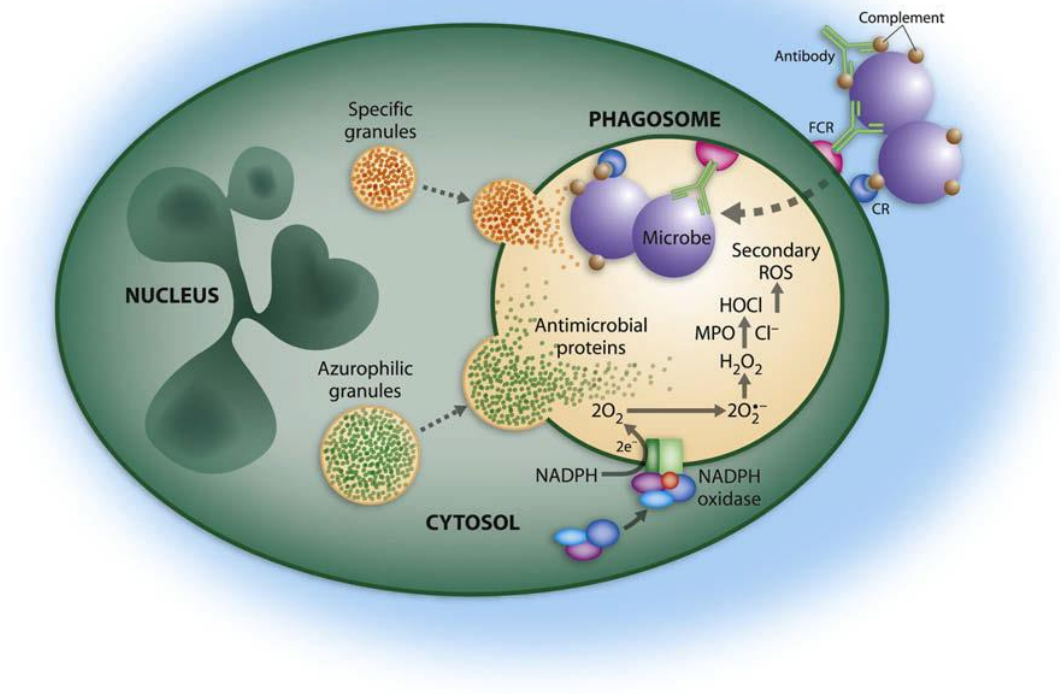

Figure 3. Polymorphonuclear leukocytes phagocytosis and microbicidal activity. Abbreviations: CR, complement receptor; FCR, Fc receptor; MPO, myeloperoxidase. (Adapted from Quinn et al., 2006).

Once *S. aureus* is phagocytosed, the phagosome is formed within the PMNs. Then, a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase is activated, this process is traditionally called respiratory burst, and generates high levels of superoxide (Quinn et al., 2006). Next, degranulation occurs when neutrophil cytoplasmic granules fuse with bacteria-containing phagosomes. Fusion of azurophilic granules with phagosomes enriches the vacuole lumen with numerous antimicrobial peptides (AMPs) and antimicrobial proteins (**Figure 3**). The PMNs are able to produce structures called neutrophil extracellular traps (NETs), composed of chromatin, histones, and azurophilic granule proteins, which have the capacity to kill bacteria, including *S. aureus* (Amulic et al., 2012).

Finally, neutrophil turnover must be regulated highly during infection because host tissues can be damaged by the inadvertent release of

cytotoxic components. Normal turnover of aging neutrophils occurs by spontaneous apoptosis and in the absence of an activating agent, but neutrophil apoptosis is accelerated significantly following phagocytosis and this phenomenon appears critical to the resolution of the inflammatory response (Kobayashi et al., 2002). Phagocytosis and killing of bacteria culminate with induction of neutrophil apoptosis (also called phagocytosis-induced cell death) and subsequent removal by macrophages, ultimately resulting in the resolution of infection.

It is therefore evident that the phagocytosis produced by PMNs is very important and decisive in the resolution of the infection.

2.4. Resistances to antibiotics in *Staphylococcus aureus*

Widespread antimicrobial resistance among *S. aureus* isolates is cause for considerable concern and has resulted in substantial increases in the cost of treatment associated with *S. aureus* infection. It is well known that the organism acquires resistance soon after the introduction of new antibiotics (Lyon and Skurray, 1987). Penicillin-resistant *S. aureus* was reported within 4 years of the introduction of penicillin G into clinical use in 1941. Then, other antibiotics such as erythromycin, tetracycline, and aminoglycosides were used for the treatment of patients infected by penicillinase-producing *S. aureus* only to result in the appearance of multi-drug resistant *S. aureus* by the 1950s.

Antibiotic resistance per se does not contribute to virulence it is simply a form of adaptation to the environment that the bacteria develop. The adaptive capacity of *S. aureus* is very important for the development of antibiotic resistance. The mechanisms of acquisition of resistance in *S. aureus* are classified into two main categories: mutation of a bacterial gene on the chromosome and acquisition of a resistance gene from other organisms by some form of genetic exchange (conjugation, transduction, or transformation). In the case of resistance acquisition, exogenous antibiotic resistance genes are found on some mobile genetic elements (plasmids, insertion sequences, transposons, or genomic islands) of resistant bacteria.

Since the appearance of bacteria resistant to antibiotics various mobile genetic elements carrying antibiotic resistance genes in staphylococci have been investigated (Projan, 2000). In *S. aureus* there are numerous genes that confer resistance to different antibiotics: penicillins (*bla*Z), aminoglycosides (*aac*A-D), tetracyclines (*tet*K and *tet*M), macrolides (*erm*A, *erm*B, *msr*A, *msr*B and *mef*A), fluoroquinolones (*gyr*A and *grl*A), lincosamides (*lin*A), folate inhibitors (*dfr*A1), phenicols (*cfr*), and ansamycins (*rpo*B) (Rahi et al., 2020).

Some of the mechanisms of resistance to antibiotics in *S. aureus* are explained below.

2.4.1. Resistance to macrolides

Emergence of resistance to macrolides occurred in staphylococci shortly after the therapeutic use of erythromycin (Chabbert, 1956). The mechanisms responsible for resistance to erythromycin in staphylococci are target site modification (Leclercq and Courvalin, 1991; Weisblum, 1995) and active drug efflux and enzymatic inactivation of the drugs. Resistance to macrolide, lincosamide and streptogramin B (MLS_B) antibiotics is mediated by the presence of *erm* genes (*erm(*A), *erm(*B) and *erm(*C)) that are responsible to the target site modification mechanism. Macrolide efflux is produced by membrane proteins encoded by the *msr(*A)/*msr(*B) genes and is specific for macrolides and streptogramin B (MS phenotype) (Lina et al., 1999; Roberts et al., 1999). The *mph(*C) gene, coding for macrolide phosphotransferase C (Mph(C)), has recently been detected in low-level resistant staphylococcal isolates of bovine (Lüthje and Schwarz, 2006) and equine (Schnellmann et al., 2006) origin.

