UNIVERSIDAD DE LA RIOJA

TESIS DOCTORAL

Título
***Staphylococcus* spp. from animals intended for human consumption, animal-derived food and humans in Spain and Senegal: genetic lineages, antibioresistance and virulence**
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Doctoral Thesis

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Olouwafêmi Mistourath Mama

Universidad de La Rioja, Logroño, 2019

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Thesis presented by **OLOUWAFEMI MISTOURATH MAMA** for the PhD degree with International Mention of the Universidad de La Rioja.

Logroño, November 2019

UNIVERSIDAD DE LA RIOJA

Departamento de Agricultura y Alimentación

Área de Bioquímica y Biología Molecular

Dra**. CARMEN TORRES MANRIQUE**, Catedrática del Área de Bioquímica y Biología Molecular de la Universidad de La Rioja

Por la presente declara que,

La memoria titulada "S***taphylococcus*** **spp. from animals intended for human consumption, animal-derived food and humans in Spain and Senegal: genetic lineages, antibioresistance and virulence"** que presenta Dña **OLOUWAFEMI MISTOURATH MAMA,** graduada en Ingeniería en Industrias Alimentarias por la Escuela Superior Politécnica de Dakar (UCAD), Senegal y Máster en Química y Biotecnología por la Universidad de La Rioja, ha sido realizada en el Área de Bioquímica y Biología Molecular de la Universidad de La Rioja, bajo su dirección y reúne las condiciones exigidas para optar al grado de Doctor.

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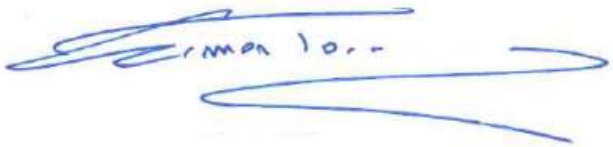

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ABBREVIATIONS

ACME	Arginine Catabolic Mobile Element
agr	Accessory Gene Regulator
AIP	Autoinducing Peptide
BHI	Brain Heart Infusion
CA-MRSA	Community-Associated MRSA
CASFM	Comité de L'antibiogramme de la Société Française de Microbiologie
CC	Clonal Complex
ccr	Cassette Chromosome Recombinase
CDDEP	Center for Disease Dynamics, Economics and Policy
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase-Negative staphylococci
CoPS	Coagulase-Positive staphylococci
Et	Exfoliative toxin
EUCAST	European Society of Clinical Microbiology and Infectious Diseases
FAO	Food and Agriculture Organization
HA-MRSA	Hospital-Associated MRSA
hl	Hemolysin
HPD	High Pig Density
ICU	Intensive Care Unit
IEC	Immune Evasion Cluster
LA-MRSA	Livestock-Associated MRSA
LPD	Low Pig Density
MALDI/TOF	Matrix-Assisted Laser Desorption/Ionization Time Of Flight
MDR	Multidrug Resistance/Resistant
MLS	Macrolide-Lincosamides-Streptogramins
MLST	Multi Locus Sequence Type
MPD	Medium Pig Density
MRSA	Methicillin-Resistant *S. aureus*
MSA	Mannitol Salt Agar

MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin-Susceptible *S. aureus*
NICU	Neonatal Intensive Cares Units
OIE	Organization of Animal Health
ORSAB	Oxacillin Resistance Screening Agar Base
PBP	Penicillin-Binding Protein
PCR	Poly Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PVL	Panton-Valentine Leukocidin
SA	*Staphylococcus aureus*
SCC	Staphylococcal Chromosomal Cassette
SCIN	Staphylococcal Complement Inhibitor
SE	Staphylococcal Enterotoxin
SFP	Staphylococcal Food Poisoning
spa	Staphylococcal Protein A
SSSS	Staphylococcal Scaled Skin Syndrome
SSTI	Skin and Soft Tissues Infection
ST	Sequence Type
SXT	Sulfamethoxazole Trimethoprim
TET^R-MRSA	Tetracycline-Resistant MRSA
TSST	Toxic-Shock Syndrome Toxin
WGS	Whole Genome Sequencing
WHO	World Health Organization

SUMMARY/ RESUMEN

SUMMARY

The first antimicrobial resistance global report on surveillance of the World Health Organization (WHO) in 2014 pointed out the need to deal with the problem under a "One Health" approach which focusses on the interactions between humans, animals and their environments, in order to enhance health and welfare. Staphylococcal species (both coagulase-positive (CoPS) and -negative (CoNS)) are normal colonizers of skin and mucous of healthy humans and animals, but also opportunistic pathogens, that can cause skin and soft tissues infections, mastitis, food intoxication, bacteraemia etc. This thesis aimed to analyse the diversity of staphylococcal species in different niches, including wild and farm animals destined for human consumption, animal-derived food and humans, to determine the antimicrobial resistance phenotype/genotype of recovered isolates, and to evaluate the occurrence of livestock-associated lineages among *S. aureus* isolates.

The first chapter studied the molecular epidemiology of staphylococcal species in nasal and/or faecal samples of non-conventional food animals such as wild boar and horses in Spain. A great diversity of staphylococcal species was observed in wild boar, being *S. aureus* (76.1%) and *S. hyicus* (16.4%) the predominant CoPS, and *S. sciuri* (39.7%) and *S. xylosus* (13%) the predominant CoNS. In horses, the predominant species were *S. aureus* (37%), *S. delphini* (21%) and *S. sciuri* (21%). Most isolates recovered from wild boar were pan susceptible (73.1% of CoPS and 77.6% of CoNS). However unusual genes involved in macrolide, lincosamide and phenicol resistance were detected among CoNS. Regarding horses' isolates, 88.7% of CoPS were pan susceptible while 48.6% of CoNS showed resistance to at least one of the antimicrobial agents. A multidrug resistance phenotype (MDR) was only observed among CoNS from both animal species. The predominant *S. aureus* lineage in wild boar was t3750-ST2328/CC133 (29.4%) and one isolate of the livestock-associated methicillin resistant *S. aureus* lineage (LA-MRSA-CC398) (t011-ST398) was identified. *S. aureus* lineage ST1640 (61.7% of *S. aureus*), is associated with horses for the first time in this study. All *S. aureus* isolates detected from horses, except those of lineage ST1460, carried the genes encoding the equid-adapted leukocidin (LukPQ) and the blocker of equine complement system activation (eqSCIN).

The second chapter focussed on staphylococci from common food-producing animals from two countries with different antibiotic use policy (calve, lamb, goat (Spain); cow and chicken (Senegal)). In Spain, the prevalence of staphylococci was higher in lamb (54%) and goat (50%) than in cow (21%) samples. In Senegal, staphylococci prevalence was higher in cow (26.8%) than in chicken (3%). In Spain, the most frequent lineage was

CC133 while the ST291 was dominant in Senegal isolates. Six species were identified among CoNS in each country, with prevalent species being *S. sciuri* and *S. simulans*. CoPS isolates from Spain were mostly pan susceptible, while those from Senegal were mostly penicillin resistant. In both countries, a relatively high percentage of CoNS isolates (32.5% (Senegal), 47% (Spain)) was resistant to at least one of the antimicrobials tested. Resistance to penicillin, tetracycline and SXT was observed among CoNS isolates of both countries. Additionally, resistance to methicillin, streptomycin, clindamycin, erythromycin, chloramphenicol and tobramycin was observed among Spain isolates. The Panton-Valentine Leukocidin (PVL) encoding genes were present in isolates of both countries, and the human virulent clone USA300 was identified among Spain isolates.

In the chapters three and four, the lineage CC398 was screened for pig-derived food *S. aureus* isolates on one hand, and among patients' blood culture *S. aureus* isolates from Spanish hospitals located in regions with different pig density degrees, on the other hand (multicentre study).

CC398 was the predominant lineage among *S. aureus* isolates of pig products (64.1%; n=23). The prevalence of MRSA-CC398 was 20.8%, observing differences in products with skin (76.5% (ear/snout)) and without skin (3.3% (fillet), 14.8% (chopped meat)); this clone was mostly typed as t011. All MRSA-CC398 isolates were *mecA*-positive, tetracycline resistant (*tet*(M) and *tet*(K)+/- *tet*(L)) and *scn*-negative. 82.6% were MDR and 17.4% harboured virulence genes. Two methicillin susceptible *S. aureus* (MSSA)-CC398 isolates were detected; both being resistant to penicillin and erythromycin/clindamycin-inducible (*erm*(T) gene). They were typed as t5452 and were *scn*-positive.

Concerning the clinical isolates, the prevalence of CC398 isolates among *S. aureus* was 4.3%. MSSA-CC398 represented 90.2% of total CC398 isolates, corresponding to 5.2% of MSSA and 3.9% of *S. aureus*. t571 and t1451 were the predominant *spa*-types among MSSA-CC398 isolates. Resistance to erythromycin/clindamycin-inducible, mediated by *erm*(T) gene, was only observed among MSSA-CC398 isolates (72.9%). All MSSA-CC398 isolates, but three, were *scn*-positive. No obvious correlation was observed between MSSA-CC398 prevalence at hospital level and the pig density degree in surrounding areas. LA-MRSA-CC398 and LA-MRSA-CC1 were detected in hospitals located in high pig density areas.

El primer informe global de la Organización Mundial de la Salud (OMS) sobre la situación de la resistencia a antibióticos, publicado en 2014, destacaba la necesidad de abordar ese problema con un enfoque "One Health", centrándose en las interacciones entre los humanos, los animales y el medio ambiente. Las especies del género *Staphylococcus,* tanto coagulasa-positivo (SCoP) como coagulasa-negativo (SCoN), son comensales de piel y mucosas de personas y animales sanos, siendo a su vez patógenos oportunistas que pueden causar infecciones de piel y partes blandas, mastitis, intoxicaciones alimentarias, bacteriemias, etc. El objetivo de esta tesis fue analizar en primer lugar la diversidad de especies estafilocócicas en distintos nichos ecológicos incluyendo los animales salvajes y los de granja destinados al consumo humano, los animales de producción y las personas. Posteriormente, determinar el fenotipo/genotipo de resistencia a antibióticos de los aislados detectados, y evaluar la prevalencia de las líneas asociadas al ganado entre los aislados de *S. aureus*.

En el primer capítulo, se estudió la epidemiología molecular de *Staphylococcus* spp. en especies animales destinadas al consumo humano minoritarias como son los jabalíes y los caballos en España. Se observó una gran diversidad estafilocócica en muestras nasales de jabalíes, siendo *S. aureus* (76.1%) y *S. hyicus* (16.4%) las especies predominantes entre los SCoP, y *S. sciuri* (39.7%) y *S. xylosus* (13%) las predominantes entre los SCoN. En los caballos, las especies predominantes fueron: *S. aureus* (37%), *S. delphini* (21%) y *S. sciuri* (21%). La mayoría de los aislados de jabalíes fueron pansensibles (73,1% de los SCoP y 77,6% de los SCoN). Sin embargo, se detectaron entre los aislados SCoN, genes inusuales codificantes de la resistencia a macrólidos, lincosamides y fenicoles. En cuanto a los aislados de caballos, un 88,7% de los SCoP fueron pansensibles mientras que un 48,6% de los SCoN mostraron resistencia a al menos un antibiótico testado. Se observaron fenotipos de multirresistencia solo en aislados SCoN en ambas especies animales. Entre los *S. aureus* de jabalíes, el linaje predominante fue el t3750-ST2328/CC133 (29,4%). También se detectó un aislado t011 del clon asociado a ganado LA-MRSA-CC398. ST1640 fue la línea genética más frecuente entre los *S. aureus* de caballos (61,7%). Todos los aislados *S. aureus* de caballos, excepto los del linaje ST1640 portaban los genes codificantes de la leucocidina específica de équidos (LukPQ) y del bloqueador de la activación del sistema complementario equino (eqSCIN).

El segundo capítulo se centró en estafilococos de animales de producción de dos países con diferentes políticas de uso de antibióticos (ternero, cordero y cabra (España);

vaca y pollo (Senegal)). En España, la prevalencia de estafilococos fue más elevada en muestras de ovino (54%) y caprino (50%), en comparación con las de vacuno (21%). En Senegal, se detectó una prevalencia mucho más alta en vacuno (26,8%) que en pollo (3%). La línea genética de *S. aureus* más frecuente en los aislados de España, fue el CC133 mientras que el ST291 fue el más abundante entre los aislados de Senegal. Se identificaron seis especies SCoN en cada uno de los países, siendo *S. sciuri* y *S. simulans* las más frecuentes en ambos países. Los aislados SCoP fueron mayoritariamente pan sensibles (España) o resistentes a penicilina (Senegal). En ambos países, un porcentaje relativamente elevado de los aislados SCoN presentaron resistencia a al menos uno de los antibióticos testados (32,5% (Senegal) y 47% (España)). Entre los SCoN de ambos países se detectaron aislados resistentes a penicilina, tetraciclina y SXT. Además, se observó resistencia a meticilina, estreptomicina, clindamicina, eritromicina, cloranfenicol y tobramicina entre los aislados de España. En aislados de ambos países, se detectaron los genes codificantes de la leucocidina de Panton-Valentine y se identificó el clon USA300 entre los aislados de España.

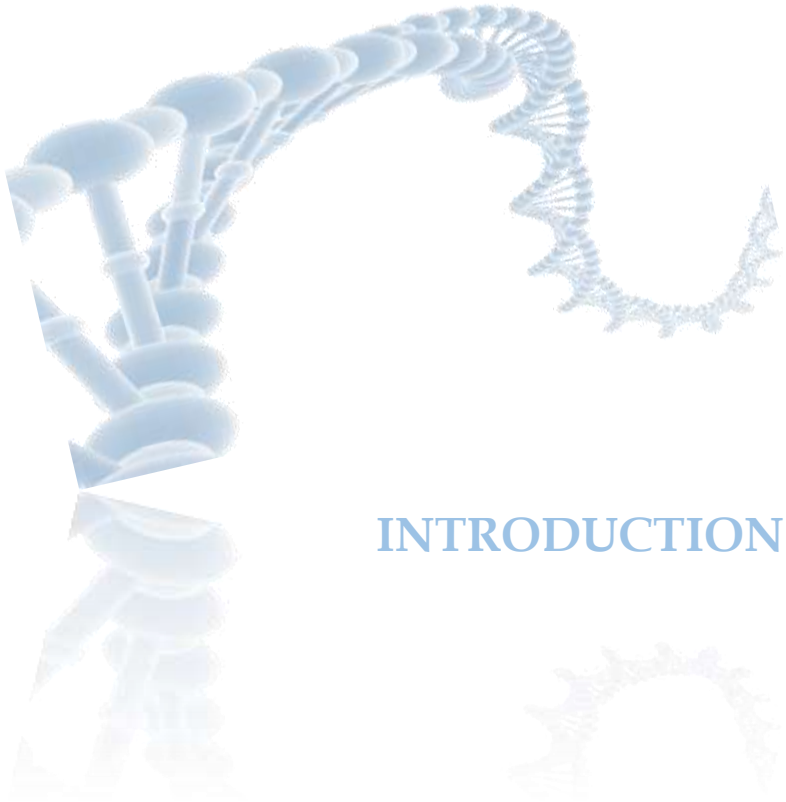
En los capítulos tres y cuatro, se analizó la presencia del linaje CC398, por un lado entre aislados *S. aureus* de productos derivados de cerdo, y por otro lado entre aislados *S. aureus* de hemocultivos de pacientes de hospitales españoles (estudio multicéntrico) situados en provincias con diferentes niveles de densidad porcina.

El linaje CC398 fue predominante entre los aislados *S. aureus* de productos derivados de cerdo (64,1%; n=23). La prevalencia de *S. aureus* resistente a meticilina (SARM)-CC398 fue del 20,8% (en productos con piel (oreja/morro, 76,6%); y sin piel (filete, 3,3%; carne picada, 14,8%); siendo este clon mayoritariamente asociado al *spa*-tipo t011. Todos los aislados MRSA-CC398 fueron *mecA*-positivos, tetraciclina-resistentes (*tet*(M) y *tet*(K)+/-*tet*(L)) y *scn*-negativo. El 82,6% de estos aislados fueron multirresistentes y el 17,4% portaban genes de virulencia. Se detectaron dos aislados sensibles a meticilina (SASM)-CC398. Ambos aislados fueron resistentes a penicilina y eritromicina/clindamicina-inducible (*erm*(T)), *scn*-positivos y tipados como t5452.

En cuanto a los aislados clínicos, el 4,3% de los aislados *S. aureus* pertenecían al linaje CC398, siendo el 90,2% de ellos SASM, lo que representa un 5,2% y 3,9% de los aislados SASM y *S. aureus*, respectivamente. Los *spa*-tipos t571 y t1451 fueron predominantes entre los aislados SASM-CC398. La resistencia a eritromicina/clindamicina-inducible mediada por el gen *erm*(T) fue observada solo en aislados SASM-CC398 (72,9%). Todos

los aislados SASM-CC398, excepto tres, fueron *scn*-positivos. No se observó una relación clara entre la prevalencia de SASM-CC398 en los hospitales y el nivel de densidad porcina de la zona correspondiente. Los aislados LA-SARM-CC398 fueron infrecuentes y detectados en hospitales situados en provincias de alta densidad porcina.

RESUMEN



INTRODUCTION

1. THE ANTIBIOTIC RESISTANCE ISSUE

According to the World Health Organization (WHO), antibiotic resistance is, today, one of the biggest threats to global health, food security and development. Antibiotic resistance is rising worldwide, and new resistance mechanisms are emerging and spreading. This issue implies less efficiency in the prevention and treatment of infectious diseases such as pneumonia, tuberculosis, bacteraemia, foodborne diseases etc., leading to higher mortality rates.

Actually, the introduction of antibiotics in clinical field started in the 1940s after the discovery of the penicillin by Alexander Fleming in 1928 (Ventola, 2015). The penicillin was successfully curing infectious diseases and the pharmaceutical industry started finding other natural substances with antimicrobial activity of different families (beta lactams, macrolides, aminoglycosides, tetracyclines etc.). Scientific researches led to the production of synthetic penicillin and a wide group of beta lactams; so that the 1950s and 1960s were considered the "era of the end of infectious diseases".

Unfortunately, in the same decade, penicillin resistance was being observed. The misuse and overuse of the antibiotics drove the evolution of the resistance mechanisms, although this process naturally occurs in bacteria, but in a very slow way (Read and Woods, 2014; Ventola, 2015). The main causes of the antibiotic resistance crisis are related to the inappropriate prescribing in humans and animals healthcare, the extensive agricultural use as growth promoters in livestock (banned in the European union since 2006, but still allowed in many other countries) and the availability of few new antibiotics (Ventola, 2015). Moreover, the dissemination of the resistance mechanisms can be also increased by an anthropic contact between a recent growing human population with wild animals, through contamination of residual water effluents, ludic activities or human consumption of hunting animals (Kasprzyk-Hordern et al., 2009; Ruiz-Fons, 2017). Furthermore, the selective pressure exerted by the environment shouldn't be neglected since many antibiotics are produced by environmental microorganisms.