2.4.2. Resistance to tetracyclines

Tetracycline resistance is widespread in *S. aureus* clinical strains. There are four tetracycline-resistance determinants, *tet*K, *tet*L, *tet*M and *tet*O. Most of the resistant strains carry either *tet*K or *tet*M gene and rarely possess *tet*L gene (Martin et al., 1986; Schmitz et al., 2001). The transposon Tn5801 carries *tet*M gene and is responsible of its dissemination. Two mechanisms

of tetracycline resistance have been identified in *Staphylococcus* species. The first mechanism involves the genes *tet*M or *tet*O which encode a protein that confers resistance to tetracycline as well as minocycline by interacting with the ribosome and promoting the release of bound tetracycline (Burdett, 1991, 1996). These genes are located in a transposon or in the chromosome. Some studies suggest that all *tet*M-positive isolates by polymerase chain reaction (PCR) are resistant to all tetracyclines including minocycline (Trzcinski et al., 2000). The second mechanism involves the *tet*K gene which encodes a protein that enhances the efflux of tetracycline; it is usually carried by small-sized plasmids, and one of them, pT181, is found inserted in type-III SCC*mec*. *S. aureus* strains carrying *tet*K only have been described as tetracycline resistant, but minocycline susceptible.

The tetM and tetK genes are the most prevalent single tetracycline resistance determinants in MRSA and MSSA. Furthermore, isolates harboring both the *tet*K and *tet*M genes display higher MIC values than the isolates containing just one of the genes (Schmitz et al., 2001).

2.4.3. Resistance to aminoglycosides

Aminoglycosides are rarely used as monotherapy against *S. aureus* due to the risk of development of resistance and toxic effects. Despite this, *S. aureus* has developed mechanisms of resistance to aminoglycoside antibiotics such as gentamicin, streptomocin and neomycin. The most widespread mechanism of resistance to aminoglycosides is the modification of the antibiotics by cellular enzymes, such as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyltransferases (AAD) and aminoglycoside phosphotransferases (APH) (Shaw et al., 1993). Further, in *S. aureus*, chromosomal mutation affecting ribosomes may confer a high level of resistance to the streptidine-containing aminoglycoside streptomycin (Lacey and Chopra, 1972), and mutations which affect the cellular permeability may provide low-level cross resistance to most aminoglycosides (Shannon and Phillips, 1982). As a treatment, it has been shown that some substance capable of carrying out transposon inactivation

of genes (i.e. *atp*A, *atp*B, *atp*G or *atp*H) encoding the subunits of the ATP synthase increase the efficacy of gentamicin against *S. aureus* (Vestergaard et al., 2016)

2.4.4. Resistance to vancomycin

The first study of *S. aureus* with reduced susceptibility to vancomycin (MIC of 8 lg/mL) was reported in Japan (Hiramatsu et al., 1997). Subsequently, eight isolates of vancomycin-intermediate *S. aureus* (VISA) were documented in the United States (Centers for Disease Control and Prevention, 1997). VISA strains have abnormal, thickened cells walls in the presence of vancomycin. Two mechanisms of resistance have been described in the strains: affinity trapping of vancomycin molecules by cell wall monomers and clogging of the outer layers of peptidoglycan by bound vancomycin molecules, and change in the structure or metabolism of teichoic acids. (Appelbaum and Bozdogan, 2004).

In 2002 occurred the emergence of the first vancomycin-resistant *S. aureus* (VRSA) in Michigan as a result of interspecies conjugative transfer of the transposon Tn1546 carrying *van*A gene between vancomycin-resistant *Enterococcus faecalis* (VRE) and *S. aureus* co-isolates in a polymicrobial infection (Weigel et al., 2003) under such selective pressure. In *S. aureus* the Tn1546 (*van*A) element is integrated into a multirresistant conjugative plasmid. The transfer of Tn1546 (*van*A) is an uncommon event with very few isolates identified, most of them from Michigan. All the donor VRE isolates analyzed from Michigan contained an Inc18- like *van*A conjugative plasmid that is the likely factor in the geographically restricted emergence of VRSA (Zhu et al., 2008). These developments are of great concern, and every effort should be made to prevent further development and spread of vancomycin resistance in staphylococci.

2.4.5. Resistance to bacitracin

Bacitracin is a polypeptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis* (Johnson et al., 1945; Azevedo et al., 1993), is active against gram-positive bacteria and used to prevent and treat skin and

ophthalmic infections. It binds to undecaprenyl pyrophosphate (UPP), resulting in inhibition of cell wall biosynthesis (Stone and Strominger, 1971). Some two-component systems (TCSs) have been demonstrated to affect the susceptibility to antibacterial agents. Gram-positive bacteria have developed several mechanisms of bacitracin resistance but the BceSR two-component system (TCS)/BceAB ABC transporter is the most efficient and well-studied. Several studies show that the TCS *Bce*RS is associated with an increase susceptibility to bacitracin, implying that this TCS may be involved in bacitracin sensing and also resistance to bacitracin. *Bce*RS senses bacitracin and also positively regulates the expression of two ABC transporters that function in bacitracin efflux (Yoshida et al., 2011). On the other hand, it has been hypothesized that *bac*A encodes undecaprenol kinase, and that when this enzyme is overproduced it is able to produce sufficient supplies of UP from undecaprenol to overcome the effects of UPP sequestration, thereby causing bacitracin resistance. The gene is not essential for in vitro growth, and seem to be implicated in virulence during an infection (Chalker et al., 2000).

2.4.6. Resistance to trimethoprim-sulfamethoxazole

Bacterial resistance to trimethoprim-sulfamethoxazole (TMP-SMX) has not been fully elucidated but it may develop independently of TMP and SMX. While SMX is a sulfonamide, TMP is a diamino pyridine and both belong to the peptide family like vancomycin and bacitracin. Sulfonamide resistance among *S. aureus* may be attributed to two mechanisms: Most resistance is likely due to chromosomally mediated overproduction of para-aminobenzoic acid (Grim et al., 2005). Although a sulfonamide-resistant plasmid has also been described in *S. aureus*, the mechanism of resistance is unclear (Lyon and Skurray, 1987). Resistance to TMP also may be chromosomally or plasmid mediated (Zinner and Mayer, 2000).

2.4.7. Resistance to chloramphenicol

Chloramphenicol shows activity against many gram-positive and gram-negative bacteria and is licensed exclusively for use in veterinary medicine.

Bacterial resistance to chloramphenicol and thiamphenicol is most commonly mediated by mono- and diacetylation via chloramphenicol acetyltransferase (CAT) enzymes. Acceptor site for acetyl groups is structurally altered in florfenicol. The resistance genes in staphylococci that confers resistance to chloramphenicol and florfenicol are *cfr* and *fex*A genes (Schwarz et al., 2004). These resistance genes are located on mobile genetic elements which may carry additional resistance genes that might facilitate their dissemination. One of the difficulties in knowing the true incidence of strains resistant to chloramphenicol is that it is not approved for the control of staphylococcal infections in animals and hence staphylococcal isolates are not routinely tested for their susceptibility to chloramphenicol. It is because of that, an unknown number of staphylococcal isolates with elevated MICs for florfenicol might remain undetected.

2.4.8. Resistance to fluoroquinolones

The increased use of fluoroquinolones has led to increasing resistance to these antimicrobials. Resistance to fluoroquinolones typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV) and of changes in drug entry and efflux. Three mechanisms have been described to justify resistance to quinolones: mutations in the genes encoding the DNA gyrase and topoisomerase IV A subunits (*gyr*A and *grl*A, respectively) (Sreedharan et al., 1990; Goswitz et al., 1992); mutations in *gyr*B, the DNA gyrase B-subunit gene; and overexpression of *nor*A, the gene encoding the NorA protein that is a membrane-based multidrug efflux protein capable of transporting fluoroquinolones as well as several other structurally unrelated compounds from the cell (Yoshida et al., 1990; Kaatz and Seo, 1995). More than one resistance mechanism may be present in a single strain. Resistance to quinolones can also be mediated by plasmids that produce the Qnr protein; although Qnr by itself produces only low-level resistance, its presence facilitates the selection of higher-level resistance mutations, thus contributing to the alarming increase in resistance to quinolones.

2.4.9. Resistance to penicillin

Penicillin was the first antibiotic mass produced for use in humans. It was initially highly effective for treatment of *S. aureus* infections, but today over 90% of human *S. aureus* strains are resistant to this antibiotic (Olsen et al., 2006). β-lactamase is an enzyme that inactivates penicillin and confers penicillin resistance by hydrolyzing the b-lactam ring of penicillin thereby inactivating the antibiotic, and/or production of a low-affinity penicillin-binding protein (PBP2a) encoded by the *mec*A gene (Chambers and DeLeo, 2009). In *S. aureus*, β-lactamase is encoded by the *bla*Z gene and the closely linked regulatory genes, blaI and blaR (Hackbarth and Chambers, 1993). This β-lactamase may be located on a plasmid, transposons or within chromosomal DNA (Malachowa and DeLeo, 2010).

2.4.10. Methicillin-resistant *Staphylococcus aureus*

Antibiotic therapy is a selective force for bacteria so that only cells that bear advantageous properties can persist, replicate and disseminate, subsequently predominating in the population. *S. aureus* is a pathogen capable of developing highly drug-resistant forms that can cause serious diseases with epidemic potential. The control and prevention of *S. aureus* infections have been complicated in by the general spread of multidrug-resistant *S. aureus* strains known as methicillin-resistant *S. aureus* (MRSA). The introduction of methicillin into clinical use in 1961 for the treatment of multi-drug resistant *S. aureus* resulted in the increase of the occurrence of MRSA in healthcare institutions worldwide (Lowy, 1998). In the same year, Jevons discovered methicillin-resistant *S. aureus* (MRSA), which by 1970s became spread all over the world (Jevons, 1961).

MRSA are increasingly resistant to all available classes of antibiotics and that make treatment of infections caused by these strains limited to vancomycin, linezolid, and daptomycin. However, resistance to these three drugs has been reported (Tsiodras et al., 2001; CDC, 2002; Mangili et al., 2005), making MRSA a tremendous public health challenge. Both methicillin-sensitive and methicillin-resistant isolates are persistent

colonizers (Casewell and Hill, 1986; Sanford et al., 1994) which makes the fight against them more difficult.

At first, MRSA infections was restricted to nosocomial environments and individuals associated to healthcare institutions, but below this ecological niche has expanded giving place to the Community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) (Saïd-Salim et al., 2003). Since 1990s, there has been an increase in infections caused by *S. aureus*, mostly caused by MRSA, in community settings among healthy individuals (Naimi et al., 2003; Fridkin et al., 2005; Zetola et al., 2005) and some of them causing serious medical complications with poor clinical outcomes (Miller et al., 2005). The number of CA-MRSA infections has risen and, in some places, has become epidemic. CA-MRSA isolates are typically less resistant to other non-β-lactams than hospital-acquired MRSA (HA-MRSA) but this is changing; some epidemic clones of CA-MRSA are increasingly being isolated from healthcare settings reducing the differences between community- and hospital-acquired infections, thereby complicating the epidemiology and control of this pathogen (Saïd-Salim et al., 2003; Chambers, 2005; Klevens et al., 2006; Moran et al., 2006; Klevens et al., 2007).

As said before, although the appearance of MRSA occurred in hospital, CA-MRSA has become a great concern around the world. Most of CA-MRSA strains exhibit typical heterogeneous type methicillin resistance, and some of the strains appear highly virulent and produce Panton–Valentine leukocidins (Gillet et al., 2002). The genetic backgrounds of HA-MRSA are rather limited (Enright et al., 2002); conversely, the diversity of CA-MRSA genotypes is comparable to the diversity of *S. aureus* in the community. All this indicate that CA-MRSA strains are not descendants of extant health-care-associated MRSA strains and have different origin of derivation (Okuma et al., 2002).

β-lactam antibiotics (eg. methicillin) are a broad group of molecules that are naturally produced by different organisms (molds belonging to *Penicillium*

spp. and *Cephalosporium spp*. for penicillins and cephalosporins, respectively, and bacteria belonging to different species for monobactams and carbapenems). The antibiotics of the β-lactam family are enzyme inhibitors, and their mechanism of action involves inhibition of bacterial transpeptidases (also called penicillin binding proteins (PBP)). This proteins are enzymes located in the cytoplasmic membrane catalyzing cell-wall assembly (De Rosa et al., 2021). PBPs are involved in the final steps of peptidoglycan biosynthesis, and they are the targets of the β-lactam antibiotics. One of the main factors of methicillin resistance in *S. aureus* is the penicillin-binding protein PBP2a encoded by the *mec* genes located on the chromosome of MRSA. It has been suggested that PBP2a takes over the biosynthesis of peptidoglycan in the presence of lethal doses of β-lactam antibiotics (Pinho et al., 2001). Because β-lactams remain the primary therapeutics for treating bacterial infections, understanding the molecular mechanism of resistance to these antibiotics is essential.

2.4.10.1. SCC*mec* cassette

The emergence of methicillin-resistant staphylococcal lineages (MRSA) is due to the acquisition and insertion of the SCC*mec* element into the chromosome of susceptible strains (MSSA). In MRSA, the acquisition of the mobile genetic element SCC*mec* leads to the acquisition of resistance to β-lactam antibiotics because this element carries the *mec* gene. This MGE also carries other genes for resistance to antibiotics and heavy metals depending on the type of SCC*mec* cassette. SCC*mec* may have been acquired by *S. aureus* from *S. sciuri* (Wu et al., 1996; Severin et al., 2005).