Since 2007, the international community adopted the "**One health**" concept, which focusses on the interactions between humans, animals and their environments in order to enhance health and welfare. In that context, the WHO works in close collaboration with the Food and Agriculture Organization (FAO) and the World Organization of Animal Health (OIE) in order to promote multisectoral answers to food security,

zoonoses and public health issues in the animal-human interface. Furthermore, in Africa, the "One Health" approach is particularly relevant because of the sanitary crisis that threatened animals' health and the ecosystem balance in previous years such as the Rift Valley epidemy of 1997 in Eastern-Africa (human and animal death). Another example is the Ebola virus epidemy which caused more than 10.000 human death in 2014 in West Africa and impacted on agriculture and food security (Bénie Bi Vroh and Seck, 2016). The virus originated from the animals and expanded in the human community.

The WHO adopted, at the 68th World Health Assembly held in 2015, the Global Action Plan on antimicrobial resistance to implement actions of surveillance of antibiotic resistance in pathogenic bacteria (World Health Organization, 2015). It was highlighted the need to study the antibiotic resistance trend in bacteria of diverse ecosystems. The bacteria of the genus *Staphylococcus* are important for study since they are commensal for humans and animals and may cause opportunistic infections and food intoxication. *S. aureus* and methicillin-resistant *S. aureus* (MRSA) are one of the microorganisms which commonly cause infections in hospitals and in the community (World Health Organization, 2014).

2. *Staphylococcus* spp. MICROBIOLOGICAL CHARACTERISTICS AND CLASSIFICATION

Staphylococcus is a genus of Gram-positive bacteria of the family of *Micrococcaceae*. They are anaerobia facultative and do not form spores. Under the microscope, they appear spherical (cocci) and non-motile, and formed in grape-like clusters. *Staphylococcus* genus was associated to human infections for the first time in 1880, by the Scottish surgeon Sir Alexander Ogston, when he found grape-like groups of cocci in a knee purulent abscess. The name *Staphylococcus*, derived from the Greek *staphyle* (bunch of grapes) and *kokkos* (grain) was given based on their morphology, differentiating them from *Streptococcus* (clustered in chain). In 1884, the doctor Friedrich Julius Rosenbach differentiated two species thanks to their colonies colour: *S. aureus* (from Latin aurum, gold) and *S. albus* (from Latin, albus, white) now named *S. epidermidis* due to its presence in human skin **(Figure 1)**.

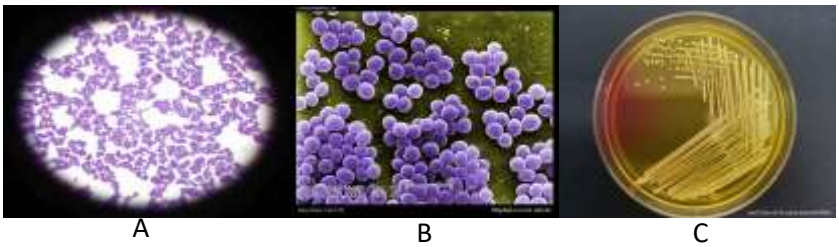


Figure 1: *S. aureus seen under microscope and on growth medium*

A: S. aureus seen under microscope (https://www.pinterest.es/pin/284923113902996739/)

B: S. aureus seen under microscope (Photo by Janice Haney Carr)

C: Yellow colonies of S. aureus in Mannitol Salt Agar (Photo by Anne Hanson and Matthew Pietraszewski, University of Maine)

Staphylococcus genus is composed of 51 species and 28 subspecies (http://www.bacterio.net) divided in two groups: coagulase-positive staphylococci (CoPS, including the most pathogenic species *S. aureus*) and coagulase-negative staphylococci (CoNS). They are classified in function of their capacity to produce the coagulase enzyme responsible for blood coagulation, transforming fibrinogen into fibrine. It is displayed in **Table 1** the staphylococcal species classified according to the coagulase production capacity.

Table 1: Species of the genus Staphylococcus classified according to their capacity to produce the coagulase enzyme. Adapted from (Becker et al., 2014b)

Coagulase-positive *Staphylococcus*	**Coagulase-negative *Staphylococcus***			
S. argenteus	*S. agnetis**	*S. edaphicus*	*S. lentus*	*S. saccharolyticus*
S. aureus	*S. argensis*	*S. epidermidis*	*S. lugdunensis*	*S. saprophyticus*
S. delphini	*S. arlettae*	*S. equorum*	*S. massiliensis*	*S. schleiferi**
S. intermedius	*S. auricularis*	*S. felis*	*S. microti*	*S. sciuri*
S. lutrae	*S. capitis*	*S. fleurettii*	*S. muscae*	*S. simiae*
S. pseudintermedius	*S. caprae*	*S. gallinarum*	*S. nepalensis*	*S. simulans*
S. schweitzeri	*S. carnosus*	*S. haemolyticus*	*S. pasteuri*	*S. stepanovicii*
	S. chromogenes	*S. hominis*	*S. petrasi*	*S. succinus*
	S. cohnii	*S. hyicus**	*S. pettenkoferi*	*S. vitulinus*
	S. condiment	*S. jettensis*	*S. piscifermentans*	*S. warneri*
	S. devriesei	*S. kloosii*	*S. rostri*	*S. xylosus*

**S. agnetis and S. hyicus are coagulase variable; S. schleiferi includes a subspecies coagulase-positive and another coagulase-negative.*

Bacteria of the genus *Staphylococcus* are mesophilic and halotolerant. Specific growth media such as Mannitol Salt Agar (MSA) and Oxacillin Resistance Screening Agar Base (ORSAB, for methicillin-resistant isolates) are used often for their isolation and identification. MSA medium contains a high concentration of sodium chloride (7.5%) (for halotolerant isolates selection), phenol red as pH indicator, and mannitol. When the mannitol is fermented, the acid pH is indicated by the change of the red colour of phenol to yellow (CoPS). The ORSAB medium is also composed of a high concentration of salt (5.5%). In this case, the fermentation of the mannitol is observed by the blue colour of the isolates CoPS, due to the blue of aniline in an acid medium. The supplementation of oxacillin in the medium inhibits the growth of methicillin-sensitive isolates. Otherwise, the identification of staphylococcal species can be performed by API Staph gallery, MicroScan, VITEK identification cards or by mass spectrometry MALDI-TOF, among others.

3. *Staphylococcus* spp. AS OPPORTUNISTIC PATHOGENS

Staphylococcal species are part of the natural microbiota of skin and mucous membranes of humans and most of mammals and birds (**Table 2**)**.** However, some of the species were described as pathogens involved in opportunistic infections of both humans and animals, being *S. aureus* the most important one (Foster, 1996; Otto, 2013).

3.1. *S. aureus*

Staphylococcus aureus is commensal for humans and animals. Thirty to 50 percent of healthy adults are colonized, with 10 to 20 percent persistently colonized (Grundmann et al., 2010). However, it is well-known for causing hospital-and community-acquired skin and lung infections. *S. aureus* is an important cause of endocarditis, osteomyelitis, septicaemia and toxic shock syndrome. *S. aureus* infection is a major cause of death in hospital-associated infections, particularly when patients have underlying conditions such as immune deficiencies or primary infections caused by other pathogens (Otto, 2013). Furthermore, it can cause foodborne diseases and food poisoning. Staphylococcal food poisoning is due to the ingestion of enough amounts of staphylococcal enterotoxins (SEs) present in contaminated food (Argudín et al., 2010). Otherwise in animals, *S. aureus* is frequently involved in mastitis in livestock, exudative dermatitis in pigs and pets and arthritis in poultry (Baba et al., 2012).

***Table 2**: Staphylococcal species with natural hosts identified. Adapted from* (Becker et al., 2014b)

Species	Natural host	Species	Natural host
S. aureus	Mammals, poultry	***S. lugdunensis***	Humans, goats, cats, dogs, chinchilla, guinea pig
S. arlattae	Poultry, goat, sheep, cow, pigs	***S. massiliensis***	Humans
S. auricularis	Humans	***S. microti***	Rats
S. capitis	Humans, cats, dogs, horses	***S. muscae***	Flies
S. caprae	Humans, goats	***S. nepalensis***	Goats, pigs, squirrel, monkeys
S. carnosus	Cows	***S. pasteuri***	Pigs
S. chromogenes	Cows, goats, horses, pigs, sheep	***S. petrasii***	Humans
S. cohnii	Humans, primates, poultry, dogs, goats, horses, clams	***S. pettenkoferi***	Humans
S. delphini	Dolphins, horses, poultry	***S. pseudintermedius***	Dogs
S. devriesei	Cows	***S. rostri***	Pigs, poultry, buffalos
S. epidermidis	Humans, cats, dogs, bovine, ovine, horses, primates	***S. saccharolyticus***	Humans, gorillas
S. equorum	Horses, bovine, ovine	***S. saprophyticus***	Humans, bovine, ovine, cats
S. felis	Cats, horses	***S. schleiferi***	Humans, dogs, cats
S. fleurettii	Goats, pigs, small mammals	***S. sciuri***	Humans, cows, goats, dogs, cats, dolphins, horses, rodents, clams, pigs, monkeys
S. gallinarum	Poultry	***S. simiae***	Primates
S. haemolyticus	Humans, dogs, cats, bovine, ovine, pigs, horses,	***S. simulans***	Humans, cows, sheep, horses
S. hominis	Humans, dogs, cats, goats, sheep, pigs	***S. stepanovicii***	Rodents, insectivorous
S. hyicus	Pigs, horses	***S. succinus***	Insectivorous, rodents, cows, poultry
S. intermedius	Poultry, horses, dogs, cats, minks	***S. vitulinus***	Horses, poultry
S. kloosii	Goats	***S. warneri***	Humans, primates, dogs, cats, ovine, horses, insectivorous, pigs, rodents
S. lentus	Goats, sheep, pigs, horses, minks, poultry, clams	***S. xylosus***	Humans, rodents, poultry, cats, clams, ovine, insectivorous, primates

3.2. Other species

Among CoPS, *S. pseudintermedius* is another species of importance which colonizes pets (Perreten et al., 2010; Paul et al., 2012; Gómez-Sanz et al., 2013b, 2014; Stull et al., 2014). *S. pseudintermedius* causes skin and urinary infections and post-operative lesions in pets and is also responsible for some human infections (Börjesson et al., 2015; Grönthal et al., 2015; Somayaji et al., 2016; Lozano et al., 2017) . It has emerged recently, because it was misidentified as *S. intermedius* during more than 30 years until 2005 (Börjesson et al., 2015).

CoNS are considered less pathogenic than CoPS, but they represent one of the major nosocomial pathogens mainly in neonatal intensive care units (NICU), and can colonize implanted foreign bodies (Becker et al., 2014b; Patel and Saiman, 2015; Osman et al., 2016). The species involved are mainly *S. epidermidis* and *S. haemolyticus*. Moreover, some species are related to genitourinary tract infections and endocarditis in humans (*S. saprophyticus* and *S. lugdunensis*) and bovine mastitis in animals (*S. chromogenes*, *S. epidermidis* or *S. simulans*) (Pyörälä and Taponen, 2009; Taponen and Pyörälä, 2009; Becker et al., 2014b).

4. VIRULENCE FACTORS AND ADAPTATION CAPACITY

Staphylococcal pathogenicity is defined as the ability of the microorganism to produce virulence factors which cause diseases or damage (colonization and cellular invasion, host cell tissues destruction, avoidance of host immune defence proliferation etc.) (Gordon and Lowy, 2008). The virulence profile of *Staphylococcus* species is related to their cell wall components (mucoid capsule, adhesin, protein A, teichoic acid), enzymes (coagulase, hyaluronidase, catalase, nuclease) and different extracellular toxins (Gordon and Lowy, 2008; Kong et al., 2016) (**Figure 2**).

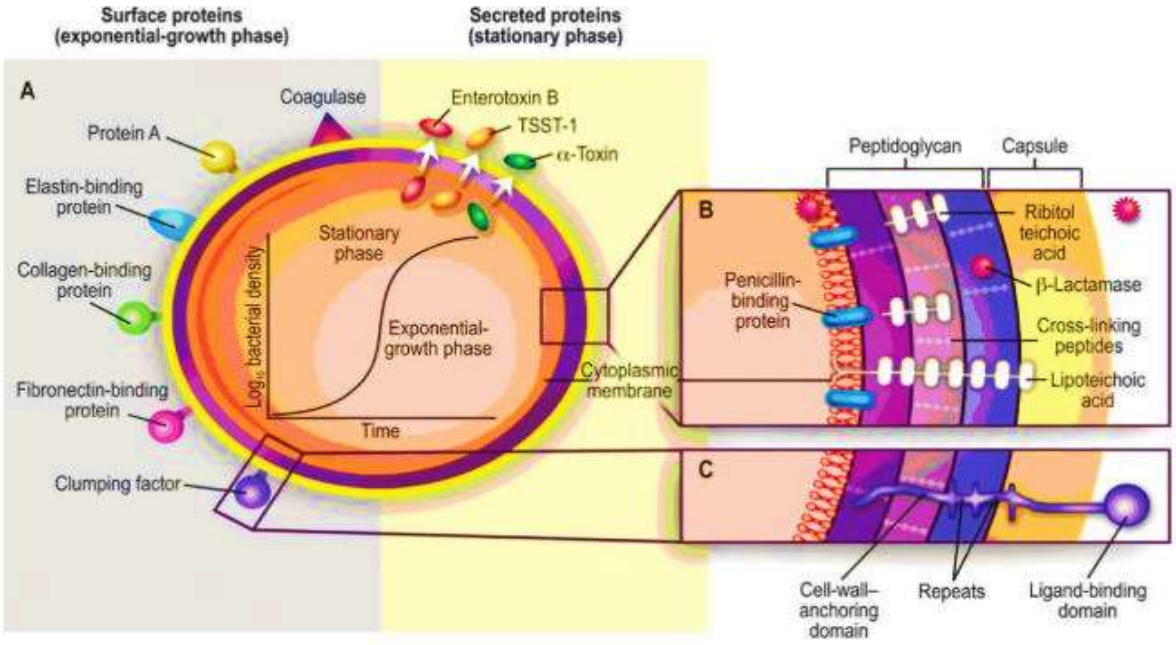


***Figure 2**: S. aureus cell structure*

Panel A shows the surface and secreted proteins. The synthesis of many of these proteins is dependent on the growth phase. Panels B and C show cross sections of the cell envelope. TSST-1 denotes toxic shock syndrome toxin 1 (Lowy, 1998).

4.1. Colonization capacity and extracellular enzymes

Staphylococci possess surface proteins, called "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs), that mediate adherence to host tissues through recognition of molecules such as collagen, fibronectin, fibrinogen and elastin. MSCRAMMs seem to be involved in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections (Wertheim et al., 2005; Gordon and Lowy, 2008). Moreover, staphylococci produce various enzymes such as protease, lipase, and hyaluronidase, that destroy tissues and enable the spread of infections to adjacent tissues. These enzymes can also take part in the formation of biofilms or in the inactivation of immune response proteins (Lowy, 1998).

4.2. Toxins

Toxins are proteins secreted by the microorganisms into the extracellular matrix during the post-exponential and early stationary phases. Among the most common toxins secreted by staphylococci, are cytotoxins (hemolysins and leukocidins), exfoliative toxins (Ets) and pyrogenic superantigen toxins (SEs and toxic-shock syndrome toxin-1 (TSST-1)).

4.2.1. Hemolysins

This family of peptides was discovered in the late 19th Century in a culture of *Staphylococcus* that showed hemolytic activity. Hemolysins are toxins that lyse red blood cells. They are classified in five types: alpha (α-hemolysin, encoded by the gen *hla*), beta (β-hemolysin, *hlb*), delta (δ-hemolysin, *hld*), gamma (γ-hemolysin, *hlg*) and gamma variant (γv- hemolysin, *hlgv* or *hlg*-2). α-hemolysin is the most studied and is associated to dermonecrotic and neurotoxic activity. In sepsis, its synergistic action on myeloid cells and platelets has been shown to kill a wide range of host animals. Upon binding of the toxin with its receptor, pore formation on cell membranes will cause necrotic cell death (Dinges et al., 2000; Kong et al., 2016). β-hemolysin is non-pore-forming, hydrolyses sphingomyelin and lyses monocytes. Even though its target cells are known, the toxin's mode of action is still unclear (Dinges et al., 2000; Kong et al., 2016). δ-hemolysin is produced by several staphylococcal strains such as *S. intermedius* and *S. epidermidis*. It is responsible for lysis of erythrocyte and a wide range of cells and organelles (Verdon et al., 2009). γ-hemolysin is hemolytic to rabbit erythrocytes and its membrane damaging activity is also apparent in leukocytes. This group of hemolysins are bi-component, capable of lysing blood red cells (Dinges et al., 2000).

4.2.2. Leukotoxins

Discovered in 1932, the Panton-Valentine Leukocidin (PVL) is the toxin most virulent produced by *S. aureus* although it is only secreted by 2-4% of the strains (Prevost et al., 1995). This toxin is part of a bicomponent Luk-Family. PVL consists of two protein subunits F and S, synthetized independently but acting synergistically on human cell membranes, leading to pore formation, alteration of the permeability and finally to the cell's destruction. Its presence is generally associated with skin and soft tissues infections (SSTIs), necrotizing pneumonia, septicaemia or endocarditis (Prevost et al., 1995; Lina et al., 1999; Balachandra et al., 2015).

S. aureus produces other leukotoxins of minor clinical relevance. The leukocidin M (*luk*M and *luk*F) are associated with the destruction of polymorphonuclear leucocytes in ruminants, generally leading to bovine and ovine mastitis (Kaneko and Kamio, 2004). LukED (*luk*E and *luk*D) causes dermonecrose but lacks an hemolytic activity (Gravet et al., 1998). Finally, LukPQ (*luk*P and *luk*Q) is a new phage-encoded leukocidin associated with horses and donkeys which preferentially destroys neutrophils in equine (Koop et al., 2017).

S. pseudintermedius also produces a bicomponent leukocidin composed of F and S subunits, similarly to *S. aureus* PVL. It is called Luk-I and destroys polymorphonuclear cells (Prevost et al., 1995; Gravet et al., 1998). It is secreted by 90% of veterinarian isolates.

4.2.3. Exfoliative toxins and pyrogenic toxins superantigen

Staphylococcal exfoliative toxins are serine protease classified in five types: EtA, EtB, EtD, EtC and EtD2: EtA, EtB and EtD are responsible for staphylococcal scaled skin syndrome (SSSS) mainly in neonates and infants, but also in adults with renal dysfunction or immunodepression. The SSSS causes skin blistering and loss of superficial skin layers, dehydration and secondary infections (Dinges et al., 2000; Kong et al., 2016). EtC and EtD2 are generally associated with animals.

The toxic-shock syndrome was first named in 1978 in paediatrics and is caused by the TSST. It is characterized by headache, disorientation, hypotension, fever, skin eruption, diarrhoea etc., and may lead to coma. SEs are secreted by *S. aureus* strains in food and are one of the most common causes of food-borne diseases. There are more than 18 SEs identified which are heat-stable and low pH-tolerant, so that they are not degraded by cooking processes (Jarraud et al., 2001; Argudín et al., 2010).