SCC is known to be a heterogeneous mobile genetic element that specifically integrates at the *orf*X site with unknown function located near the origin of replication. This may provide advantage to the instant utilization of imported antibiotic resistance genes. Its specific insertion or excision is mediated by the recombinase chromosome cassette complex (*ccr*) and by the presence of direct and inverted repeats at the ends of the SCC*mec* (Wang and Archer, 2010). *Ccr* recombinases are encoded by three

phylogenetically distinct *ccr* genes, *ccr*A, *ccr*B, and *ccr*C, with nucleotide similarities among them below 50%. In general, *ccr* genes with more than 85% of nucleotide identities are assigned to a same allotype. Thanks to DNA sequencing, new allotypes of the three types of recombinases are continually being discovered that give rise to new types of SCC*mec* cassette (Wu et al., 2015).

The combination of classes of *mec* gene complexes and *ccr* gene complexes forms the basis of their classification (Ito et al., 2004; IWG-SCC, 2009), and are essential elements for the functionality of the SCC*mec*. The SCC*mec* element contains the *mec* gene (*mec*A, *mec*B and *mec*C) along with the genes that control its expression, *mec*R1 (which encodes the signal that transduces the MecR1 protein) and *mec*I (encoding the repressor protein MecI), and acts as a carrier to exchange genetic information between *Staphylococcus* strains.

In addition, in this mobile genetic element the so-called non-essential, very heterogeneous J-regions (J1-3) are located, varying in length and composition and separated by the *mec* gene complex and the *ccr* complex. These regions contain various genes or pseudo genes whose presence does not appear essential or useful for the bacterial cell; the notable exceptions are resistance genes for non-β-lactam antibiotics or heavy metals, some of which are derived from plasmids or transposons.

Since the initial description of SCC*mec* in 1999 (Katayama et al., 2000), up to 14 different SCC*mec* have been described in MRSA (labeled I-XIV) and subtypes have been described, but the extreme plasticity of the SCC element results in the continual permutation and identification of new “types” (Hanssen and Ericson Sollid, 2005; Deurenberg et al., 2007; Lakhundi and Zhang, 2018; Urushibara et al., 2020). Numerous multiplex PCR schemes have been proposed for rapid typing (Oliveira and De Lencastre, 2002; Kondo et al., 2007; Milheiriço et al., 2007a; Stephens et al., 2007). Subsequently an International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWC-SCC) has been

assembled, and Oliveira et al. (Oliveira et al., 2006) have created a web-based resource for SCC*mec* typing by comparative sequencing (http://www.ccrtyping.net).

While health-care-associated MRSA strains carry either type-I, -II or -III SCC*mec*, CA-MRSA strains typically carry SCCmecIV, V, or VII elements. Type-IV SCC*mec* (defined by carriage of class B *mec* gene complex and type-2 *ccr* gene complex) rarely seen in health-care-associated MRSA strains, is the dominant SCC*mec* in CA-MRSA. No resistance genes against non-β-lactam antibiotics are found in types-IVa and -IVb SCC*mec*. Several strains isolated from outpatients' clinic in 1981 in Japan possessed type-IV SCCmec, but it was distinct from the above subtypes; this new subtype having greater size than subtypes IVa and IVb, was designated IVc. So far, no CA-MRSA strains have been associated with this subtype of type-IV SCC*mec* (Ito et al., 2003). ***Table 2*** shows the general characteristics of differentiation between MRSA isolates.

HA-MRSA	Frequently associated genetic lines: ST5, ST22, ST36, ST45, ST239, ST250. History of hospitalization or recent surgical intervention. SCC*mec* type I, II, III. Not related to an increased frequency of PVL.
CA-MRSA	Frequently associated genetic lines: ST1, ST8, ST59, ST80, ST93. No history of hospitalization but close contact between individuals. Related to a higher frequency of PVL.
LA-MRSA	Frequently associated genetic lines: ST5, ST9, ST97, ST130, ST398. Carriers, use of antimicrobials. Not related to an increased frequency of PVL.

Table 2. General characteristics of differentiation between MRSA isolates. (Adapted from: Porrero, 2014a).

2.4.10.2. The recently discovered *mec*C gene

The *mec*C gene represents a fourth of the hitherto known phylogenetic branches of the *mec* genes. In 2011, the novel SCC*mec* element type XI in

S. aureus was discovered carrying the *mec*C gene (García-Álvarez et al., 2011; Shore et al., 2011a). SCC*mec* XI has structural similarities to other known SCC*mec* elements and is integrated within the same chromosomal locus, *orf*X site. The *mec*C gene only shares 70% identity with *mec*A gene on the nucleotide level and the proteins encoded by these genes PBR2a and PBP2c show 63% identity; despite this both can mediate beta-lactam resistance in *S. aureus*. Besides, *mec*C mediates oxacillin and cefoxitin resistance, like *mec*A, irrespective of the genetic strain background. Transcription of *mec*C has been observed to be oxacillin inducible, indicating the presence of a functional beta-lactam-dependent regulatory system in *S. aureus* strains harboring the SCC*mec* XI element (Ballhausen et al., 2014).

The majority of known *mec*C-MRSA belongs to the ST-CC130/spa-CC 1535 clonal lineage with t843 as the most prominent spa type (Becker et al., 2014). The occurrence of *mec*C as part of the SCC*mec* XI element in different not closely related CCs indicates that SCC*mec* XI might have been independently acquired several times. It has been observed that *mec*C-MRSA strains exhibit the usual, strain-dependent pathogenic potential as exhibited by other *S. aureus* lineages. Human *mec*C-MRSA strains belonging to MLST CC130, CC1934 and CC599 carried typical *S. aureus* virulence markers including genes encoding hemolysins (*hla*, *hlb* and *hld*), immune evasion factors (*aur*, *spl*A, *spl*B and *spl*E) and biofilm factors (*ica* operon).

2.5. *Staphylococcus aureus* and MRSA in veterinary medicine

S. aureus is not solely a human bacterial pathogen but colonizes and causes infections in multiple hosts including dairy cows, sheep, poultry and rabbits (Fitzgerald, 2012). Even though *S. aureus* has been found to be rather host specific (Herron-Olson et al., 2007), it has been reported that a single mutation, which occurred naturally, was sufficient to allow a radical change in bacterial host tropism in *S. aureus* (Viana et al., 2015a). Therefore, the capacity for pathogens to switch host species leading to

epidemic spread in new host populations is a major veterinary and public health concern.

In veterinary medicine, *S. aureus* stands out for its involvement in infections of the mammary gland of lactating females that results in significant economic loss to the dairy industries. In sheep and goats, it is responsible for acute gangrenous mastitis, as well as skin disorders such as dermatitis and folliculitis. In cattle, breast infection tends to progress to chronic or subclinical forms. *S. aureus* also causes serious economic losses in rabbit breeding, since it affects all production levels, mainly causing mastitis and pododermatitis in breeding females and pyoderma in rabbits, although otitis and rhinitis are also common (Corpa et al., 2009). It is transmitted between animals by direct contact and through secretions or utensils. The high prevalence of carriers could increase the incidence of staphylococcus on farms (Selva et al., 2015) since these animals do not have lesions and are not usually eliminated.