4.3. Immune evasion mechanism

The human innate immune system is responsible for the discrimination between self and foreign molecules, whether pathogen or not. It is mainly composed of phagocytes and the complement system. *S. aureus* possesses a human innate immune system evasion mechanism, which enables its adaptation and survival during the first stages of infection and/or colonization. *S. aureus* innate immune evasion cluster (IEC) is composed of up to five genes (*scn*, *chp*, *sak*, *sea*, *sep*), which combinations result in seven types of IEC (**Figure 3**) (Van Wamel et al., 2006).

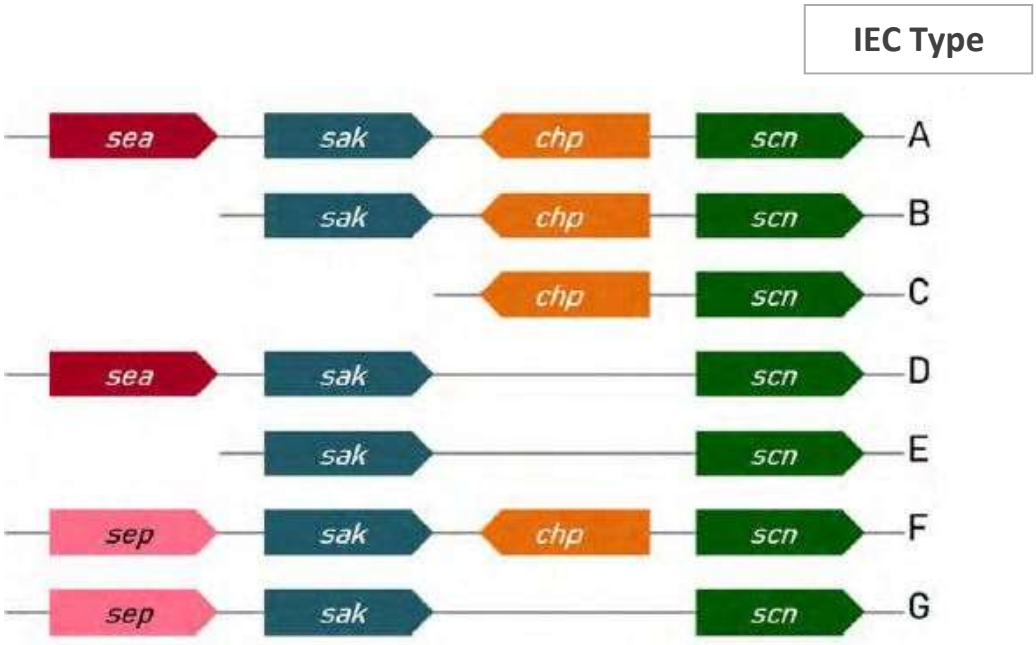


***Figure 3**: IEC type. Adapted from Van Wamel et a., 2006*

These genes are carried by the bacteriophage of the family 3 (φ3), also known as β-hemolysin-converter. They are integrated in the 3'terminal of *hlb*, being able to truncate *hlb* expression. Staphylococcal complement inhibitor (SCIN) (encoded by *scn* gene) is the most important component of IEC. SCIN prevents the ability of human neutrophils to phagocytose *S. aureus*. CHIPS (encoded by *chp* gen) is a bacterial chemokine receptor modulator that inhibits neutrophil chemotaxis. Both SCIN and CHIPS are important virulence factors that protect *S. aureus* from innate immune defence systems (Van Wamel et al., 2006). The staphylokinase (encoded by *sak* gene) has antiopsonic activities and directly destroys defensins. SEA and SEP (encoded by *sea* and *sep*, respectively) are enterotoxins that can have a synergic effect with the other IEC mechanisms (Rahimpour et al., 1999). It should be noted that 90% of *S. aureus* of human origin carry the phage φ3 containing the gene *scn*, so that *scn* is considered a marker of human origin (Van Wamel et al., 2006).

Moreover, it has been recently described an equine variant of SCIN, eqSCIN (encoded by *scn-eq*), which is a potent blocker of equine complement system activation and subsequent phagocytosis of bacteria by phagocytes (De Jong et al., 2018). Whereas SCIN-A from human *S. aureus* isolates exclusively inhibits human complement, eqSCIN represents the first animal-adapted SCIN variant that functions in a broader range of hosts (horses, humans, and pigs); it is carried by the phage φSaeq1 (De Jong et al., 2018).

In the **Table 3**, are displayed selected virulence factors of *S. aureus* and their clinical implications.

4.4. Host-related risk factors

The pathogenicity of a microorganism also depends on risk factors related to the host. Persons colonized with *Staphylococcus* are at increased risk for subsequent infections. In fact, hosts with higher risks of staphylococcal infections are those weaken by a chronic disease or an immunodepression. Important host-related risk factors are as follows i.) the loss of primary barriers integrity such as the skin and mucous, considered the main defence mechanism against staphylococcal infections; ii.) clinical factors which involve an immune deficiency system; iii.) the presence of foreign material such intravenous catheters, which are rapidly coated with serum constituents (fibrinogen or fibronectin) and enable staphylococci to adhere through MSCRAMMs, facilitating colonization, etc. (Lowy, 1998; Gordon and Lowy, 2008).

***Table 3**: Selected virulence factors of S. aureus and associated clinical implications*

Type of virulence factors	Selected factors	Associated clinical syndromes
Involved in attachment	MSCRAMMs (e.g., clumping factors, fibronectin-binding proteins, collagen, and bone sialoprotein-binding proteins)	Endocarditis, osteomyelitis, arthritis, prosthetic-device and catheter infections
Involved in persistence	Biofilm accumulation (e.g., polysaccharide intercellular adhesion)	Relapsing infections. Cystic fibrosis, syndromes as described above for attachment
Involved in evading/destroying hosts defenses	Leukocidins (e.g., PVL), capsular polysaccharides, protein A, CHIPS	Invasive skin infections and necrotizing pneumonia, abscesses
Involved in tissue invasion/penetration	Proteases, lipases, nucleases,	Tissue destruction and metastatic infections
Involved in toxin-mediated disease and/or sepsis	Enterotoxins, toxic shock syndrome toxin-1, exfoliative toxins	Food poisoning, toxic shock syndrome, scaled skin syndrome, bullous impetigo, sepsis syndrome
With poorly defined role in virulence	Coagulase, ACME, bacteriocin	

***Note**: ACME, arginine catabolic mobile element; CHIPS, chemotaxis inhibitory protein of staphylococci; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; PVL, Panton- Valentine leukocidin; table adapted from Gordon (Gordon and Lowy, 2008)*

5. ANTIBIOTIC RESISTANCE

Antibiotics aim to destroy the bacteria by interfering with essential cellular processes, leading to cellular death (bactericide) or inhibition of the microorganism growth (bacteriostatic). However, bacteria have demonstrated the ability to quickly respond to the antibiotics with the development of diverse resistance mechanisms. Staphylococcal resistance mechanisms include enzymatic inactivation of the antibiotic, alteration of the target with decreased affinity for the antibiotic, trapping of the antibiotic and efflux pumps. The mechanisms involve different cellular structures or metabolic pathways. They can be innate (intrinsic resistance of the species) or acquired (spontaneous mutation or horizontal genetic transference)(Pantosti et al., 2007). It should be highlighted that the acquired resistance is the most important since the resistance encoding genes may be transferred between commensal, pathogenic and environmental bacteria. In the **Figure 4**, it is displayed the targets of antibiotics commonly used against *S. aureus* as well as the resistance mechanisms developed by the microorganism. The main resistance mechanisms will be described according to the groups of antibiotics (beta lactams and no beta lactams) in the next point.

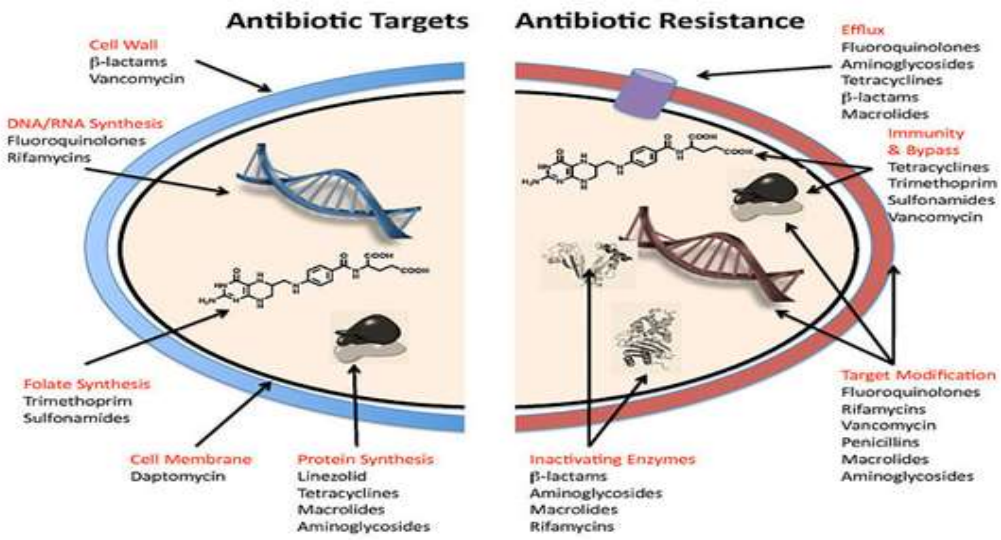


***Figure 4**: Different antibiotics targets and associated resistance mechanisms* (Wright, 2010)

5.1. Resistance to Beta lactams

Beta lactam antibiotics are the most used class of antibacterial agents in the infectious disease including penicillin, cephalosporins, monobactams and carbapenems. Penicillin is the first natural β-Lactam to be used clinically, and for staphylococcal infections treatment. But few years after its discovery, it appeared resistance mechanisms mediated

by the gene *blaZ* (encoding a β-lactamase enzyme) which inactivates the penicillin β-Lactam ring by hydrolytic excision (**Figure 5**). It was then necessary to find other β-lactam antibiotics, such as methicillin and oxacillin, which are semi-synthetic.

These antibiotics interrupt bacterial cell-wall formation as a result of covalent binding to essential penicillin-binding proteins (PBPs, transpeptidase), enzymes which are involved in the terminal steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria (Bush and Bradford, 2016). Every bacterial species has its own distinctive set of PBPs that can range from three to eight enzymes per specie. In the case of *Staphylococcus* species, they possess four intrinsic PBPs (PBP1-PBP4).

The PBP2 is the main target of β-Lactam antibiotics, since it is the only PBP with transglucosylase activity in addition to transpeptidase. In fact, the other PBPs lack the glycosyltransferase which is necessary for the peptidoglycan synthesis. Staphylococcal resistance to methicillin is due to an altered PBP2, named PBP2a which has a low affinity for β-Lactam antibiotics, unlike the other PBPs (**Figure 5**) (Sauvage et al., 2008).

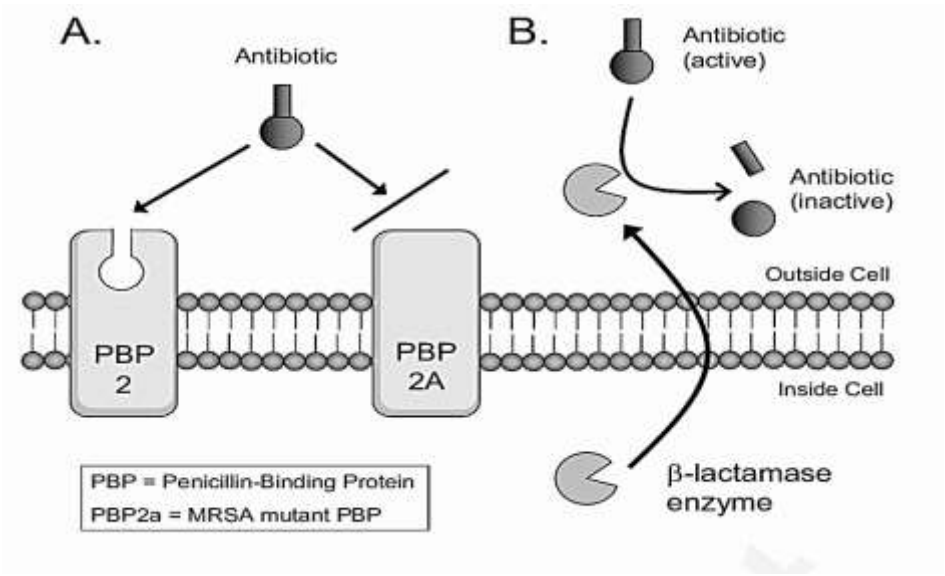


***Figure 5**: Diagram of the two principal antibiotic resistance mechanisms observed in MRSA bacteria*
A: Expression of alternate form of penicillin-binding protein PBP2, called PBP2a, with reduced binding affinity for antibiotic. B: Production and release of β-lactamase enzyme which cleaves and inactivates antibiotic molecules (Murphy and Walshe, 2011).

PBP2a is the product of the gene *mecA* and its regulatory genes *mecI* and *mecR1*. The *mecA* complex is included in a 30–60 kb element, denominated staphylococcal

chromosomal cassette *mec* (SCC*mec*). SCC*mec*, a mobile genetic element of horizontal transference, is most common in CoNS and can be considered an antibiotic resistance island as it can integrate additional mobile elements or resistance genes (Pantosti et al., 2007). Two *mecA* gene homologues with 80% and 91% nucleotide identities were found in *Staphylococcus sciuri* and *Staphylococcus vitulinus* species, respectively. The *mecA* gene homologue of *S. sciuri* is considered to have a ubiquitous presence among its species group (*S. sciuri*, *S. fleurettii*, *S. lentus*, *S. vitulinus*) and to be the evolutionary precursor of the *mecA* gene (Tsubakishita et al., 2010; Becker et al., 2014b).

Recent studies have demonstrated that resistance to methicillin was mediated in staphylococci not only by *mecA* but also by homologous genes, *mecC* and *mecB*. The *mecC*, initially termed $mecA_{LGA251}$, was discovered in 2011 in England in a *S. aureus* (LGA251) genome located in a novel staphylococcal cassette chromosome *mec* element, designated type-XI SCC*mec* (García-Álvarez et al., 2011). It is thought to originate from CoNS (*S. sciuri* and *S. stepanovicii*) (Paterson et al., 2014). The *mecC* was 69% identical to its homologue *mecA* at the DNA level, and the encoded protein PBP2c was 63% identical to PBP2a. It was observed that the PBP2c exhibits a higher binding affinity for oxacillin. Moreover, the *mecC*-positive strains fail to be detected by current *mecA* laboratories detection methods, so that they were considered methicillin-susceptible (Ballhausen et al., 2014; Skov et al., 2014). Although the origins of *mecC* MRSA are not yet clear, there is good evidence that contact with animals poses a zoonotic risk and that *mecC* MRSA can be transmitted between species (Patterson et al., 2014). On the other hand, the *mecB* gene was first described in a *Macrococcus caseolyticus* in 2009 (Becker et al., 2014a). However, is was recently reported a plasmid-encoded, and thereby transferable, methicillin resistance encoded by *mecB* in an isolate of the genus *Staphylococcus* (Becker et al., 2018).

5.2. Resistance to Non-beta lactams

For staphylococcal infections, other alternatives to beta lactams do exist and include diverse families of antibiotics such as aminoglycosides, macrolide-lincosamide-streptogramines (MLS), tetracyclines glycopeptides, mupirocins, fucidins, diaminopyrimidines, phenicols, oxazolidinones and fluoroquinolones. Their mechanisms of action and the resistance mechanisms used by *Staphylococcus*. spp are summarized in **Table 4**. Unfortunately, strains with decreased susceptibility (or high-level resistance) to antibiotics of last resort (used for SARM severe infections), such as vancomycin have been described (Gardete and Tomasz, 2014). Furthermore, other

antibiotics including those of last generation are getting less and less efficient. It is the case of linezolid, daptomycin, clindamycin, etc. (Chatterjee and Otto, 2013). Some bacteria, MRSA included, show a multidrug resistance (MDR) phenotype meaning that they are resistant to antibiotics of at least three distinct families; which makes staphylococcal treatment more difficult.

***Table 4**: Mechanisms of action to antibiotics vs resistance mechanisms in Staphylococcus, adapted from* (Reygaert, 2013, 2018)

Antimicrobial agents	**Mechanism of action**	***Staphylococcus* resistance genes**	***Staphylococcus* Resistance mechanism**
-Aminoglycosides Amikacin Gentamicin Tobramycin Streptomycin	Inhibit protein synthesis (bind to 30S Ribosomal Subunit)	*aac(6´)-Ie-aph(2")-Ia,* *ant4´-Ia,* *ant6'-Ia*, *ant*(3)(9), *str*, *aphA3´*	Aminoglycoside modifying enzymes, modify target
Tetracyclines	Inhibit protein synthesis (bind to 30S Ribosomal Subunit)	*tet*(K), *tet*(L), *tet*(M), *tet*(O)	Active efflux Ribosomal protection, competitive binding
-Macrolides, Lincosamides and Streptogramins	Inhibit protein synthesis (bind to 50S Ribosomal Subunit)	*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(T), *erm*(Y), *msr*(A)/*msr*(B), *mph*(C), *lnu*(A), *lnu*(B), *lnu*(C), *cfr*	Methylation of ribosome, decreased binding
-Phenicols chloramphenicol	Inhibit protein synthesis (bind to 50S Ribosomal Subunit)	*cfr*, cat_{pC194}, cat_{pC221}, cat_{pC223}, *fexA*, *fexB*	Acetylation of drug, inactivation

Antimicrobial agents	Mechanism of action	*Staphylococcus* resistance genes	*Staphylococcus* Resistance mechanism
-Glycopeptides Vancomycin	Inhibit cell wall synthesis	*VanA*	Modification of target
-Mupirocins Mupirocin	Inhibit protein synthesis (bind to isoleucyl-tRNA synthetase)	Mutation in *mupA*	Change of target
-Fucidin Fusidic acid	Inhibit protein synthesis (alter elongation factor G)	*fusB*, *fusC*	Target protection
-Sulfonamides Trimethoprim/Sulfame-thoxazole	Inhibit DNA replication (inhibit dihydrofolate reductase)	*dfrA*, *dfrD*, *dfrG*, *dfrK*	Target enzyme modification
-Quinolones Ciprofloxacin	Inhibit DNA replication (alter DNA gyrase and topoisomerase IV)	Mutation in *gyrA*, *grla*, Change in the expression of *norA*, *sdrM*	Mutation in the target, active efflux

It is speculated that a lot of resistance genes originated from CoNS. Among them, the genes *vga*, responsible for resistance to lincosamides and streptogramines are frequent in *S. epidermidis*, *S. haemolyticus*, *S. lentus*, *S. cohnii* and *S. simulans* (Bhargava and Zhang, 2012; Lozano et al., 2012a) while the *lnu* genes (resistance to lincosamides) were described in *S. aureus*, *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis* and *Lactobacillus spp.* (Lüthje et al., 2007; Lozano et al., 2012a). The gene *fexA*, encoding resistance to chloramphenicol and florfenicol, was first found on a bovine *S. lentus* plasmid. Moreover, it was observed that it is functionally active in *E. coli* suggesting a potential transfer of *fexA*-mediated resistance between different bacterial species and genera (Kehrenberg and Schwarz, 2004). The gene *cfr* responsible for resistance to oxazolidinones, phenicols, lincosamides and

streptogramins was initially detected on a bovine *S. sciuri* plasmid and later in other CoNS (Kehrenberg and Schwarz, 2004, 2006; He et al., 2014). Other genes like *erm*(T) (resistance to macrolides, lincosamides and streptogramins) or *dfrK* (resistance to trimethoprim-sulfamethoxazole) are thought to be initially hosted by CoNS, although they are frequently detected in livestock-associated *S. aureus* (Kadlec et al., 2012).