There are numerous strains of *S. aureus*, which can be characterized by different techniques. Traditionally, it has been differentiated between high and low virulence strains, the most frequent clones in rabbit breeding being ST121 and ST96 respectively (Vancraeynest et al., 2006; Viana et al., 2011). However, in recent years, both veterinarians and farmers have expressed concern about the appearance of particularly virulent staphylococcal outbreaks and the difficulty of treating these outbreaks, probably due to the great adaptability of *S. aureus*, leading to the appearance of new clones. This seems evidence that the classification is more complex than described.

Population genetic studies from multiple hosts and geographic locales reveal a clonal population structure, with strong evidence of host-specialization among clones (Reinoso et al., 2004). Clones with distinctive genetic backgrounds are responsible for most infections within a host type. The molecular basis of host-specialization in *S. aureus* remains unknown despite its clinical importance from a human and animal health perspective.

2.5.1. *Staphylococcus aureus* in rabbit production

In the last 30–40 years, rabbit farming intensification has coincided with a highly virulent epidemic clone of *S. aureus* emerging, that it has already mentioned, known as ST121, which is associated with the most chronic staphylococcal rabbit infections, mainly skin abscesses, pododermatitis and mastitis on commercial rabbitries. This strain does not affect only rabbits, Clonal Complex 121 strains are also associated with distinct clinical entities in humans (Kurt et al., 2013), and are a particular common cause of human skin and soft tissue infections (McCaig et al., 2006). However, other less common lineages can be involved in staphylococcal rabbit infections, such as ST96, which is the second commonest lineage defined by multi-locus sequence typing (MLST) involved in rabbit staphylococcal infections. In experimental infections of the dermis in rabbits, *S. aureus* ST96 strains are not capable of producing abscesses, however, these strains are isolated from lesions in farms. In experimental mammary gland infections in lactating females, inoculation of ST96 clones did cause infection but this infection generated different immune response than the one produced by ST121 strains and the injuries were less severe too (Penadés et al., 2020). This suggests that certain conditions exist in rabbit farms that make it easier for *S. aureus* to cause infection in animals. These conditions can be both animal conditions (immunosuppression due to disease or production stress, treatments with antibiotics or other substances…), and environmental conditions (temperature changes, cleaning and disinfection of farms...).

Farmers and veterinarians have also warned about an increase in the frequency and virulence of staphylococcal infections in rabbit farms in the last years. This increase in virulence may be due to the appearance of new clones of *S. aureus* with virulence factors that favor its adaptation to the host and the appearance of antibiotic resistant strains that may be selected when treating animals with antibiotics. Epizootic rabbit enteropathy (ERE) is a fatal condition to infected animals and threaten the rabbit production industry (Puon Peláez et al., 2018). This disease makes antibiotic treatment necessary in the fattening phase of rabbits even when there is a trend in the

reduction in the use of antibiotics in animal production, and therefore this generalized use of antibiotics favors the appearance of resistant strains of *S. aureus*, which is a public and animal health problem.

2.5.2. MRSA in livestock and wild animals

As explained above, one of the most important resistances, especially in this bacterium, is resistance to methicillin. The clonal complex of *S. aureus* resistant to methicillin (MRSA), widely related to livestock (LA-MRSA), is CC398. This clonal complex was first described in 2005, it usually colonizes the pig (Armand-Lefevre et al., 2005; Conceicão et al., 2017), but it has also been isolated in staphylococcal infections in horses, cows and chickens (Graveland et al., 2011). Before this, finding MRSA infections have been considered sporadic infections produced by isolates of human origin (Vanderhaeghen et al., 2010; Fitzgerald, 2012; Chatterjee and Otto, 2013), and due to persistent contact between individuals (Weese, 2010; Fitzgerald, 2012; McCarthy et al., 2012). The importance of the finding of this clone MRSA CC398 related to the swine sector is that it was the first description of a MRSA reservoir in slaughter animals (Fluit, 2012; Petinaki and Spiliopoulou, 2012) thus this clone plays an important role as a reservoir of transmission to humans (Frana et al., 2013). It belongs to a differenced genetic line previously no detected and it represents the ability of MRSA clones to adapt to other hosts. Currently it is mainly associated with occupational exposure but can be highly pathogenic. In addition, its presence in foods of animal origin has been investigated, and although it has been detected, contact transmission is considered to be the most frequent mechanism and therefore constitutes an occupational risk (Verkade and Kluytmans, 2014). In industrial rabbit farming, only one case of MRSA has been described, in 2014 in a farm in Italy, produced by the clonal complex associated with CC398 livestock (Agnoletti et al., 2014).

The great dissemination of LA-MRSA ST398 raised the need to know its origin. The first data pointed to a porcine origin since ST398 MSSA was detected in pigs (Armand-Lefevre et al., 2005; Guardabassi et al., 2007).

However, subsequent massive sequencing studies suggest that the ancestral origin of CC398 is human, having subsequently adapted to pigs (Price et al., 2012). Other clones of MRSA for which inter-species jumps have been observed and that define slaughter animals as a reservoir of MRSA have been CC5 (in poultry), CC97 and CC130 (both in cattle).

In Europe, a broad animal host-spectrum of MRSA comprising companion and wild-living animals as well as livestock have been discovered posing the question of the impact of those strains as source for zoonotic infections. In the case of cattle, CC97 is a genotype whose frequency of isolation is increasing in humans and its origin is bovine (Spoor et al., 2013). Besides CC130 is one of the genetic lines in which the *mec*C gene has been most frequently detected in both humans and animals, having recently analyzed the potential transmission between small ruminants and people (Harrison et al., 2013). In the last years, this *mec*C gene type has been found so widespread among livestock, companion, and wildlife animals. Following the first description of *mec*C-MRSA in specimens of bovine sources (dairy cattle, beef cattle and milk), it was found also in isolates recovered from specimens of other livestock animals, such as sheep and commercial rabbits. The most widely distributed clonal complex in *mec*C-MRSA is CC130; it has been found in several animal species including those with economic impact such as cattle and other commercial animals. Also, other CCs that have been found in *mec*C-positive isolates, such as CC49 and CC425, have been associated with infections and colonization in animals (García-Álvarez et al., 2011; Meemken et al., 2013; Simpson et al., 2013; Vandendriessche et al., 2013).

Staphylococci harboring *mec*C, seem to be widely distributed among wildlife animals too. Those isolates were recovered from animals colonizing quite different habitats and belonging to different classes like birds and mammals. This reinforces the hypothesis that wild rodents, insectivores and small carnivores may serve as reservoir for MRSA and that cassettes of the SCC family may be introduced into livestock or companion animals e.g. via rodent-colonizing bacterial species (Becker et al., 2014). Moreover, it has

been demonstrated by analysis of the soil resistome that bacteria that express resistance to antibiotics are widespread.

The ability of MRSA to adapt to more than one host, including the human species, together with a high prevalence of MRSA in certain animal species, suppose a risk to the population as slaughter animals act as a reservoir of MRSA (32). Therefore, studies of the dissemination of virulence factors and resistance to antibiotics are necessary to explain the appearance of new *S. aureus* clones and the success in their dissemination.

3. The host: Characteristics of the rabbit as a production animal

Rabbit meat production worldwide is not very important, assuming less than 0.5% of world production. However, according to FAO sources, Spain is among the 4 largest producers of rabbit meat behind China, Italy and France. Rabbit meat production is located mainly in the Mediterranean, where there is a greater demand for rabbit meat: Italy, Spain and France are the main producers and consumers. In Spain and Portugal, this production has become more concentrated and intensified in recent years, it has evolved from more or less traditional production systems to other more intensive ones due to relevant advances in genetic selection, reproductive management and feeding systems (Pascual, 2010). Farms now house large numbers of animals that are exposed to sanitary problems.

Due to their growth capacity, reproductive characteristics, and ease of handling on farms, medium-sized breeds of rabbit are the most widely used in the production of rabbit meat. The California and White New Zealand breeds are the most used. Rabbit breeding is done primarily by selecting lines for desired characters. The different rabbit lines arise from small populations in which the founder animals are animals with very outstanding values for the desired characters, coming from several populations with a good productive level or a large commercial population. These lines are generally subjected to a selection program with clear selection objectives and effective selection methods. These are lines in which there is more uniformity than in a breed, the results are more predictable, are productively

specialized and are usually owned by companies or institutions. Genetic selection for reproduction has worked, with programs resulting in an effective increase of between 0.05 and 0.13 live-born kits per generation of selection (Gómez et al., 1996; García and Baselga, 2002a, 2002b).

The Spanish rabbit production is evolving to farms of more than two hundred does and it is compulsory to increase productivity, because production costs and sale prices are very close. Nowadays, the use of more productive animals is increasing (Rosell, 1996); the demand of crossbred does and the use of males from specialized paternal lines is becoming higher and higher. Two public institutions work since 1976 in the development of new lines of rabbits under the scope of their use in a three-way scheme of crossbreeding; they are the Unit of Rabbit Science (Barcelona, I.R.T.A.) and the Department of Animal Science (Valencia, U.P.V.). Consequently, the efforts have been focused on the foundation and selection of maternal and paternal lines. Specialized maternal lines are devoted to produce crossbred does by crossing two of them, while paternal lines supply the sires to be mated to the crossbred does (Baselga et al., 1999). The rabbit lines selected in Spain currently are: Line A (UPV), Line C (IRTA), Line P (IRTA), Line R (UPV), Line V (UPV), Line HH (UPV) and line LP (UPV).

Litter size and food conversion rate are still the most important traits in rabbit meat production. Litter size is difficult to improve by selection and intensive research is being carried out to increase it. Food conversion rate is expensive to measure, but further research is needed about implementing records of this trait in selection programmes. The R line (Pink), is a paternal line selected for 25 generations for its average daily gain of the kits between the 4th and 9th weeks of life that is related to food conversion and easier to measure. However the selection by certain parameters has sometimes been accompanied by undesired side effects (Rauw et al., 1998) such as higher disease incidence (Dourmad et al., 1994) or reproductive problems. In a study in which reproductive parameters were evaluated in R line, it was observed that the rabbits in production showed serious reproductive problems that were attributed to the effect of selection by GMD (Vicente et

al., 2012). In addition to the productive objectives, health may be considered one of the main concerns of current rabbit production under commercial conditions, with high replacement rates and the frequent appearance of digestive disorders (Rosell and de la Fuente, 2009). In fact, one of the main objectives in animal production is the maintenance of a balance between productivity and animal health. Therefore, studies are needed in production animals to evaluate whether selection for productive parameters affects their health.

3.1. Cryopreservation in genetic selection

Embryo cryopreservation is a mechanism that consists of freezing embryos at low temperatures to keep them viable in the long term and it is used to carry out the selection of production animals among other reasons because it allows to conserve unique genetic material which might otherwise be lost. In addition to this, cryopreservation makes it possible to evaluate the genetic improvement of the selected traits and of other traits that may be indirectly altering. This is because thanks to cryopreservation, animals of any generation can be obtained at any given time.

However, the cryopreservation procedure may induce differences in environmental effects that in fact could cause changes in embryo, fetuses, and postnatal development (Armbrust and Eisen, 1994). The effect of the genotype on the efficiency of cryobanked embryos in the rat, mouse, and rabbit has been observed (Rall et al., 2000; Lavara et al., 2011). As an example, cryopreserved embryos from paternal rabbit lines (selected for growth characteristics) showed reduced survival rate compared with cryopreserved embryos from maternal lines (selected for reproductive characteristics) (Vicente et al., 2003). Even though, little is known about the possible effect of embryo cryopreservation and transfer on postnatal growth development in mammals and these effects may affect the results when cryopreservation is used to evaluate some parameter.

3.2. The flow cytometry tool as a way to assess the immune response

Fluorescence-activated flow cytometry is one of the most powerful technologies that is routinely used in immunology. It enables identification, characterization, and isolation of defined leukocyte subsets. This technology has also revealed that there are hundreds of phenotypically distinct cell types in the peripheral blood (De Rosa and Roederer, 2001). The ability to discriminate between these cell types is crucial to our understanding of cellular immunity and disease pathogenesis.

Two physical parameters (forward and side scatter) and two fluorescence parameters are sufficient for simple discrimination between peripheral blood cells (Lanier et al., 1983). Forward- and side-scatter characteristics can be used to distinguish monocytes and granulocytes (high side scatter and high forward scatter) from platelets and erythrocytes (low side scatter and low forward scatter). Lymphocytes and circulating dendritic cells show intermediate forward and side scatter and can be further segregated by lineage (that is, B cells, T cells or natural killer (NK) cells) using one or more fluorescence parameters. Within T-cell populations, various markers have been proposed to distinguish effector- and memory-cell populations. These markers are often paired (Davis et al., 1995; Perfetto et al., 2004), (e.g. CD14 with CD45 expressed by granulocytes). Panels of well characterized monoclonal antibodies (mAb) provide a powerful tool to analyse leukocyte populations.

The rabbit is an example of a species where there is a critical need for flow cytometry mAb reagents (NCBI Rabbit Genome Resources, USA). To date, however, only a few mAbs have been developed to meet this need and this makes necessary to look for antibodies from other species to complete the panel of mAbs. The probability of finding a mAb that recognizes an epitope conserved on orthologous leukocyte differentiation molecules (LDM) is greater between closely related species than between distantly related species (Brodersen et al., 1998) and although they are few, some mAbs recognize cell receptors from another species and allow LDM studies to be

carried out in a broader range of species (Davis and Hamilton, 2008). The use of flow cytometry for the evaluation of the immune system of rabbits is much less widespread than in other species due to this limitation in the existing monoclonal antibodies (mAbs) for this species, but more and more studies show its usefulness for the use of the rabbit as a research animal (Davis and Hamilton, 2008) for the study of diseases that affect rabbits (Guerrero et al., 2011) and for the comparison of different lines of selected rabbits based on different productive parameters (Ferrian et al., 2013).