6. MOLECULAR TYPING OF *S. aureus*

The molecular typing is an essential key for epidemiological investigation of hospital-onset *S. aureus* infections, to follow and identify outbreak related strains and to distinguish epidemic from endemic or sporadic isolates. It also aims to identify predominant clones in a determined region or ecosystem, as well as their genetic specificity or their way of dissemination. Molecular typing tools are necessary to understand the evolution of resistance to antibiotics. The most reliable typing methods of *S. aureus* used in this work will be detailed below.

6.1. *Spa*-typing

This method is an important sequence-based tool in the study of strain origin, clonal relatedness and epidemiology of *S. aureus* outbreaks, developed in 1996 (Frénay et al., 1996). It involves the amplification and sequencing of the polymorphic region X of staphylococcal protein A gene (*spa*), which consists of a variable number of direct repeats (Frénay et al., 1996; Harmsen et al., 2003). The protein A is a cell wall component able to bind immunoglobulins and interfere with opsonization and phagocytosis. Its variable region is subject to spontaneous mutations, as well as loss and gain of repeats that are assigned to numerical code. The different combinations of the repeats are given a *spa*-number attributed to *S. aureus* strains and registered on the website https://www.spaserver.ridom.de/. The technic is automatized and realized by the software "RidomStaph-Type".

6.2. Multilocus sequence typing (MLST)

This method was developed in 1998 (Maiden et al., 1998) and is based on the analysis of sequences of internal fragments (about 450pb) of seven housekeeping gene encoding metabolic enzymes. It was applied for *S. aureus* in 2000, analysing the following genes: Carbamate kinase (arcC), Shikimate dehydrogenase (aroE), Glycerol kinase (glpF), Guanylate kinase (gmk), Phosphate acetyltransferase (pta), Triosephosphate isomerase (tpi) and Acetyl coenzyme A acetyltransferase (yqiL) (Enright et al., 2000).

The sequence of each locus is compared to the allelic sequences previously determined and stored on the database of https://pubmlst.org/. A number is assigned to each locus sequence. Isolates that have identical sequences at all seven genetic loci are considered a clone and assigned a unique sequence type (ST). Sequence types that differ by single nucleotide polymorphisms at fewer than three loci are considered closely related and are grouped into clonal complexes (CC). This is accomplished by application of the eBURST algorithm (Chambers and Deleo, 2009).

It should be noted that there is a tight relation between the *spa*-type and the MLST, although this last one is more expansive, with less discrimination capacity than *spa*-type.

6.3. Locus *agr* typing

Staphylococci are able to sense the bacterial cell density, or quorum, and to respond with genetic adaptations, thanks to one main system, which is called accessory gene regulator (*agr*) (Le and Otto, 2015). The system *agr* was described for the first time in a *S. aureus* strain (Peng et al., 1988), but is widely spread in the staphylococcal genera. The up-regulation of *agr* is important for the timing of virulence factor expression during infection and the development of acute diseases, pneumonia, SSTIs etc., while down-regulation is implicated in chronic staphylococcal infections involving biofilm formation (Le and Otto, 2015).

The *agr* locus is 3.5 kb in size and consists of two divergent transcriptional units, RNAII and RNAIII whose transcription is driven by the P2 and P3 promoters, respectively (Peng et al., 1988). The RNAII locus contains four genes, *agrA agrB*, *agrC* and *agrD* (**Figure 6**). This last one encodes an autoinducing peptide (AIP), precursor of extracellular quorum signal of *agr*. The *agrB* gene product is a transmembrane endopeptidase, which combined to a*grD*, is responsible for the maturation and secretion of the AIP. The *agrC* and *agrA* genes encode a two-component signal transduction system (where *agrC* acts as the signal receptor and *agrA* as the response regulator) that serves as a quorum-sensing regulon to autoinduce RNA III (Shopsin et al., 2003; Gomes-Fernandes et al., 2017). RNAIII is the principal intracellular effector of the *agr* response, responsible for the control of a*gr* targets (Le and Otto, 2015).

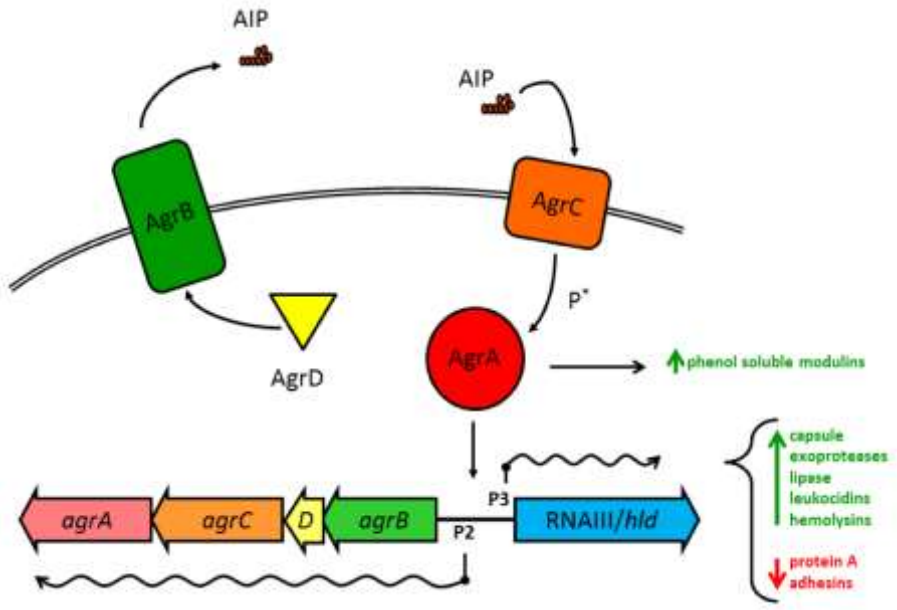


***Figure 6**: The structure and function of the agr operon in S. aureus.*(Gray et al., 2013)

S. aureus strains have been divided into four *agr* specificity groups (*agr*I, *agr*II, *agr*III and *agr*IV) (Shopsin et al., 2003). Isolates of one *agr* group can activate the *agr* response in isolates of the same group, but they usually inhibit it in members of the other groups. This system may influence host ecology by enhancing or inhibiting the ability of an *S. aureus* isolate to colonize (or compete) in the presence of resident strains, including other staphylococci (Shopsin et al., 2003).

6.4. Staphylococcal chromosomal cassette (SCC*mec*) typing

The discovery of SCC*mec* as host of the genes *mecA* was an advance for understanding the biology of methicillin resistance and the evolutionary relationships among MRSA (Itou et al., 2000). SCC*mec* is a mobile genetic element inserted into the chromosome and it is composed of two major elements: the *mec* gene complex and the cassette chromosome recombinase (*ccr)* gene complex. The *mec* gene complex comprises the genes *mecA* or *mecC*, its regulation genes *mecI* (a repressor) and *mecR1* (a sensor inducer) and an insertion sequence, IS431*mec* (Hiramatsu et al., 2002). The recombinase genes, *ccrA* and *ccrB*, are specifically involved in the recombination events (integration and excision) of SCC*mec* in staphylococcal genome. The combination of the *ccr* isotypes and the class of *mec* gene complexes is used to classify the SCC*mec* (Itou et al., 2000).

To date, thirteen types have been reported for the SCC*mec* element (I-XIII); the type XIII was recently found in a SARM ST152 which presented a new gen *ccr*C2 (Baig et al., 2018). Various multiplex PCRs were implemented to determine the SCC*mec* type

(Berglund et al., 2005; Kondo et al., 2007; Takano et al., 2008), as well as an online database which predict SCC*mec* type (https://cge.cbs.dtu.dk/services/SCCmecFinder).

7. EPIDEMIOLOGY AND GENETIC LINEAGES of *S. aureus*

As mentioned below, *S. aureus* has the capacity to colonize its hosts and adapt to new ecosystems. Based on MLST and CC molecular typing, MRSA genetic lineages determined to date were classified in three major groups according to their environment of predilection:

- HA-MRSA (hospital- or healthcare-associated MRSA)
- CA-MRSA (community-associated MRSA)
- LA-MRSA (Livestock-associated MRSA)

The prevalence of MRSA is variable in function of the geographic location (**Figure 7**). Data of the CDDEP (Center for Disease Dynamics, Economics and Policy) showed that among *S. aureus* invasive infections of 2017, SARM represented 25% in Spain, 39% in Portugal, 44% in Romania and 15% in Poland. The rate was of 45% in the United States (2016) and 31% in Mexico (2017). Data in Asia and Africa were also variable (38% in China in 2017; 63% in Pakistan in 2017; 63% in Nigeria in 2017 and 19% in Ghana in 2016). European countries show a general trend towards increasing MRSA prevalence from the north to the south of the continent, with <5% of MRSA from invasive infections in northern Europe (for example, the Netherlands, Norway, etc.) compared with 25–50% in southern Europe (for example, Portugal, Spain, Italy and Greece) (Lee et al., 2018).

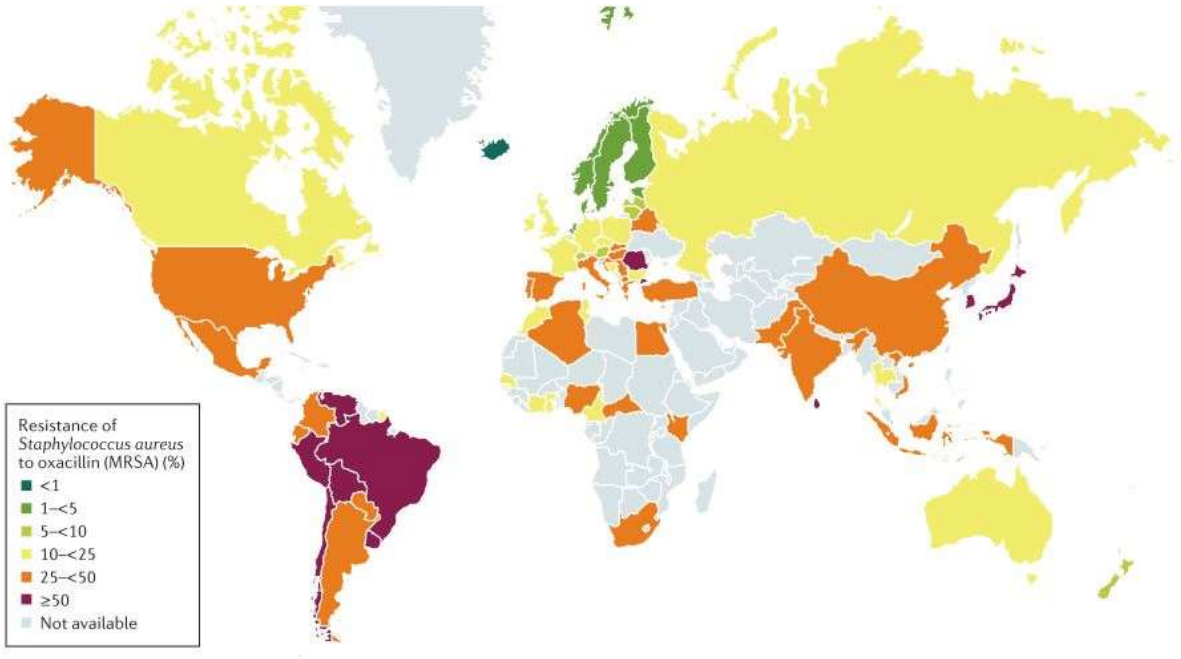


***Figure 7**: Worldwide prevalence of MRSA*

Data include aggregated resistance rates for invasive isolates (includes intermediate resistance) from blood and cerebrospinal from inpatients of all ages during 2014-2016. Data extracted from CDDEP. Adapted by (Lee et al., 2018).

7.1. Hospital- or healthcare-associated MRSA (HA-MRSA)

MRSA was initially a healthcare-associated pathogen dominated by distinct lineages and often associated with multidrug resistance. According to (Bal et al., 2016), individuals who have had lengthy hospitalization, intensive care unit (ICU) admission, residency in a nursing home, antibiotic exposure surgery, haemodialysis, chronic wounds or indwelling invasive devices have an increased risk of infection with HA-MRSA. The first case of MRSA in a patient was detected in an hospital of England in 1961 (Jevons, 1961) soon after introduction of methicillin into clinical practice. During the following decade, it was reported the firsts hospital outbreaks caused by MRSA in many countries (England, Denmark, France, Switzerland and USA). By the 1980s, HA-MRSA represented a serious concern in the nosocomial setting worldwide (Aires-de-Sousa, 2017). It was observed that only a few epidemic clones were causing disease worldwide, namely the Iberian, Hungarian, Brazilian, New York/Japan and Paediatric clones (Oliveira et al., 2002). The Iberian clone (CC8) was first reported in Spain in 1989 and later in many other countries (Portugal, Italy, the UK, Germany, etc.). The Hungarian clone (CC8) appeared in hospitals in Hungary and Taiwan. The Brazilian clone (CC8) was first described in 1992 in Brazil and then in other countries such as

Portugal, Argentina, Uruguay, etc. The New York/Japan (CC5) clone was identified as the dominant MRSA in hospitals in metropolitan New York, other cities in the US, and in one hospital in Tokyo, Japan. The paediatric clone (CC5) was first reported in a paediatric hospital in Portugal in 1992 and then in Poland, USA, Argentina and Colombia.

In Europe, HA-MRSA mainly belong to the clonal complexes CC5, CC8, CC22, CC30 and CC45 (Oliveira et al., 2002). In Africa, the prevalent CCs in hospitals are CC5, CC22, CC30, CC45 and CC88 (Abdulgader et al., 2015). The predominant CCs in Latin-America are CC8, CC5 and CC30, in Australia, CC8 and CC22 and in Asia, CC5 and CC8 (Rodríguez-Noriega and Seas, 2010; Chen and Huang, 2014; Aires-de-Sousa, 2017).

7.2. Community-associated MRSA (CA-MRSA)

A new source for nosocomial outbreaks was identified in 1982 in the USA with the apparition of the CA-MRSA in drug abuser patients with no hospitalization history (Saravolatz et al., 1982). A few years later, it was described CA-MRSA in healthy patients with no contact with the hospital area in Australia (Udo, 1993). In fact, MRSA is considered CA when identified in the outpatient setting or within the first 48 h following hospital admission in an individual with no medical history of MRSA infection or colonisation, admission to a healthcare facility, dialysis, surgery or insertion of indwelling devices in the past year (Bal et al., 2016). Populations or settings in which outbreaks of CA-MRSA infection have been reported include sports teams, military personnel and prisons (Lee et al., 2018). The emergence of CA-MRSA is a worldwide threat, both in the community and in healthcare facilities, since CA-MRSA is more virulent compared to HA-MRSA.

CA-MRSA strains most commonly cause SSTIs, which are often associated with abscesses or pus formation and account for ~90% of cases (Lee et al., 2018). CA-MRSA is both phenotypically and genotypically different from HA-MRSA. In contrast to HA-MRSA, CA-MRSA strains are susceptible to multiple antibiotics, but they are resistant to β-lactam agents. CA-MRSA strains belong to clonal types unrelated to HA-MRSA clones (Deurenberg et al., 2007). The main molecular markers of CA-MRSA are: (i) presence of *lukF-lukS* genes, (ii) staphylococcal cassette chromosome *mec* (SCC*mec*) type IV, V or VII and (iii) accessory gene regulator genotype I or III (Deurenberg et al., 2007; Nastaly et al., 2010). In the **Table 5**, are displayed the main differences between the genetic linages of CA-MRSA and HA-MRSA.

The CA-MRSA has been spreading in Europe since the 1990s, being the CC80 the predominant lineage. CC80 emerged in sub-Saharan Africa in the 1980s before rapidly disseminating into Europe, as a result of human migration (Bal et al., 2016). In the USA, CA-MRSA was first documented in children during the late 1990s. The isolates belonged to a single clone, designated USA400 a member of CC1. The USA400 clone has later been replaced by USA300, (belonging to CC8), which is now the most common CA-MRSA in America. A significant overlap between clones of the HA-MRSA and CA-MRSA is reported so that the differentiation of the two groups is becoming blur (Nastaly et al., 2010; Bal et al., 2016).

Table 5: comparison of CA- and HA-MRSA, adapted from (Nastaly et al., 2010)

Characteristic	CA-MRSA	HA-MRSA
Clinical manifestation	SSTIs, necrotizing pneumonia, sepsis	Pneumonia, urinary tract, bloodstream, surgical site
Risk groups	Young, drug users, prisoners, athletes, soldiers, etc.	Elderly people, healthcare workers, preterm neonate, long-term hospitalized patients
Transmission	Person-to-person spread: shared facilities (towel, pool, etc)	Person-to-person spread: healthcare staff
Resistance to antimicrobial agents	Susceptible to multiple antibiotics but resistant to β-lactam	Resistant to multiple antibiotics
SCC*mec* type	IV, V, VII	I, II, III
***agr* type**	I, III	I, II
PVL toxin	Usually present	Absent
Genetic lineages associated	CC1, CC8 (ST8), CC30 (ST30), CC59, CC80, CC88, CC93	CC5, CC8 (ST239), CC22, CC30 (ST36), CC45

7.3. Livestock-associated MRSA (LA-MRSA)

Infections with LA-MRSA can occur in people who have direct contact with farm animals in factory farms (especially associated to pig but also detected in cow and poultry production systems). This affects, for example, farmers, veterinarians or slaughterhouse employees. The first report of MRSA infections in animals was a case of

bovine mastitis in Belgium in the early 1970s (Devriese et al., 1972). Since then, increasing numbers of reports have been published on MRSA infection and colonization in both companion and food-chain animals pointing MRSA as an important veterinary and zoonotic pathogen.

In Europe, CC398 MRSA is the dominant lineage in livestock while in Southeast Asia, CC9 MRSA is the predominant type. In addition to these two clonal complexes, several other MRSA lineages including CC1, CC5, CC97, CC121, CC130 and ST425 have been reported from livestock (Bal et al., 2016; Aires-de-Sousa, 2017; Zarazaga et al., 2018). The predominant lineages in Africa are CC5, CC80 and CC88 (Lozano et al., 2016). The prevalent LA-MRSA lineage in Europe (CC398) will be further described.