3.3. Immune system and its relation to the success of pathogen infections

The immune system must ensure effective defence against pathological microbes and toxic or allergenic proteins and avoid inflammatory responses that produce excessive damage of self-tissues or that might eliminate beneficial, commensal microbes (Josefowicz et al., 2012). During the early stages of infection, the effective destruction of pathogens depends on the adequate coordination of a complex series of cellular and molecular processes in the host, given the logarithmic growth of microorganisms during these first steps of infection (Rigby and DeLeo, 2012). The immune system uses a complex array of protective mechanisms to control and usually eliminate these organisms and toxins.

The immune response includes the innate and adaptive systems, and although the innate mechanisms were considered part of a nonspecific inflammatory response (including neutrophil and macrophage phagocytosis and complement activation) it has considerable specificity. This is because it is directly directed towards molecular components present on the surface of microorganisms known as pathogen associated molecular patterns (PAMPs) recognized through interaction with the pattern recognition receptors (PRRs). Effective sensing of PAMPs rapidly induces host immune responses via the activation of complex signalling pathways that culminate in the induction of inflammatory responses mediated by various cytokines

and chemokines, which subsequently facilitate the eradication of the pathogen (Takeuchi and Akira, 2010).

The innate immune system also mounts an effective defence against infectious agents through the initiation of adaptive immunity, which is long-lasting and has immunological memory. Adaptive immunity is mediated via the generation of pathogen (antigen)-specific B and T lymphocytes through a process of gene rearrangement of germ-line gene elements to form intact T cell receptor (TCR) and immunoglobulin (B cell antigen receptor; Ig) genes (Chaplin, 2010).

In *S. aureus* infections, the innate response is the most important in determining the evolution of an infection since adaptive immunity alone is not sufficient for optimal defense against *S. aureus* infections (Chan et al., 2017).

The innate and adaptive immune systems usually act together, with the innate response representing the first line of host defense, and with the adaptive response becoming prominent after several days, as antigen-specific T and B cells have undergone clonal expansion. Synergy between both is essential for an intact, fully effective immune response even when they immune responses are fundamentally different in their mechanisms of action (Chaplin, 2010).

3.3.1. Polymorphonuclear neutrophils and its role in the innate immune response

The innate immune system includes different cell types, such as blood monocytes and tissue macrophages, polymorphonuclear cells (neutrophils, eosinophils, and basophils), dendritic cells, and NK cells, and although these are all considered the host's first line of defense, neutrophils play the central role of bacteria-host interaction (Kin and Sanders, 2006).

Polymorphonuclear neutrophils (PMN) belong to the innate immune system and constitute the main defense against invading bacteria and fungi. Neutrophils are short-lived granulocytes derived from pluripotent

hematopoietic stem cells in the bone marrow (Weissman et al., 2001) and they are classically underappreciated professional phagocytes. The killing mechanisms of PMN are: phagocytosis, degranulation and NETs (Kolaczkowska and Kubes, 2013).

During the PMN recruitment, some circulating neutrophils roll along the walls of postcapillary venules, by interacting with endothelial cells, surveying connective tissue, mucosal membranes, skeletal muscle, and lymphatic organs for signs of tissue damage, inflammation, or invading microorganisms (Alexander and Klaus, 2012). This PMN searches for the presence of host- and/or pathogen-derived chemotactic signals or chemoattractants that are produced by a variety of host cells in response to damage or the presence of invading pathogens. These signals direct neutrophil chemotactic movement out of intravascular circulation to sites of damage or infection within tissues, resulting in a rapid influx and accumulation of PMN (Rigby and DeLeo, 2012).

Once at the site of infection, the PMN bind and ingest invading microorganisms by a process known as phagocytosis, a critical first step in removal of bacteria during infection. Neutrophils recognize PAMPs that are recognized directly by PRRs expressed on the surface of the neutrophil. Engagement of such receptors activates signal transduction pathways that prolong cell survival, facilitate adhesion and phagocytosis, induce release of cytokines and chemokines, elicit degranulation, and promote reactive oxygen species (ROS) production and release, ultimately contributing to microbicidal activity (Kobayashi et al., 2005).

The process of neutrophil phagocytosis triggers synthesis of a number of immunomodulatory factors (Kobayashi et al., 2003) that modulates subsequent neutrophil responses, and coordinates early responses of other cells types such as monocytes, macrophages, dendritic cells, and lymphocytes, thereby providing an important link between innate and acquired immune responses. Additionally, phagocytosis has been shown to accelerate programmed cell death (apoptosis) of neutrophils (Kobayashi et

al., 2003) a phenomenon that critical to the resolution of the inflammatory response (Kennedy and DeLeo, 2009).

Moreover, phagocytosis is accompanied by the generation of microbicidal ROS (oxygen-dependent) and fusion of cytoplasmic granules with microbe-containing phagosomes (degranulation). Degranulation enriches the phagosome lumen with antimicrobial peptides and proteases (oxygen-independent process), which in combination with ROS create an environment non-conducive to survival of the ingested microbe(s) (Rigby and DeLeo, 2012).

Lastly, PMNs can also release NETs (extracellular neutrophil traps), which are composed of DNA, histones, antimicrobial peptides, and proteases that envelop bacteria for destruction, minimizing tissue damage (Brinkmann et al., 2004; Fuchs et al., 2007).

3.3.2. Monocytes and macrophages

Macrophages are long-lived cells with potent endocytic, phagocytic, and secretory functions, able to modulate their properties upon contact with different cell types as well as extracellular matrix. In response to inflammation and infection, bone marrow-derived blood monocytes flock to tissues attracted by chemotactic signals, and differentiate into macrophages and dendritic cells (DCs) in situ (Gordon et al., 2014). Growth factors such as M-CSF, GM-CSF and cytokines are responsible of local proliferation and self-renewal of macrophages (Sieweke and Allen, 2013).

The effects of macrophages on the local tissues include trophic as well as cytocidal interactions with neighbouring cells, the remodelling of matrix, as well as phagocytic clearance of dying cells and other homeostatic and defense functions. Together with perivascular macrophages, tissue macrophages regulate the recruitment of PMNs and monocytes to the site of infection and participate in the elimination of dead cells. They also produce ROS and RNS, antimicrobial peptides, and chelating proteins that

deprive bacteria of essential nutrients for their metabolism (Brandt et al., 2018).

3.3.3. T and B lymphocytes and the adaptative immune response

T and B lymphocytes play a crucial role in the cellular and humoral immunity, respectively and T lymphocytes influence the immune response of B lymphocytes (Xing et al., 2017). B lymphocytes are involved in humoral immunity by secreting antibodies (Parra et al., 2016) and can differentiate into plasma cells and memory B lymphocytes cell types. Activated B lymphocytes can behave as antibody-producing cells when binding to an antigen, but they can also participate as antigen presenting cells (APCs). In addition, they also intervene in immune defense through the secretion of cytokines (Drouet-Viard and Fortun-Lamothe, 2002; Hua and Hou, 2013).