7.3.1. MRSA-CC398

MRSA-CC398 was firstly detected in pigs in 2004 (Armand-Lefevre et al., 2005). In the following years, isolates of MRSA-CC398 were found in many European countries, essentially in pigs so that the clone was initially named pig-associated. It was later described in other production animals and pets (Crombé et al., 2013). The possible transfer of these strains to humans constitute a risk for human health. Indeed, this lineage is often associated with colonization and infection of humans working in the livestock industry, such as farmers, veterinarians, slaughterhouse, personnel, etc. (Crombé et al., 2013; Smith and Wardyn, 2015; Becker et al., 2017).

MRSA-CC398 strains show several specific genotypic and phenotypic characteristics. They are non-typeable by standard pulse field gel electrophoresis (PFGE) using *Sma*I enzyme (Bens et al., 2006); but are typeable with the enzymes XmaI (Bens et al., 2006), ApaI (Kadlec et al., 2009), BstZI, SacII (Rasschaert et al., 2009) or Cfr9I (Argudín et al., 2010). The MLST is useful for determination of the CC, but the *spa*-typing has revealed geographic clustering in Europe (Lozano et al., 2012b). The most prevalent CC398 associated *spa*-types are t011, t034, and t108, among others. A specific PCR is developed on the basis of the gene *sau1-hsdS1*, for the rapid detection of CC398 isolates (Stegger et al., 2011). MRSA-CC398 strains often carry SCC*mec* type IVa or V, but other SCC*mec* elements (VII, IX, X) have also been detected. They are resistant to tetracycline (mediated by *tet*(M) gene alone or in combination with other *tet* genes), which is a marker for its detection (Lozano et al., 2012b; Price et al., 2012; Benito et al., 2014b). They also often present co-resistance against other antibiotics (macrolides, lincosamides, trimethoprim, among others).

Since its discovery, MRSA-CC398 has rapidly become an emerging cause of human infections, most often associated with livestock exposure. Previous studies suggested a possible transmission of CC398-MRSA via direct contact (between animals and humans) and a dissemination through pig farms environment (air, animals feed, etc.), (Broens et al., 2012; Ferguson et al., 2017). Up to date, MRSA-CC398 origin remains unclear. The first data suggested a porcine origin, but another hypothesis based on complete genome sequencing points a human origin, with a later jump to the pig host, associated to specific genetic changes (Price et al., 2012).

CC398 origin hypothesis

At the beginning of MRSA-CC398 emergence, MSSA-CC398 had only been detected in pigs and pig farmers; so the association between exposure to pigs and human carriage leads to the hypothesis of a pig origin of this lineage (Voss et al., 2005). It was thought that MSSA-CC398 might initially have colonized pigs and then acquired *mecA* from other staphylococci that colonize pigs or pig farmers (Van Loo et al., 2007; Vanderhaeghen et al., 2010). A few years later, the implementation of the next-generation sequencing and microarray assays have allowed bacterial Whole Genome Sequencing (WGS) and comparative genome analysis. Which contributed to a deeper understanding of the origin and evolution of *S. aureus* CC398 genetic lineage, leading to the dismiss of the initial pig-origin hypothesis.

Indeed, WGS analysis of 89 isolates MSSA- and MRSA-CC398 of both animal and human origins was performed (Price et al., 2012). The results suggested that LA MRSA-CC398 has evolved from human-adapted MSSA-CC398. The jump from humans to animals would have been accompanied by the acquisition of methicillin and tetracycline resistance on one hand, and by the loss of the phage φ3, that carries the IEC genes on the other hand. It should be noted that tetracycline is characteristic of animals isolates because of its common use in farms. Consequently, two distinct phylogenetic groups are established among CC398 isolates:

- MSSA-CC398, a human-adapted ancestor subpopulation (methicillin and tetracycline susceptible, IEC-positive)
- and MRSA-CC398, a livestock-adapted derived subpopulation (methicillin and tetracycline resistant, *mecA*-positive, *tet*(M)-positive and IEC-negative).

New variant of CC398 isolates

It has been demonstrated that the isolates MRSA-CC398 of animal origin present the same characteristics as those detected in humans with any type of relation with livestock (*tet*(M)-positive and IEC-negative) (Price et al., 2012). This finding suggests a transmission from animals to humans which could occur through animal-human direct contact, derived-meat manipulation, environment etc. **(Figure 8)** (Smith, 2015). However, it has been recently reported cases of MRSA-CC398 colonization and infections in humans which lack contact with livestock and any other risk factor, suggesting a later transmission between humans (Benito et al., 2014b; Larsen et al., 2015; Lekkerkerk et al., 2015).

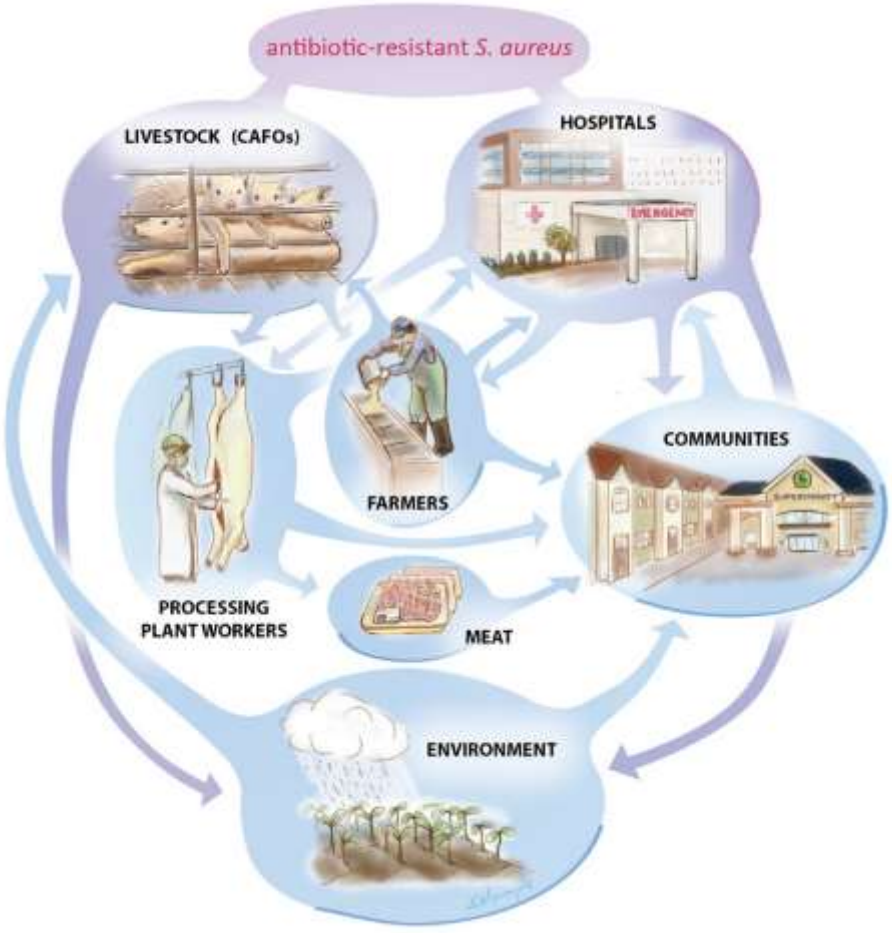


Figure 8: Interconnexion of different ecosystems, S. aureus possible ways of dissemination, adapted from (Smith, 2015)

Moreover, a possible adaptation of MRSA-CC398 to humans is worth considering with the acquisition of IEC genes, normally missing in the animal subpopulation. Indeed, IEC genes have been reported in MRSA-CC398 from animals and humans (Cuny et al., 2015; Pérez-Moreno et al., 2017). IEC re-acquisition by LA-MRSA-CC398 strains

could constitute an emerging public health problem. It would represent an evolutionary step towards LA-MRSA-CC398's adaptation to human hosts, and might enhance its invasiveness and ability to be transmitted to humans (Pérez-Moreno et al., 2017).

Moreover, the emergence of infections (frequently septicaemia) associated with MSSA-CC398 in humans without animal contact, have been reported worldwide mainly in France, North-eastern USA, China and the Caribbean. The strains were characterized as *spa*-type t571, tetracycline-susceptible and PVL-negative. In France, MSSA-CC398 represent 7.5% of all MSSA endocarditis cases (Chroboczek et al., 2013). In China, CC398/t571 was predominant (11%) among MSSA human clinical isolates while CC9 was the most common LA-MRSA in pigs (Chen et al., 2010). Later, comparative genome analysis revealed that the strains characteristics differed from LA-MRSA CC398: presence of phage φ3 (*scn*-positive), lack of *tet*(M) and a greater virulence potential and highly transmissibility among humans. These strains seem to have evolved from the human-associated livestock-independent subpopulation (Uhlemann et al., 2012). Furthermore, they harbor a chromosomally encoded *erm*(T) gene that can be considered a marker for MSSA-CC398 detection (Vandendriessche et al., 2011; Cuny et al., 2013). In addition, the ability of t571 isolates to acquire PVL encoding genes is a big threat. Indeed, lethal necrotizing pneumonia caused by MSSA ST398 *spa*-type t571 (PVL-positive, *tet*(M)-negative) has already been reported (Rasigade et al., 2010).

7.4. MRSA in Food

S. aureus is a leading cause of gastroenteritis resulting from the consumption of contaminated food. Indeed, staphylococcal food poisoning (SFP) is due to the ingestion of enough amounts of preformed enterotoxins (Le Loir et al., 2003). Foods that have been frequently involved in staphylococcal intoxication are essentially of animal origin, e.g. meat and meat products, poultry and egg products, milk and dairy products, etc. (Argudín et al., 2010). Contamination is mainly associated with improper handling of cooked or processed foods via manual contact or through respiratory secretions. During slaughtering of animals, MRSA already present in/on the animal from which the food originates can be transferred to the carcasses and the slaughterhouse environment, and consequently the meat of these animals may become contaminated (Kluytmans, 2010).

Food contamination is even more worrisome since MRSA strains have been detected from animal-derived food worldwide **(Figure 9)**. Many of them were able to produce enterotoxins or to carry SEs encoding genes (Sergelidis and Angelidis, 2017). However,

methicillin resistance is unrelated to the production of enterotoxins, and SFP is not a disease that is treated with antibiotics. Therefore, enterotoxigenic MRSA may possess a similar potential to cause SFP as any other enterotoxigenic MSSA. The main risks related to the presence of MRSA in foods are the dissemination of the pathogen in the community (Wendlandt et al., 2013). As previously reported, any of the MRSA groups earlier described (HA, CA, and specially LA-MRSA-CC398) can contaminate animal-derived-food, and cause food poisoning outbreaks (Kluytmans et al., 1995; Jones et al., 2002; Lozano et al., 2009; Wendlandt et al., 2013; Larsen et al., 2016; Sharma et al., 2018; Wu et al., 2019).

In view of the above and due to the high rate of international commercialization, food may serve as a vehicle for *S. aureus* and MRSA. Hygienic practices at all stages of the food production chain should be improved in order to prevent the dissemination of food-borne antimicrobial-resistant bacteria.

JUSTIFICATION OF THE THESIS PROJECT

Antimicrobial resistance is a big threat for public health as it limits options for bacterial infections prevention and treatment, leading to an increase of mortality. The first antimicrobial resistance global report on surveillance of the WHO in 2014 included bacteria which are causing some of the most common infections in different settings; in the community, in hospitals or transmitted through the food chain. *S. aureus* resistant to methicillin (MRSA) was one of the selected bacteria, due to its high occurrence and spread in hospital cares in different countries and the underlying clinical and economic burden.

The antimicrobial resistance involves not solely infectious agents, but also non-pathogenic bacteria which are present in diverse ecological niches and are potential reservoir for resistance and virulence genes. Staphylococcal species (both coagulase positive (CoPS) and negative (CoNS)) are normal colonizers of skin and mucous of healthy humans and animals but also opportunistic pathogens that can cause skin and soft tissues infections, mastitis, food intoxication, bacteraemia etc. They can acquire genetic elements with resistant genes and transfer them to other species. CoNS are thought to be the origin methicillin resistance encoding gene.

Staphylococci can be also found in the environment elements (air, soil, water, etc.) and in food. People, animals and the environment are interconnected so that the dissemination of antimicrobial resistance bacteria should be studied under a global perspective. However, most of epidemiological studies in Europe and Africa, focussed on *S. aureus* from clinical settings, and in a lesser extend of the farm environment.

The present doctoral thesis is a compendium of scientific papers which aims at studying staphylococci epidemiology analysing both CoPS and CoNS from conventional and unconventional animals of the food chain in Spain and Senegal (hunting animals, equine, livestock, etc.), and from derived food. The work also studied the spread of *S. aureus* livestock-associated clonal lineages in human infections. In this thesis, the antimicrobial resistance phenotypes and the resistance genes involved were studied. Virulence factors and host-adapted mechanisms were analysed as well. Molecular typing is an important technique used to evaluate genetic lineages. This thesis project intends to increase scientific knowledge of *Staphylococcus* epidemiology and to give evidences on impact of antimicrobial resistance spread and poor hygiene practices in food safety issues.

OBJECTIVES/ OBJETIVOS

The main objectives of this thesis are:

1. To know the diversity of species, the genetic lineages, the antimicrobial resistance phenotypes/genotypes as well as the virulence and host-adaptation factors of *Staphylococcus* spp. from unconventional animals of the food chain (hunting and companion animals) destined for human consumption in Spain. **(Chapter 1)**
   1.1. Wild boar (papers 1 and 2)
   1.2. Horses (paper 3)
2. To know the diversity of species, the genetic lineages, the antimicrobial resistance phenotypes/genotypes as well as the virulence and host-adaptation factors of *Staphylococcus* spp. from food-producing animals at slaughterhouse level in Spain and Senegal. **(Chapter 2:** papers 4 and 5**)**
3. To analyse the genetic lineages of *S. aureus* from animal-derived food samples in Spain and to determine the frequency of CC398 among MRSA and MSSA isolates. **(Chapter 3:** paper 6, in revision**)**
4. To determine the frequency of the lineage CC398, generally associated to livestock, among invasive MSSA and MRSA isolates in Spanish hospitals and to study the antimicrobial resistance phenotypes/genotypes as well as the virulence and host-adaptation factors of selected isolates. **(Chapter 4:** paper 7 (in revision) and paper 8 (in preparation)**)**

Los objetivos de esta tesis son:

1. Estudiar la diversidad de especies, las líneas genéticas, y los fenotipos/genotipos de resistencia a antibióticos, así como los factores de virulencia y de adaptación al huésped de *Staphylococcus* spp. aislados de animales non-convencionales de la cadena alimentaria (animales de caza y de compañía) destinado al consumo humano en España. (**Capítulo 1)**
   1.1. Jabalíes (artículos 1 y 2)
   1.2. Caballos (artículo 3)
2. Estudiar la diversidad de especies, las líneas genéticas, y los fenotipos/genotipos de resistencia a antibióticos, así como los factores de virulencia y de adaptación al huésped de *Staphylococcus* spp. aislados de animales de producción a nivel de matadero en España y Senegal. (**Capítulo 2:** artículos 4 y 5**)**
3. Analizar las líneas genéticas de *S. aureus* de alimentos de origen animal en España y determinar la frecuencia del linaje CC398 en aislados SARM y SASM. (**Capítulo 3:** artículo 6, en revisión**)**
4. Determinar la frecuencia del linaje CC398, generalmente asociado a ganado, en aislados invasivos SASM y SARM en hospitales españoles y estudiar el fenotipo/genotipo de resistencia a antibióticos, así como los factores de virulencia y de adaptación al huésped de aislados seleccionados. (**Capítulo 4:** artículo 7 (en revisión) y 8 (en preparación))

RESULTS

CHAPTER 1: *Staphylococcus* spp. FROM HUNTING AND COMPANION ANIMALS DESTINED FOR HUMAN CONSUMPTION IN SPAIN

As mentioned earlier, antibioresistance is an issue which involves diverse ecosystems (humans, animals, food and the environment); though, it should be analysed under a global and multidisciplinary perspective ("One Health" concept). The epidemiology of staphylococci in animals has gained interest in the last years, not only because of their importance in veterinary medicine but also because of the emergence of some clonal lineages associated with animals and their increasingly zoonotic potential. Most works found in the literature, analysed CoPS (mainly *S. aureus*) from pets or farm animals. Little is known about wild animals' carriage, especially those destined for human consumption. These animals may be subjected to less antimicrobial selective pressure, but they could be a reservoir for staphylococci or a vehicle for their transmission. The risk of zoonotic transmission is increased when wild animals are part of the food chain, implying more contact with humans. Several works studied staphylococcal epidemiology in companion animals but very few analysed the impact on food safety, because they are rarely considered for human consumption. The first chapter of this thesis studied the molecular epidemiology of staphylococcal species in wild boar (papers 1 and 2) and in horses (paper 3) in Spain, where they are unconventional sources of meat.

Nasal samples of 371 healthy wild boars were collected in commercial hunting events (known as "monterías") during a hunting period (October-December 2016) from six Spanish regions by the SaBio group of the Hunting Resources Research Institute (IREC /CSIC-UCLM-JCCM), Ciudad Real (Spain) for staphylococci recovery and characterization. CoPS were analysed in paper 1 and CoNS were studied in paper 2. Sixty-six of the animals carried CoPS (17.8%) while 136 carried CoNS (36.6%).

Among the 67 CoPS isolates, *S. aureus* was predominant (n=51), followed by *S. hyicus* (n=10) and *S. pseudintermedius* (n=6). A high rate of antimicrobial susceptibility was generally found among *S. aureus* (74.5% were susceptible to all antimicrobials tested). However, one isolate was penicillin/methicillin- and tetracycline-resistant with characteristics of the LA-MRSA-CC398 (ST398-t011, *agr*I, *blaZ*, *mecA*, *tet*(M) and *tet*(K) genes). A great diversity of *S. aureus* genetic lineages (17 STs) was observed among wild boar with predominance of ST2328/CC133 (29%), mainly associated with t3750. *S. hyicus* isolates were all susceptible to antimicrobials tested, while *S. pseudintermedius* isolates were mostly streptomycin-resistant and carried the *lukF/S-I* and *siet* virulence genes.

Seventeen species were identified among 161 CoNS isolates recovered, being the predominant species, *S. sciuri* (39.7%), *S. xylosus* (13%) and *S. chromogenes* (10.5%). A 22.4% of CoNS showed resistance to at least one antimicrobial tested. Tetracycline resistance phenotype was the most frequently detected (10.5%), mostly mediated by *tet*(K) gene. Other genes, involved in beta lactams, macrolides, lincosamides, chloramphenicol and SXT resistance were also detected [*mecA*, *erm*(B), *erm*(F), *mphC*, *erm*(43), *msr*(A)/*msr*(B), *lnu*(A), *dfrG*, *fexA*, and $catp_{C221}$].

Generally, these works described wild boar as source for a great diversity of both CoPS and CoNS isolates. CoNS are here analysed for the first time in wild boar. The detection of the LA-MRSA-CC398 in wildlife is worrisome and points out the easy capacity of adaptation of this lineage in diverse ecosystems. Moreover, wild boar can act as vehicle for staphylococci and antimicrobial resistance genes, especially if they are intended for human consumption.

Paper 3 analysed staphylococcal isolates from 80 nasal and faecal samples of 73 healthy horses destined for human consumption in Spain. *Staphylococcus* isolates were detected in 90% of nasal samples and in 66% of faecal samples. Eight species were identified among 90 isolates recovered, with predominance of *S. aureus* (37%), *S. delphini* (21%) and *Staphylococcus sciuri* (21%). *S. aureus* strains were all MSSA, 82.3% were susceptible to all the antibiotics tested, and the remaining showed resistance to (gene detected) streptomycin (*ant(6)-Ia*), penicillin (*blaZ*), and SXT (*dfrA*, *dfrG*). The predominant *S. aureus* lineage, ST1640 (61.7%), is associated with horses for the first time in this study. *S. aureus* isolates, except those of lineage ST1640, carried the gene encoding the equid-adapted leukocidin (LukPQ) and the blocker of equine complement system activation (eqSCIN). The *scn* gene was absent in all *S. aureus* isolates. The toxic shock syndrome toxin-encoding gene was detected in some *S. aureus* isolates. All *S. delphini* isolates were susceptible to all antimicrobials tested. Multidrug resistance was observed among *S. sciuri* isolates, but not among *S. aureus*.

This work highlighted the great diversity of CoPS and MRCoNS in an animal species very close to human, and here destined for human consumption. A potentially emergent *S. aureus* lineage (ST1640) in horses is detected but its origin is unclear. The detection of isolates carrying important virulence factors is a food safety and public health issue as they can be transferred to food and consequently to humans.

The three papers presented below reported with more details the results described above and their interpretation, as well as the methodology used.

Paper 1: Mama, O.M., Ruiz-Ripa, L., Fernández-Fernández, R., González-Barrio, D., Ruiz-Fons, J.F., Torres, C., 2019. High frequency of coagulase-positive staphylococci carriage in healthy wild boar with detection of MRSA of lineage ST398-t011. *FEMS Microbiol. Letters.* 366, fny292. doi.org/10.1093/femsle/fny292

Paper 2: Mama, O.M., Ruiz-Ripa, L., Lozano, C., González-Barrio, D., Ruiz-Fons, J.F., Torres, C., 2019. High diversity of coagulase negative staphylococci species in wild boars, with low antimicrobial resistance rates but detection of unusual genes. *Comp. Immunol. Microbiol. Infect. Dis*. 64, 125–129. doi: 10.1016/j.cimid.2019.03.006

Paper 3: Mama O.M., Gómez P., Ruiz-Ripa L., Gómez-Sanz E., Zarazaga M., Torres C., 2019. Antimicrobial Resistance, Virulence, and Genetic Lineages of Staphylococci from Horses Destined for Human Consumption: High Detection of *S. aureus* Isolates of Lineage ST1640 and Those Carrying the *lukPQ* Gene. *Animals*. 9, 900. doi.org/10.3390/ani9110900

PAPER 1

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RESEARCH LETTER – Food Microbiology

High frequency of coagulase-positive staphylococci carriage in healthy wild boar with detection of MRSA of lineage ST398-t011

Olouwafemi Mistourath Mama[1], Laura Ruiz-Ripa[1], Rosa Fernández-Fernández[1], David González-Barrio[2], Jose Francisco Ruiz-Fons[2] and Carmen Torres[1,*,†]

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One sentence summary: Wild boar frequently carry coagulase-positive-staphylococci (*S. aureus*, *S. pseudintermedius* and *S. hyicus*), specially relevant are *S. aureus* (both methicillin-susceptible and methicillin-resistant) of emergent lineages of interest in human and animal medicine.
Editor: Stefan Schwarz
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ABSTRACT

The objective of this study was to determine the frequency and diversity of coagulase-positive staphylococci (CoPS) in nasal samples of healthy wild boar, to study their resistance phenotypes/genotypes and to check the occurrence of the MRSA-ST398. Nasal samples of 371 wild boars were collected in Spain for staphylococci and MRSA recovery. Staphylococci identification was performed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF). The susceptibility to 11 antimicrobials was tested by disc-diffusion and the presence of resistance genes by PCR. Molecular typing and virulence factors determination were carried out by PCR and sequencing. The rate of CoPS carriage (*Staphylococcus aureus*, *Staphylococcus hyicus* and *Staphylococcus pseudintermedius*) in wild boar was of 17.8% (13.7%, 2.7% and 1.6%, respectively). Susceptibility to all tested antimicrobials was shown in 74.5% of *S. aureus* and one strain was MRSA [lineage ST398-t011-*agrI*, carrying *blaZ*, *mecA*, *tet*(M) and *tet*(K) genes]. A total of 22 *spa*-types and 17 STs were detected among *S. aureus*, including: ST398/CC398 ($n = 1$), ST2328-ST133/CC133 ($n = 20$), ST425/CC425 ($n = 7$), ST5/CC5 ($n = 5$), ST1/CC1 ($n = 3$), ST130/CC130 ($n = 2$) and ST88/CC88 ($n = 1$). Two *spa*-types (t02, t15) and four STs (ST455, ST796, ST797, ST798) were detected among the six *S. pseudintermedius* isolates recovered, and all of them carried the *lukF/S-I* and *siet* virulence genes. All *S. hyicus* isolates were susceptible to antimicrobials tested.

Keywords: *S. aureus*; *S. pseudintermedius*; *S. hyicus*; MRSA; CC398; wild boar

INTRODUCTION

Coagulase positive *Staphylococcus* (CoPS) species are part of the natural microbiota of skin and mucous membranes of humans and animals (Seinige, Von Altrock and Kehrenberg 2017). The *Staphylococcus aureus*, *Staphylococcus pseudintermedius* and *Staphylococcus hyicus* (coagulase variable)

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PAPER 2

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High diversity of coagulase negative staphylococci species in wild boars, with low antimicrobial resistance rates but detection of relevant resistance genes

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ABSTRACT

This work was focused to determine the prevalence and the species diversity of coagulase-negative staphylococci (CoNS) in wild boars, and to study their antimicrobial resistance phenotype and genotype. Nasal samples of 371 wild boars from six Spanish regions were collected for CoNS recovery. The identification was performed by MALDI-TOF mass-spectrometry. Antimicrobial susceptibility for eight antimicrobial agents was studied by disc-diffusion method and the presence of 31 antimicrobial resistance genes by PCR.

CoNS were detected in nasal samples of 136/371 animals tested (36.6%), and 161 isolates were obtained (1–3/animal); a high diversity of species was found (n = 17), with predominance of *S. sciuri* (n = 64), *S. xylosus* (n = 21) and *S. chromogenes* (n = 17). Among CoNS isolates, 22.4% showed resistance to at least one antimicrobial tested. Tetracycline-resistance phenotype was the most frequently detected (10.5%), generally mediated by *tet*(K) gene [associated or not with *tet*(L)]. Other relevant resistance genes were identified including unusual ones [*mecA*, *erm*(B), *erm*(F), *mphC*, *erm*(43), *msr*(A)/*msr*(B), *lnu*(A), *dfrG*, *fexA*, and cat_{pC221}].

This is the first study in which CoNS isolates from wild boars are analysed. The knowledge of antimicrobial phenotype and genotype of CoNS in natural ecosystems is highly important since these staphylococcal species can act as vectors of relevant antimicrobial resistance mechanisms.

1. Introduction

Coagulase-negative staphylococci (CoNS) constitute a very heterogeneous group differentiated from other *Staphylococcus* spp. such as *S. aureus* or *S. pseudintermedius* by its lack of coagulase production [1]. They are generally found living naturally on skin and mucous membranes of humans and animals (mammals and birds), and they can also be found on foodstuffs [2–4]. *S. saprophyticus*, for example, is a CoNS species which seems to be part of the gastrointestinal microbiota of cattle and pigs, and is a common contaminant of respective foods, such as raw beef and pork [3]. CoNS can be inoffensive commensals or invasive opportunistic pathogens [5]. Thus, considered as opportunistic, they represent one of the major nosocomial pathogens mainly in neonatal intensive care units (NICU) and food poisoning, and can colonize implanted foreign bodies [3,5–7]. Staphylococcal species, most notably *S. epidermidis* and *S. aureus*, cause 60%–70% of infections in NICU [8]. Besides, an inadequate and abusive therapeutic use of antibiotic drugs in humans and animals, combined to their use as growth promoters in livestock (still allowed in some countries, but not in the European Union), lead to bacterial resistance acquisition [9,10]. Thereby, a continuous loss of CoNS susceptibility towards most of the antimicrobials commonly used has been reported for decades [3].

While the species diversity and antimicrobial resistance characteristics of CoNS from humans and food-producing animals and derived foodstuffs have been extensively investigated, scarce information about CoNS in wildlife does exist. According to few studies, CoNS have been found in wild animals such as birds of prey, lynxes and gray treefrogs [11–13]. In Spain, wild boars are part of the population diet and these animals are known to be a reservoir of *S. aureus* [14]. However, the presence of CoNS in wild boars has not been studied before. Our work was aimed to determine the prevalence of CoNS in these animals in Spain, and to study the species diversity and their antimicrobial resistance phenotypes and genotypes.

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PAPER 3

animals

MDPI

Article

Antimicrobial Resistance, Virulence, and Genetic Lineages of Staphylococci from Horses Destined for Human Consumption: High Detection of *S. aureus* Isolates of Lineage ST1640 and Those Carrying the *lukPQ* Gene

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Simple Summary: Staphylococci are opportunistic pathogens which colonize humans and animals. Zoonotic transfer of staphylococcal species between domestic animals and humans is common and can occur through direct contact, the environment, and animal-derived food processing, implying a risk of the spread of antimicrobial resistance mechanisms and virulence factors into different ecosystems. Our work aimed at studying the diversity of staphylococcal species in nasal and faecal samples of healthy horses intended for human consumption and their resistance and virulence determinants. Staphylococci were detected in 90% and 66% of nasal and faecal samples tested, respectively. Eight staphylococcal species were detected, with the most prevalent ones being *Staphylococcus aureus* (all isolates were methicillin-susceptible), *Staphylococcus delphini*, and *Staphylococcus sciuri*. The predominant *S. aureus* lineage, ST1640, is associated with horses for the first time in this study. *S. aureus* isolates, except those of lineage ST1640, produced equid-adapted leukocidin (LukPQ) and blocker of equine complement system activation (eqSCIN). The toxic shock syndrome toxin-encoding gene was also detected in some *S. aureus* isolates. Multidrug resistance was observed among *S. sciuri* isolates, but not among *S. aureus*. Measures of hygiene and control should be implemented during horse slaughter and meat processing.

Abstract: This work aimed to determine the frequency and diversity of *Staphylococcus* species carriage in horses intended for human consumption, as well as their resistance and virulence determinants. Eighty samples (30 nasal; 50 faecal) were recovered from 73 healthy horses in a Spanish slaughterhouse. The samples were cultured for staphylococci and methicillin-resistant staphylococci (MRS) recovery. The phenotype/genotype of antimicrobial resistance was analysed for all isolates. The *spa*-type and sequence-type (ST) were determined in *Staphylococcus aureus* strains; moreover, the presence of virulence and host-adaptation genes (*tst*, *eta*, *etb*, *pvl*, *lukPQ*, *scn-eq*, and *scn*) was studied by PCR. *Staphylococcus* species were detected in 27/30 (90%) and 33/50 (66%) of nasal and faecal samples, respectively. Ninety isolates belonging to eight species were recovered, with predominance of *S. aureus* (n = 34), *Staphylococcus delphini* (n = 19), and *Staphylococcus sciuri* (n = 19). *S. aureus* strains were all methicillin-susceptible (MSSA), 28/34 were susceptible to all the antibiotics tested, and the remaining six showed resistance to (gene-detected) streptomycin (*ant* (6)-*Ia*), penicillin (*blaZ*),

and trimetroprim/sulphametoxazole (SXT) (*dfrA*, *dfrG*). The lineage ST1640/t2559 was predominant ($n = 21$). The genes *lukPQ* and *scn-eq* were present in all but the ST1640 isolates. Three *S. sciuri* isolates were multidrug-resistant. Healthy horses in Spain seem to be a reservoir for virulent MSSA and the lineage ST1640, although the presence of the latter in horses is described for the first time in this study. Moreover, the equine-adapted leukocidin gene *lukPQ* is frequent among *S. aureus* strains. A large variety of staphylococcal species with low antibiotic resistance rate were also observed.

Keywords: healthy horses; staphylococci; MSSA; ST1640; *lukPQ*

1. Introduction

Staphylococci are commensal bacteria that generally colonize nares, skin, and mucous membranes of humans and of wild and domestic animals, although some species are opportunistic pathogens [1–6]. Horses have been described as carriers of staphylococcal species and methicillin-resistant staphylococci (MRS) [7–10]. Coagulase-positive staphylococci (CoPS) such as *Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus delphini*, and *Staphylococcus pseudintermedius* are frequently reported as colonizers or infectious agents in horses [8–12]. Coagulase-negative staphylococci (CoNS) have been described as causative agents of mastitis, wound infections, and skin abscesses in various animals, including horses [13].

Staphylococcal infections are a major issue in both human and veterinary medicine, and their role in severe diseases has increased with the acquisition of antimicrobial resistance mechanisms [11,13]. Moreover, *S. aureus* has a large variety of virulence factors, such as staphylococcal enterotoxins, toxic shock syndrome toxin (TSST-1), or leukocidins, among others [14]. Leukocidins are a family of bicomponent pore-forming toxins involved in *S. aureus* pathogenicity [15]. To date, six leukocidins have been identified, including Panton Valentine leukocidin (*lukF/lukS-PV*), LukMF', and the novel equid-adapted leukocidin LukPQ, which are related to phage-encoded genes mainly found in humans, ruminants, and equines, respectively [15,16]. LukPQ, encoded by the 45-kb prophage φSaeq1, was found to be strongly associated with *S. aureus* from horses and donkeys. This leukocidin preferentially destroys neutrophils with higher efficiency than its closest fellow, LukED [15]. It was recently revealed that the prophage φSaeq1 also encodes a novel variant of staphylococcal complement inhibitor SCIN-A (termed eqSCIN, encoded by *scn-eq*) which shares 57.8% amino acid identity with SCIN-A (encoded by *scn*) from human *S. aureus* [17]. eqSCIN is a potent blocker of equine complement system activation, which plays an important role in *S. aureus* host adaptation. Whereas SCIN-A isolates exclusively inhibit human complement, eqSCIN represents the first animal-adapted SCIN variant that functions in a broader range of hosts (horses, humans, and pigs) [17].

The presence of staphylococcal species in horses is of public health concern since the potential transfer of *Staphylococcus* spp. and their resistance and virulence genes between healthy humans and domestic animals has been evidenced [18–20]. Direct contact may be a way of transmission, but other vehicles, such as the environment and food, should be taken into consideration. In Spain, horse meat is used for human meat consumption; hence, it is important to determine the diversity of staphylococcal species colonizing the mentioned animal species. In that context, this work aimed to identify the different species of staphylococci present in nares and faeces of healthy horses destined to human consumption, as well as the antimicrobial resistance phenotype and genotype of the recovered isolates, and the virulence traits for *S. aureus* species.

2. Material and Methods

2.1. Sample Recovery

A total of 80 samples (nasal: $n = 30$ and faecal: $n = 50$) were recovered with sterile swabs from 73 healthy horses intended for human consumption and kept in Amies transport medium (Copan, Murrieta/USA). Seven animals were tested for both types of samples. Animals came from 19 Spanish regions before they were transported to a slaughterhouse located in Northern Spain, where samples were taken in February 2012.

2.2. Staphylococcus spp. Isolation, Identification, and DNA Extraction

The samples were first inoculated in Brain Heart Infusion (BHI, supplemented with NaCl 6.5%) broth (Conda, Madrid/Spain) and incubated at 37 °C for 24 h. After growth, the bacterial culture was distributed on plates of mannitol–salt–agar (Conda, Madrid/Spain) and oxacillin resistance screening agar base (Oxoid, Hampshire/England) for staphylococci and MRS recovery, respectively. Up to four colonies/plate with staphylococcal morphology were isolated and subjected to the DNase agar test (Conda, Madrid/Spain). Identification was performed by PCR (for CoPS isolates) [21] and by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker, MA, USA) (for all the staphylococci).

The DNA extraction was performed as follows: one colony was resuspended in 45 µL of milli-Q water and 5 µL of lysostaphin (1 mg/mL). The suspension was warmed in a water bath at 37 °C during 10 min. Then 45 µL of milli-Q water, 150 µL of Tris (0.1 M, pH 8.5), and 5 µL of proteinase K (2 mg/mL) were added to the suspension before it was heated again in a water bath at 60 °C for 10 min and at 100 °C for 5 min. Finally, centrifugation was performed at 12,000 rpm for 3 min and the supernatant was kept for further experiences.

2.3. Antimicrobial Susceptibility and Resistance Genes

Susceptibility testing to penicillin, cefoxitin, gentamicin, tobramycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ciprofloxacin, linezolid, and trimethoprim/sulfamethoxazole (SXT) was performed by disk-diffusion method according to the Clinical Laboratory Standards Institute recommendations [22]. Susceptibility to streptomycin was also tested (CASFM 2018). The presence of the following antimicrobial resistance genes was determined by PCR, in accordance with the identified resistance phenotypes: beta-lactams (*mecA*, *blaZ*), tetracycline (*tet*(K), *tet*(L), and *tet*(M)), macrolides-lincosamides (*erm*(A), *erm*(B), *erm*(C), *erm*(T), *msr*(A), *lnu*(A), *lnu*(B), and *vgaA*), streptomycin (*str* and *ant*(6)-Ia), chloramphenicol (*fexA* and *fexB*), and SXT (*dfrA*, *dfrD*, *dfrG* and *dfrK*) [23–27].

2.4. Molecular Typing

For the *S. aureus* isolates, *spa*-typing was performed as previously described [28]. Multilocus sequence typing (MLST) was determined for representative isolates (one isolate of each *spa*-type, except for the *spa*-type t2559 for which three associated isolates were chosen). For this purpose, PCR and sequencing of seven housekeeping genes (www.pubmlst.org) were performed to define the sequence type (ST) and the clonal complex (CC). Additionally, detection of *agr* allotypes was carried out by two multiplex PCRs in all isolates [29]. For *S. delphini* isolates, a PCR with specific primers was performed to classify them in two groups (A or B) as previously described [30].

2.5. Virulence Genes

For *S. aureus* isolates, the presence of the genes encoding the toxic shock syndrome toxin (*tst*) and exfoliative toxin A (*eta*) and B (*etb*) was studied by PCR [26]. In addition, the genes encoding the leukocidins of Panton-Valentine (*lukF*/*lukS*-PV) and LukPQ were studied by PCR and sequencing [15,26]. The presence of *scn* (gene for SCIN-A) [31] and *scn-eq* (gene for eqSCIN) was analysed by PCR and sequencing. The *scn-eq* PCR was performed using a pair of primers designed in this study (eqSCN-F: TGCTGCTTTTGCTTTGTATCC and eqSCN-R: TGCAGGAGTTTTAGTTGCAGTTTT) and the following conditions: 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 2 min at 61.5 °C, and 3 min at 72 °C, with a final extension at 72 °C for 10 min.