T lymphocytes are involved in cellular immune response and are subdivided into helper T lymphocytes (Th) and cytotoxic T lymphocytes (CTLs), defined by the expression of CD4 and CD8 glycoprotein, respectively (Xing et al., 2018). CD25 is strongly expressed on CD4-positive regulatory T cells and is not detected on resting CD8-positive cells. However, all activated T cells express the CD25 protein. Helper T lymphocytes are activated by recognizing antigenic peptides bound to major histocompatibility complex (MHC) class II molecules, and exhibit predominantly helper functions. Furthermore, Th cells play a central role in defense against a wide variety of pathogenic microorganisms, helping B cells to produce antibodies and regulating CTL and macrophages (Cosmi et al., 2014). On the contrary, cytotoxic T lymphocytes are restricted to recognition by MHC class I molecules, and perform cytotoxic functions (Xiong and Bosselut, 2012); some of the most important functions are avoiding autoimmune reactions and stopping the effector response against exogenous antigens, when the response itself becomes dangerous for the host (Cosmi et al., 2014).

In summary, when an infection occurs there are many factors that determine its success or failure. The two central elements are the pathogen and the host, but many more intrinsic and external factors intervene. That is why if we study the pathogen or the host alone, we would lack a large amount of information derived from the interaction between the two. In fact, the evolution of organisms individually would not make sense if the environment in which they are found is not taken into account. This evolution also occurs in parallel and simultaneously, when an organism develops a virulence factor, its host develops over time other mechanisms to defend itself. Therefore, a continuous fed-back occurs between the pathogen and the host and that is why more studies that associate both elements are necessary.

OBJECTIVES

OBJECTIVES

The pathogen *S. aureus* is most important in human and veterinary medicine, and specifically in rabbit farming. This situation is further aggravated by the emerging increase in antibiotic-multiresistant bacteria. One of the most important antibiotic-multiresistant bacteria is the MRSA strain that appeared first in hospitals, and later in livestock animals, wild animals and the environment. In rabbits this dangerous clone has not been described in detail, and only one MRSA isolate has been reported on a farm in Italy (Agnoletti et al., 2014). On rabbit farms, other strains like ST121 clones are more prevalent (Viana et al., 2011), but the widespread use of antibiotics and the spread of new mutants of these clones (Pérez-Fuentes, 2019) may change this situation. Moreover, *S. aureus* (and even some MRSA) have been described in wild animals (Loncaric et al., 2013). Given the high percentage of rabbit *S. aureus* carriers on farms (Selva et al., 2015) and the problems that bacteria cause, it would also be interesting to know what happens in wild rabbits because they could play an important epidemiological role as reservoirs for *S. aureus*.

Finally, not only bacteria, but also the host and its immune response, play a key role in infections developing. In fact it has been described how different genetic lines of rabbits show differences in their immune system, and even distinct behaviour, when facing differing immunological challenges (Guerrero et al., 2011; Ferrian et al., 2012). Therefore, the study of the immune system and its response when facing experimental *S. aureus* infection would provide us with valuable information to generate more resistant rabbit genetic lines for staphylococcal infections.

In order to face this serious problem, four works were we carried out in this thesis whose objectives are detailed below:

1. Characterisation of livestock-associated methicillin-resistant *Staphylococcus aureus* isolates obtained from commercial rabbitries.

S. aureus is a widely distributed bacterium that is extremely capable of adapting. The amount and variability of virulence factors make it an able bacterium to cause many serious diseases. Its great resistance in the environment and the presence of carriers make *S. aureus* a bacterium that affects public and animal health. As described above, in recent years there has been increasing concern about the spread of virulent and resistant *S. aureus* strains, such as LA-MRSA, among farm animals. Apart from losses in animal production, this concern is due to the fact that they represent a reservoir of transmission to man. The appearance of these virulent and resistant strains in recent years coincides with the growing concern about staphylococcus due to the appearance of outbreaks on rabbit farms that are more frequent, virulent and difficult to eliminate. For all these reasons, the aims of this study were to:

- evaluate the presence of MRSA in a *S. aureus* collection obtained from staphylococcal lesions on different Spanish and Portuguese rabbit farms;

- compare the isolated strains using MLST, SCC*mec* and *agr* typing;

- determine the antibiotic resistance profile and virulence factors.

2. Characterisation of Methicillin-resistant *Staphylococcus aureus* in wild lagomorphs located in high density areas

However, commercial rabbits are not the only places affected by *S. aureus* as *mec*C-MRSA strains have also been isolated in wild animals. Wild rabbits could act as a reservoir for possible transmissions to wild fauna, production rabbits and as a reservoir of transmission to hunters. The role of *S. aureus* as a possible pathogen or coloniser in wild populations of lagomorphs has not yet been studied in a mass systematic manner. In high density areas, the interaction between rabbits and other animals is more marked and,

therefore, the probability of *S. aureus* transmission increases. For these reasons, the following objectives were set in this study to:

- know the role of wild rabbits and hares as a reservoir of *S. aureus* in wild lagomorph high density areas;

- characterise the *S. aureus* strains isolated from wild rabbits and hares to compare them to those obtained previously on commercial farms;

- study the prevalence of MRSA strains in wild populations.

3. Phenotypic and genotypic study of the SCC*mec* cassette element in MRSA and MSSA strains isolated from rabbits

The emerging increase in antibiotic-resistant bacteria in recent years has raised global concern as clones of bacteria resistant to all antibiotics have already appeared among farm animals given their widespread use. This very much complicates their treatment and creates dangerous clones that could act as a reservoir of human transmission. *S. aureus* is a dangerous bacterium because of its excellent capacity to develop resistance to antibiotics. In this species, one of the clones that caused most concern was MRSA strains, not only because they present resistance to methicillin, but also because these clones are usually multiresistant. Given this increasing importance of antibiotic resistances, especially methicillin resistance, and its relation with mobile genetic elements, the objectives of this work were:

- the genotypic study of mobile genetic element SCC*mec* in a selection of *S. aureus* strains isolated from rabbits;

- the phenotypical study of the antibiotic resistances presented by strains MSSA and MRSA.

4. Effect of selection by growth rate and vitrification of embryos on the rabbit (*Oryctolagus cuniculus*) immune system and its response after a *Staphylococcus aureus* experimental infection

On rabbit farms, it is very important to maintain a balance between productivity and animal health to obtain good results. Animals' susceptibility to suffer from a disease depends on many factors, among which the most important are the productive state and genetics (selection to which animals are subjected). This selection for specific characters sometimes causes other characteristics to be indirectly altered, such as health. Therefore, it is necessary to control other relevant aspects when selecting animals. One of the mechanisms that controls the effects of selection on selected characters and other characters is embryo cryopreservation, which allows animals from previous generations to be obtained. However, this technique has been found to affect the animals obtained by it and, therefore, its effects must also be monitored. With the R line, a paternal line of rabbits selected by daily weight gain, increased frequency of diseases has been observed compared to other maternal lines. Therefore, the following objectives were set in this work to:

- evaluate the effect of selection by growth rate on the immune system at two levels (maintaining immune competence under conventional conditions and mounting immune response to an immunological challenge with *S. aureus*);

- evaluate the effect of embryo vitrification when mounting the immune response when facing an immunological challenge with *S. aureus.*