3. Results

Staphylococcus isolates were detected in 27/30 (90%) of nasal samples and in 33/50 (66%) of faecal samples. The seven animals tested for both types of samples were positive for staphylococcal species in both cases.

A total of 90 isolates of eight species were detected in the positive nasal/faecal samples: *S. aureus* ($n = 34$), *S. delphini* ($n = 19$), *Staphylococcus sciuri* ($n = 19$), *Staphylococcus simulans* ($n = 4$), *Staphylococcus fleurettii* ($n = 2$), *Staphylococcus lentus* ($n = 2$), *Staphylococcus saprophyticus* ($n = 2$), *Staphylococcus xylosus* ($n = 2$), *Staphylococcus haemolyticus* ($n = 2$), *Staphylococcus schleiferi* ($n = 2$), *Staphylococcus vitulinus* ($n = 1$), and *Staphylococcus hyicus* ($n = 1$) (Tables 1 and 2). Twenty-four samples harboured at least two isolates of either distinct species, distinct antibiotic resistance phenotypes or different *spa*-types.

3.1. S. aureus Isolates: Molecular Characteristics, Antimicrobial Resistance, and Virulence Determinants

S. aureus was detected in 17/30 (56.6%) and 16/50 (32%) of the nasal and faecal samples, respectively. The 34 isolates recovered (nasal origin: $n = 18$; faecal origin: $n = 16$) were ascribed to five *spa*-types (t2559, t3269, t127, t1294 and t549), four STs (ST1640, ST1, ST816 and ST1660), and four *agr*-types (I, II, III and IV) (Table 1). One isolate per sample was detected except for one nasal sample which harboured two *S. aureus* isolates of different *spa*-types (t2559 and t1294). Most of the isolates were susceptible to all the antimicrobials tested ($n = 28$; 82.4%), while the remaining six showed the following resistance phenotypes (number of isolates; genes detected): streptomycin (3; *ant*(6)-*Ia*), penicillin+streptomycin (1; *blaZ*, *ant*(6)-*Ia*, *str*), and penicillin + SXT (2; *blaZ*, *dfrA*, *dfrG*). In addition, three nasal isolates (all ST816) hosted the gene *tst*. Moreover, all isolates but those of the lineage ST1640 harboured the genes *lukPQ* and *scn-eq*. The isolates of lineage ST1640 were collected from animals which came from six regions, most of them of Southern Spain.

The major *S. aureus* lineage in horses found in this study was the ST1640 associated to nine and 12 strains of nasal and faecal origins, respectively. In addition, the antimicrobial resistance rates, phenotypes and genotypes are similar in isolates of both origins (Table 1). Concerning the virulence genes, the isolates of the lineage ST1640 were the only ones which lacked the LukPQ determinants. None of the isolates of this study harboured the *scn* gene, a marker of the human immune evasion cluster (IEC).

Table 1. Characteristics of 34 *Staphylococcus aureus* isolates recovered from nasal and faecal samples of horses at a slaughterhouse.

Sample Source	Number of Strains	ST/CC [a] (Number of Strains)	*Spa*-type (Number of Strains)	*Agr*-Type	Antimicrobial Resistance		Virulence Genes (Number of Strains)
					Phenotype [b] (Number of Strains)	Genotype (Number of Strains)	
Nasal	18	ST1640 (9)	t2559 (9)	IV	STR (1)	*ant(6)-Ia*	-
					SUSCEPTIBLE (8)	-	-
		ST1/CC1 (5)	t3269 (3)	III	SUSCEPTIBLE (3)	-	*lukPQ* (3), *scn-eq* (3)
			t127 (2)	III	PEN, SXT (1)	*blaZ*, *dfrG*	*lukPQ*, *scn-eq*
					SUSCEPTIBLE (1)	-	*lukPQ*, *scn-eq*
		ST816/CC479 (3)	t1294 (3)	II	SUSCEPTIBLE (3)	-	*tst* (3), *lukPQ* (3), *scn-eq* (3)
		ST1660/CC9 (1)	t549 (1)	II	PEN, STR	*blaZ*, *ant(6)-Ia*, *str*	*lukPQ*, *scn-eq*
Faecal	16	ST1640 (12)	t2559 (12)	IV	STR (1)	-	-
					SUSCEPTIBLE (11)	-	-
		ST1/CC1 (3)	t127 (2)	III	PEN, SXT (1)	*blaZ*, *dfrA*, *dfrG*	*lukPQ*, *scn-eq*
			t386 (1)	III	SUSCEPTIBLE (1)	-	*lukPQ*, *scn-eq*
		ST133/CC133 (1)	t2420 (1)	I	STR	-	*lukPQ*, *scn-eq*
					SUSCEPTIBLE	-	*lukPQ*, *scn-eq*

[a] The multilocus sequence typing (MLST) was performed for one isolate of each *spa*-type, and for three isolates of *spa*-type t2559; [b] PEN: penicillin; STR: streptomycin; SXT: trimethoprim/sulphametoxazole.

Table 2. Characteristics of 56 non-*aureus* staphylococcal isolates recovered from nasal and faecal samples of horses at the slaughterhouse.

Sample Source	(Number of Strains)	Species (Number of Strains)	Antimicrobial Resistance	
			Phenotype [c] (Number of Strains)	Genotype (Number of Strains)
Nasal	30	*S. delphini* [a] (11)	SUSCEPTIBLE (11)	-
		S. sciuri (10)	STR (1)	*str (1)*
			PEN, FOX (2)	*mecA* (2)
			PEN, FOX, TET (2)	*mecA* (2), *tet*(K) (2), *tet*(L) (2)
			PEN, FOX, CLI (1)	*mecA*, *lnu*(A) (1)
			PEN, FOX, STR, TET [e] (1)	*mecA*, *str*, *tet*(K), *tet*(L) (1)
			SUSCEPTIBLE (3)	-
		S. fleurettii (2)	SUSCEPTIBLE (2)	-
		S. lentus (2)	ERY, CLI [d] (1)	*erm*(A), *erm*(B), *msr*(A) (1)
			PEN, FOX, STR (1)	*mecA*, *str* (1)
		S. saprophyticus (2)	SUSCEPTIBLE (2)	-
		S. xylosus (2)	CLI (1)	-
			SUSCEPTIBLE (1)	-
		S. haemolyticus (1)	SUSCEPTIBLE (1)	-
Faecal	26	*S. delphini* [b] (8)	SUSCEPTIBLE (8)	-
		S. sciuri (9)	PEN, FOX (6)	*mecA* (6)
			PEN, FOX, STR, TET [e] (1)	*mecA*, *str*, *tet*(K), *tet*(L) (1)
			ERY, CLI [d], STR, CHL [e] (1)	*erm*(C), *str*, *fexA* (1)
			SUSCEPTIBLE (1)	-
		S. simulans (4)	SUSCEPTIBLE (4)	-
		S. schleiferi (2)	SUSCEPTIBLE (2)	-
		S. haemolyticus (1)	SUSCEPTIBLE (1)	-
		S. vitulinus (1)	SUSCEPTIBLE (1)	-
		S. lyticus (1)	SUSCEPTIBLE (1)	-

[a] type B: $n = 10$; type A: $n = 1$; [b] type B: $n = 7$; type A: $n = 1$; [c] PEN: penicillin, FOX: cefoxitin, ERY: erythromycin, CLI: clindamycin, TET: tetracycline, STR: streptomycin, CHL: chloramphenicol; [d] inducible resistance phenotype. [e] multidrug resistance phenotype.

3.2. Non-aureus Staphylococcus Species: Molecular Characteristics, and Antimicrobial Resistance

Among the other species identified, one belonged to *Staphylococcus intermedius* group (SIG), *S. delphini*, and was present in 11 (36.6%) of the nasal samples and in eight (16%) of the faecal samples. Nineteen *S. delphini* isolates were detected in total (type B: $n = 17$; type A: $n = 2$). All isolates were susceptible to the antimicrobials tested (Table 2).

The 37 remaining isolates belonged to coagulase-negative staphylococci (CoNS) group. CoNS were present in 16 (53.3%) of the nasal samples and 15 (30%) of the faecal samples. One isolate per sample was recovered except for five samples which harboured two or three isolates of distinct species. Resistance to at least one antimicrobial agent was detected in 52.6% and 44.4% of the nasal and faecal isolates, respectively. Seventy percent of the nasal resistant isolates and 100% of the faecal resistant isolates belonged to the predominant species *S. sciuri*. The other species with resistant isolates were *S. lentus* and *S. xylosus*. Moreover, three *S. sciuri* isolates showed a multidrug resistance (MDR) phenotype, meaning that they were resistant to one agent in three or more antimicrobial categories (Table 2). Globally, the following rates, phenotypes, and genotypes of antimicrobial resistance were reported among CoNS isolated from horses (detection rate; resistance genes detected): penicillin and cefoxitin (37.8%; *mecA*), erythromycin (5.4%; *erm* (A), *erm* (B), *erm* (C) or *msr* (A)), clindamycin (10.8%; *lnu* (A)), tetracycline (10.8%; *tet* (K) and *tet* (L)), streptomycin (13.5%; *str*), and chloramphenicol (2.7%; *fexA*).

3.3. Comparison of Nasal and Faecal Samples of Seven Healthy Horses

Seven animals could be tested for both nasal and faecal samples. The results are displayed in Table 3. All the animals that carried *S. aureus* in their nostrils also had this microorganism in their faeces ($n = 5$). In 3/5 cases, the *S. aureus* isolates belonged to the same genetic lineage ST1640. More than one staphylococcal species was detected in five of seven nasal samples, while faecal samples predominantly carried a single staphylococcal species (*S. aureus* in 5/7 cases). The lineage ST1640 was predominant in both nasal and faecal samples.

Table 3. Comparison of staphylococci recovered from nasal and faecal samples from seven healthy horses.

Animal	Nasal Samples			Faecal Samples		
Animal Code	Species Detected (Number of Strains)	Type A/B or *spa*-type /ST	Antimicrobial Resistance Phenotype [a]	Species Detected (Number of Strains)	Type A/B or *spa*-Type/ST	Antimicrobial Resistance Phenotype [a]
1	*S. delphini* (1)	Type B	SUSCEPTIBLE	*S. simulans* (1)	-	SUSCEPTIBLE
	S. haemolyticus (1)	-	SUSCEPTIBLE			
25	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE	*S. aureus* (1)	t2420/ST133	SUSCEPTIBLE
26	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE
	S. sciuri (1)	-	PEN, FOX, STR, TET			
27	*S. aureus* (1)	t549/ST1660	PEN, STR	*S. aureus* (1)	t127/ST1	PEN, SXT
	S. delphini (1)	Type A	SUSCEPTIBLE	*S. delphini* (1)	Type B	SUSCEPTIBLE
28	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE	-	-	-
29	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE
	lentus (1)	-	PEN, FOX, STR			
30	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE	*S. aureus* (1)	t2559/ST1640	SUSCEPTIBLE
	S. sciuri (1)	-	SUSCEPTIBLE			

[a] PEN: penicillin; FOX: cefoxitin; TET: tetracycline; STR: streptomycin; SXT: trimetroprim-sulphametoxazole.

4. Discussion

High *S. aureus* occurrence has been detected among both nasal and faecal samples of healthy horses destined for human consumption (56.6% and 32%, respectively). According to previous works on healthy horses from various farms in Germany and Denmark, the occurrence of *S. aureus* in nasal samples was much lower (6.7% and 13.5%, respectively) [32,33]. Alternatively, a recent Italian study showed that the prevalence of methicillin resistant *S. aureus* (MRSA) in horses tested in slaughterhouses (7%) was significantly higher than those tested on farms and racecourses [34]. In our study, however, no MRSA was detected among the population tested. Islam and collaborators observed that 63.3% of the *S. aureus* strains recovered were MSSA strains, mostly assigned to ST1/t127 and ST1660/t549 [33]. However, the lineages ST1, ST1660, and ST133 are also frequent among MSSA from horses [32,33,35]. Our strains were mostly associated to ST1640/t2559 (n = 21), although ST1 (n = 8), ST1660 (n = 1), and ST133 were also detected. To our knowledge, the lineage ST1640/t2559 is here detected for the first time among horse samples. Nonetheless, the *spa*-type t2559 was previously found (associated to CC5/CC30) in the nostrils of patients of general practitioners with no sign of infections in the Netherlands [36]. The lack of the *scn* gene suggests that none of the strains were of human origin.

Interestingly, all strains but those of the lineage ST1640/t2559 harboured the equine-adapted leukocidin determinant *lukPQ* (prevalence of 38%) and the *scn-eq* gene. These findings suggest that the ST1640 might have jumped recently from another source to the equine environment. On the other hand, an international equid collection study reported *lukPQ* values ranging from 0% to 50%, indicating either (1) the absence of these genes may also be a common feature among horse isolates or, again, (2) a reflection of an early phase of those isolates in the adaptation to this host [15]. Otherwise, it was revealed that *lukPQ* and *scn-eq*, both encoded by the prophage φSaeq1, are prone to occur together and were associated with the clonal complexes CC1, CC133, CC1660, CC350, and CC522 [15,17]. These findings are confirmed by our results (*lukPQ* and *scn-eq* genes associated with CC1, CC133, CC1660, and ST816). The phage-encoded leukocidin LukPQ displays a high toxicity towards equine neutrophils, while the eqSCIN blocks complement activity in equine serum, which implies an important role in the evasion of *S. aureus* of the equid host defence mechanism [15,17]. Moreover, LukPQ has a broad host range as at high concentrations it is capable of lysing bovine and to some extent human neutrophils. Its transmission to human *S. aureus* strains could enhance its pathogenicity. The toxic shock syndrome gene *tst* was detected in strains of ST816, even though *tst* is generally observed among small ruminant isolates [4,35,37]. The presence of these virulence factors in healthy horses destined for human consumption might be of concern for food security and public health since it can spread through handling and processing.

Regarding the antimicrobial resistance, a low prevalence of resistant strains among *S. aureus* isolates was observed (17.6%, n = 4). They showed resistance to penicillin, streptomycin, and SXT, which are antibiotics frequently used in veterinary medicine [38].

Other staphylococcal species were identified from the horses studied, with predominance of *S. delphini* and *S. sciuri*. *S. delphini* is described as a colonizer of a wide variety of animal species (Equidae, Mustelidae, dolphins, pigeons, cinerous vulture, among others) [9,39–41]. Here, the *S. delphini* group B revealed a predominance in horses. A similar trend was observed by Stull and collaborators in Canada [9], as well as in wild birds in Spain [40]. The high susceptibility to the antibiotics observed among our strains was in accordance with previous results in donkeys and might be due to a lower selective pressure exerted on these animal species [9,41]. Unfortunately, data on antimicrobial therapy or exposure level of these animals were not available.

S. sciuri was the predominant species with resistance to methicillin, as previously reported among equine staphylococcal isolates [42]. This species hosts a native *mecA* homologue (*mecA1*) estimated to be the origin of the *mecA* gene for MRS [42]. Three of our strains showed an MDR phenotype. Those resistance genes could be disseminated among horses and humans through contact and derived food manipulation, which would be a risk for animals and human health. In fact, CoNS and methicillin resistant CoNS of the species detected in this study (*S. epidermidis*, *S. haemolyicus*, *S. sciuri*, *S. xylosus*,

or *S vitulinus* among others) are frequently isolated from healthy and infected horses and, sometimes, among equine personnel [13,32,42,43].

On the other hand, comparison of staphylococcal carriage between the nostrils and the faeces among several animals in this study indicate a higher carriage rate in nasal samples. These results are in agreement with former data, which describe human and animal skin and mucosa, especially the nares, as the most frequent carriage site for staphylococci [3,44]. Remarkably, our results indicate that the gut and nasal microbiota of these animals is similar when referring to staphylococcal species.

5. Conclusions

This study provides data on the staphylococcal carriage of healthy horses. A high prevalence of MSSA, mostly susceptible to the antibiotics tested but carrying important virulence genes (*lukPQ*, *scn-eq*, and *tst*), is highlighted. The detection and predominance of the *S. aureus* lineage ST1640 in horses is noteworthy, as it represents its first description in horses. Furthermore, a high diversity of species among non-*S. aureus* isolates was observed, including CoPS and MRCoNS. Due to current evidence on the influence of animal-derived food in the dissemination of staphylococci and their resistance and virulence genes, strict measures of hygiene and control must be taken for horses at slaughter and for meat processing.

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CHAPTER 2: *Staphylococcus* spp. FROM FOOD-PRODUCING ANIMALS AT SLAUGHTERHOUSE LEVEL IN SPAIN AND SENEGAL

Livestock are natural hosts of staphylococcal species. Antimicrobial use as growth promotors in food-producing animals (in the past or now, depending on country´s legislation) or for prophylaxis, may impact on these microorganism resistance profiles, leading to a higher risk of resistance gene spread into farms or animal-derived foods. Slaughter process may alter animals' staphylococcal carriage and can be a stage for contamination of derived meat. Zoonotic transmission may also occur during the process from animals to the personnel. Therefore, it is important to evaluate the carriage rate of staphylococci in food-producing animals at slaughterhouse level as well as their antimicrobial resistance and virulence genetic characteristics, and to follow strict hygienic and practices measures. In this chapter, we describe isolates recovered from common food-producing animals such as calve, lamb, goat, cow and chicken in two countries with different antibiotic control policy.

In the paper 4, nasal samples of 117 farm animals (calve, 72; lamb, 37 and goat, 8) were collected from one slaughterhouse in La Rioja, Spain, during the period of June-September 2009. Staphylococci (CoPS and CoNS) were identified in 50%, 54% and 21% of goat, lamb and calve samples, respectively. Among the 13 CoPS isolates recovered (12 *S. aureus* and 1 *S. pseudintermedius*), two were multidrug-resistant-MRSA and corresponded to the pathogenic and invasive clone USA300 responsible for human infections (t5173/ST8/*agr*-I/SCCmec-IVa/ACME-positive/PVL positive). Both isolates were *scn*-positive suggesting a human origin. MSSA harboured either *tst* or enterotoxin genes. The predominant *S. aureus* lineage was ST133. Six species were identified among 47 CoNS isolates with predominance of *S. simulans* (53.2%) and *S. sciuri* (23.4%). Fifty-three percent (53%) of CoNS were resistant to at least one antibiotic including six MDR isolates. The antibiotic with higher resistance rates were streptomycin (27%), tetracycline (23.4%) and clindamycin (19.1%).

In paper 5, nasal samples of 149 cows and 199 chickens (348 animals) were collected in the General Society of Slaughterhouses and a local market in Senegal. Staphylococci were present in 3 and 26.8% of chicken and cow samples, respectively. CoPS were all MSSA and penicillin resistant. They belonged to four genetic lineages: ST291 23 (n = 3); ST152 (n = 2); ST15 (n = 1) and ST6 (n = 1). All harboured a virulence gene and the PVL determinants were detected in 3 isolates. *tst* and enterotoxin genes were also detected.

The *scn* gene was absent in these isolates. Six species were identified among CoNS with predominance of *S. sciuri* (40%) and *S. simulans* (27.5%). They were all methicillin resistant and higher resistance rate were observed for tetracycline (27.5%) and SXT (10%).

Although the studies were performed in two countries and on different animal species, *S. aureus* was the prevalent species among CoPS , and *S. simulans* and *S. sciuri* were predominant among CoNS. Moreover, *tst*, enterotoxins and PVL genes were detected at relatively high rates in both studies leading to the conclusion that food-producing animals, especially livestock are reservoir for *S. aureus* carrying important virulence genes. Furthermore, the contamination of animals with human virulent clones is worrisome as they can be transferred through the food chain. Methicillin resistance seems to be rare in staphylococci from these animal species, which is good news for public health. However, resistance to tetracycline is high in both countries.

More information about these two works and the characteristics of recovered isolates are displayed in papers 4 and 5 presented below:

Paper 4: Mama, O.M., Gómez-Sanz, E., Ruiz-Ripa, L., Gómez, P., Torres, C., 2019. Diversity of staphylococcal species in food producing animals in Spain, with detection of PVL-positive MRSA ST8 (USA300). *Vet. Microbiol*. 233, 5-10. doi: 10.1016/j.vetmic.2019.04.013.

Paper 5: Mama, O.M., Dieng, M., Hanne, B., Ruiz-Ripa, L., Diop, C.G.M., Torres, C., 2019. Genetic characterisation of staphylococci of food-producing animals in Senegal. PVL detection among MSSA. *BMC Veterinary Research*. 15, 395. doi:10.1186/s12917-019-2137-9.

PAPER 4

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Diversity of staphylococcal species in food producing animals in Spain, with detection of PVL-positive MRSA ST8 (USA300)

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ABSTRACT

This work aimed to determine the prevalence, diversity, antibiotic-resistance phenotype/genotype and virulence factors in staphylococci of farm-animals. Nasal samples of 117 farm-animals (calve: 72; lamb: 37; goat: 8) were collected from one slaughterhouse in La Rioja/Spain and cultured for staphylococci and methicillin-resistant *Staphylococcus* (MRS) recovery. Identification was performed by MALDI-TOF. Antimicrobial resistance phenotype/genotype was determined by susceptibility testing and specific PCRs. Molecular typing (*spa*-typing, multilocus-sequence-typing, *agr*-typing, SCC*mec*), and detection of 12 virulence genes and human Immune-evasive-cluster (IEC) genes were performed by PCR/sequencing in *S. aureus*. Two marker genes of arginine catabolic mobile element (ACME) were determined by PCR (USA300-MRSA detection). Staphylococci were identified in 50%, 54% and 21% of goat, lamb and calve samples, respectively. Among the 13 *S. aureus* isolates recovered, 11 were susceptible to all antimicrobials tested, and two were multidrug-resistant-MRSA [beta-lactams (*blaZ*, *mecA*), macrolides [(*msr*(A)/*msr*(B)] and fluoroquinolones]. The MSSA harboured either *tst* or enterotoxin genes, while the MRSA harboured the *lukF/lukS-PV* genes. Five sequence-types were detected. The two MRSA strains (from lamb and goat) were typed as t5173/ST8/*agr*-I/SCC*mec*-IVa/ACME-positive, corresponding to USA300 clone, and were IEC-B-positive. Among the 47 coagulase-negative staphylococci (CoNS), six species were identified, predominating *S. simulans* (n = 25) and *S. sciuri* (n = 11). Fifty-three percent of CoNS showed resistance to at least one antimicrobial agent (six multidrug-resistant strains), and the following resistance phenotypes/genotypes were detected: streptomycin [27.6%; *ant(6)-Ia*, *str*], tetracycline [23.4%; *tet*(M), *tet*(L), *tet*(K)], clindamycin [19.1%; *lnu*(A), *vgaA*], erythromycin [10.6%; *erm*(C), *msr*(A)/*msr*(B)], chloramphenicol (8.5%; *fexA*), tobramycin (6.4%), penicillin-cefoxitin (4.3%; *blaZ*, *mecA*), and SXT (2.1%). The detection of the MRSA-USA300 lineage in food animals is worrisome and should be further monitored.

1. Introduction

Staphylococcal species are divided in two groups: coagulase-positive staphylococci (CoPS, as *S. aureus*, *S. pseudintermedius* or *S. intermedius*, among others), and coagulase-negative staphylococci (CoNS, as *S. sciuri*, *S. epidermidis*, or *S. saprophyticus*, among others). They generally colonize the skin and mucous membranes of humans and animals (mammals and birds), but are also opportunistic pathogens (Kluytmans, 2010). In humans, staphylococci may be responsible for either skin and soft tissue infections, pneumonia or food intoxication. In livestock, especially among bovines, they cause mastitis, among others (Pantosti, 2012). Staphylococci are also common contaminants of animal-derived foods, such as raw meats or milk-derived products (Lozano et al., 2009; Kluytmans, 2010; Becker et al., 2014). In that context, livestock is a source of staphylococci that can be transmitted to humans, highlighting the need to study and characterize the species from food producing animals.

Methicillin resistant *S. aureus* (MRSA) of the lineage CC398 is a colonizer of pigs, humans exposed to pig farming and other farm species. MRSA CC398 is also implicated in farm-related infections, and it is therefore designated as livestock associated (LA) MRSA (Benito et al., 2014b; Pantosti, 2012). Furthermore, other *S. aureus* clonal lineages such as CC130, CC599, CC59, CC9, CC133 or CC425 are animal-adapted lineages, causing infections in animals and zoonosis in humans

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PAPER 5

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RESEARCH ARTICLE Open Access

Genetic characterisation of staphylococci of food-producing animals in Senegal. PVL detection among MSSA

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Abstract

Background: Food-producing animals can be a vehicle for staphylococcal species as well as their virulence and antimicrobial resistance genes. This work aimed to analyse the diversity of staphylococcal species in food-producing animals in Dakar/Senegal, and to determine the antimicrobial resistance phenotype/genotype and virulence factors of recovered isolates. Nasal samples of 149 cows and 199 chickens (348 animals) were collected from one slaughterhouse and a local market respectively, and were inoculated on selective media for staphylococci recovery. For *S. aureus* isolates, molecular typing (*spa*-type, MLST) was performed by PCR/sequencing, and the presence of 27 virulence genes (exfoliative and toxic shock toxins, PVL, haemolysins and enterotoxins) as well as the gene *scn* were analysed by PCR. Susceptibility to twelve antibiotics was studied by disc-diffusion method for all staphylococci; the resistance genes involved were screened by PCR.

Results: *Staphylococcus* spp. was present in 3 and 26.8% of chicken and cow nasal samples, respectively. Seven *S. aureus* isolates and forty isolates of other staphylococcal species were identified. *S. aureus* isolates were recovered from cow ($n = 6$) and chicken ($n = 1$) samples, belonging to four genetic lineages: t084/ST15 ($n = 1$); t10579/ST291 ($n = 3$); t355, t4690/ST152 ($n = 2$); and t6618/ST6 (n = 1). All *S. aureus* were methicillin-susceptible, penicillin-resistant (*blaZ*), and two of them were also tetracycline-resistant [*tet*(K)]. All the isolates carried at least one of the virulence genes tested. The PVL genes were detected in three ST15 and ST152 isolates. They all harboured haemolysins encoding genes and lacked the *scn* gene. The other staphylococci recovered were *S. sciuri* ($n = 16$), *S. simulans* ($n = 11$), *S. hyicus* ($n = 5$), *S. haemolyticus* ($n = 4$), *S. chromogenes* ($n = 3$), and *S. hominis* (n = 1); they were all methicillin-susceptible and 27.5% tetracycline-resistant [*tet*(K) and *tet*(L)].

Conclusions: A low prevalence of *S. aureus* was detected among food-producing animals, all susceptible to methicillin. However, the presence of virulence genes (*lukF/lukS-PV*, *eta*, *tst*, *sea* and *see*) is worrisome to the extent that they could be transferred to derived food and therefore, to humans.

Background

Staphylococcus species are common colonizers of skin and mucous membranes of humans and different animal species, but can become opportunistic pathogens causing skin and soft tissues infections (SSTIs) and mastitis, among others [1]. Furthermore, they are current contaminants of animal-derived food, being responsible for food intoxication [2]. *S. aureus* in particularly can express a large variety of pathogenicity factors, such as the staphylococcal enterotoxins, toxic shock syndrome toxin (TSST-1), and Panton-Valentine leucocidin (PVL), among others [3]. In fact, PVL is the most important toxin produced by *S. aureus*; it destroys membranes of host defence cells and erythrocytes by the synergetic action of two specific proteins named LukS-PV and LukF-PV [4]. PVL is though involved in severe skin infections, haemolysis, leucocyte destruction and necrosis [4] . PVL-positive methicillin susceptible *S. aureus* (MSSA) is considered endemic in the African continent [5]. The toxin has been detected worldwide in MSSA

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and methicillin resistant *S. aureus* (MRSA) isolates of diverse ecosystems, including humans (clinical or community-associated isolates) [6], wildlife [7], farm animals and animal-derived food [8]. The presence of the toxin in such different environments and in MRSA is a concern for public health and food safety, mainly for African regions, especially in those areas in which access to healthcare is limited. Moreover, *S. aureus* strains adapted to humans carry an innate immune evasion cluster (IEC) system that protects them against the human immune system [9]. The IEC consists of several genes, the combination of which gives a determined IEC type. The gene *scn*, since present in all the IEC types, is considered to be a marker-gene for the detection of the IEC [9]. The presence of this system in a *S. aureus* isolate would suggest a human origin.

In general, studies about molecular epidemiology of staphylococci in African countries are being mainly performed in hospital environments [10, 11] and in the food sector [12], but there is scarce data related to farm animals [8]. Furthermore, those studies essentially focus on *S. aureus* species and most of them are performed in the Northern, Central and Southern regions of Africa [8]; consequently, data from West Africa is scarce, especially regarding Senegal. In Senegal, a study was carried out in 2012 on *S. aureus* from pigs and pig farmers, which highlighted the predominance of the clonal complexes CC152 and CC15, the low rate of resistance to methicillin and the frequent detection of PVL toxin [13], and a few years ago a review revealed that CC398, an emergent Livestock-associated *S. aureus* lineage in Europe [2, 14–17], was almost absent in animals and food in Africa [8]. In the said context, this study attempts to provide new information on molecular diversity, antimicrobial resistance and virulence determinants for *S. aureus* and other staphylococcal species in other food-producing animals (such as cow and chicken,) in Senegal (West Africa), and proposes an analysis of the potential occurrence of the lineage CC398 in the area.

Results

Staphylococcus species detection

Staphylococcus spp. were present in 3 and 26.8% of chicken and cow nasal samples, respectively. *S. aureus* was detected in seven of the tested animals (six cows and one chicken) (Table 1), whereas other staphylococcal species were found in cows ($n = 35$ isolates) and chickens ($n = 5$ isolates) (Table 2). One *Staphylococcus* isolate was obtained from all positive samples, except for one cow sample with two isolates (*S. aureus* and *S. hyicus*). A total of 47 staphylococci were recovered: *S. aureus* ($n = 7$), *S. sciuri* ($n = 16$), *S. simulans* ($n = 11$), *S. hyicus* ($n = 5$), *S. haemolyticus* ($n = 4$), *S. chromogenes* ($n = 3$), and *S. hominis* (n = 1).

Staphylococcus aureus isolates: antimicrobial resistance, molecular typing and virulence

All seven *S. aureus* isolates were susceptible to cefoxitin and were therefore considered as MSSA. The six isolates of cow origin showed resistance for penicillin (with *blaZ* gene) and two of them also to tetracycline (with *tet*(K) gene). The *S. aureus* isolate of chicken origin showed susceptibility to all antimicrobials tested.

Five *spa*-types were detected among the *S. aureus* isolates, associated with four sequence-types (STs): t084/ST15 ($n = 1$); t10579 /ST291 ($n = 3$); t355, t4690 /ST152 ($n = 2$); and t6618 /ST6 (n = 1) (Table 1). All the isolates were negative for the clonal complex (CC) 398 specific PCR.

The genes encoding for PVL were detected in 2 out of 7 MSSA isolates (28.6%), specifically in t355/ST152 and t4690/ST152 isolates from chicken and cow origins, respectively. The *eta* and *tst* virulence genes were found in isolates of cow origin: 1) *eta* gene in one isolate t084/ST15; and 2) *tst* gene in three t10579/ST291 isolates. The enterotoxin genes *sea* and *see* were present in one isolate of lineage t6618/ST6 recovered of a cow. All the isolates hosted haemolysin encoding genes. In addition, all *S. aureus* isolates lacked the *scn* gene (IEC-negative). This data is summarised in Table 1.

Table 1 *S. aureus* from nasal samples of healthy cows and chicken: phenotypic and genotypic characteristics

				Antimicrobial resistance			
Origin	Strain	ST/CC	*Spa*-type	Phenotype[a]	Genotype	Virulence genes	*scn*
Cow	C10068	ST6/CC6	t6618	PEN-TET	*blaZ, tet*(K)	*sea, see, hla, hlb, hld*	negative
Cow	C10067	ST15/CC15	t084	PEN-TET	*blaZ, tet*(K)	*eta, hla, hld*	negative
Cow	C10064	ST291	t10579	PEN	*blaZ*	*tst, hla, hlb, hld, hlg*	negative
Cow	C10066	ST291	t10579	PEN	*blaZ*	*tst, hla, hlb, hld, hlg*	negative
Cow	C10063	ST291	t10579	PEN	*blaZ*	*tst, hla, hlb, hld, hlg*	negative
Cow	C10065	ST152/CC152	t4690	PEN	*blaZ*	*lukF/lukS-PV, hla, hlb, hld, hlg*	negative
Chicken	C10056	ST152/CC152	t355	Susceptible	–	*lukF/lukS-PV, hla, hlb, hld, hlg*	negative

[a]*PEN* penicillin, *TET* tetracycline

Table 2 Non-*aureus* staphylococcal species in nasal samples of healthy cows and chicken: phenotypic and genotypic characteristics

Origin	Species	Antimicrobial Resistance	
		Phenotype[a]	Genotype
(n° of positive animals)	(n° of isolates)	(n° of isolates)	(n° of isolates)
Cow (35)	*S. sciuri* (16)	TET (2)	*tet*(K) (2)
		TET (1)	*tet*(K)
		Susceptible (13)	–
	S. simulans (8)	TET (2)	*tet*(K) (2)
		Susceptible (6)	–
	S. haemolyticus (3)	SXT (1)	*dfrG*
		Susceptible (2)	–
	S. chromogenes (3)	Susceptible (3)	
	S. hyicus (5)	TET (1)	*tet*(K)
		Susceptible (4)	–
Chicken (5)	*S. simulans* (3)	PEN-TET (1)	*blaZ*, *tet*(K)
		TET-SXT (2)	*tet*(L) (2), *dfrK* (2)
	S. haemolyticus (1)	TET-SXT	*tet*(K), *tet*(L), *dfrK*
	S. hominis (1)	ERY-TET	*tet*(L), *msr*(A)/*msr*(B)

[a]*PEN* penicillin, *TET* tetracycline, *ERY* erythromycin, *SXT* trimethoprim/sulfamethoxazole

Non-aureus staphylococci: antimicrobial resistance phenotype and genotype

Among the 40 non-*S. aureus* isolates, 32.5% showed resistance to at least one antimicrobial agent tested. The following resistance rates and genotypes were observed: tetracycline [27.5%; *tet*(K), *tet*(L)], trimethoprim/sulfamethoxazole (SXT) (10%; *dfrG*, *dfrK*), penicillin (2.5%; *blaZ*), erythromycin [2.5%; *msr*(A)/*msr*(B)] and clindamycin (2.5%). It should be noted that resistance to tetracycline was mediated only by *tet*(K) gene for the isolates from cow origin, and by either *tet*(K) or *tet*(L) for the isolates recovered from chicken; furthermore, *dfrG* was present in one isolate from cow, whereas *dfrK* was detected in isolates of chicken origin.

Discussion

Farm animals are a source for staphylococcal species as well as for their resistance genes and virulence factors [28]. The transmission to humans could occur either through direct contact or via animal derived food [1], hence the importance of analysing staphylococci from food-producing animals.

In this study, the frequency of detection of *S. aureus* was low in cows (4%) and chickens (0.5%). A similar study from Nigeria showed a rate of 2.6% in cattle from slaughterhouses [29]. A higher detection rate was observed in other animals intended for human consumption, such as pigs in Senegal (12.3%) [13] as well as goat and sheep, according to studies carried out in Tunisia [28, 30].

Among the *S. aureus* detected in our work, the most frequently detected lineage was ST291, followed by ST152; however, a similar study performed in Senegal on isolates of pigs and pig farmers showed a predominance of the lineages ST15 and ST152, containing the PVL genes [13]. The sequence type ST291 is a ST398 double locus variant, which encodes two specific subunits, *sauI-hsdS1* and *sauI-hsdS2*, located in GIα and GIβ genomic islands respectively, whereas CC398 isolates encode a single *sauI-hsdS1*, located in GIα [31]. Furthermore, *sauI-hsdS1* of ST291 showed 60% nucleotide similarity to the CC398 *sauI-hsdS1;* consequently, the CC398 specific PCR cannot identify ST291 isolates as part of the CC398 cluster [25, 31], as was the case in our study. The lineage ST291 has been previously described as the major lineage in cattle with mastitis in Egypt [32]; they were all MSSA harbouring *scn* and PVL genes, unlike our isolates. The lineage ST15, mainly associated to MSSA isolates, frequently harbours PVL and enterotoxins [5] and is highly prevalent in African countries, according to the findings of healthcare institutions [33, 34]. Nevertheless, this lineage has also been found in animals (cattle, poultry and donkeys) [3, 8, 30]. The clonal complex CC152 was reported as one of the major clonal complexes in many African countries (healthcare environment) (Madagascar, Morocco, Cameroon, Gabon, Niger, Nigeria, Ghana, Mali and Senegal) [13, 33]. The lineage ST152 is sporadically associated to community-associated (CA) MRSA in some European countries, whereas ST152-MSSA is a particularly frequent clone in Western and Central Africa [33, 35]. PVL is the most important toxin secreted by *S. aureus* and is involved in severe skin infections and life-threatening diseases. This toxin is found all over the world, mainly among CA *S. aureus* isolates [6, 35]. Nonetheless, it was also described in isolates from farm/wild animals (linked to the lineages ST5, ST8, ST15, ST80, ST152, and ST121) [36–38] and animal-derived food (linked to the lineages ST8, ST121, and ST152) [3, 39]. PVL is very frequently harboured by MSSA isolates in Africa, where PVL-positive *S. aureus* is considered endemic [5]. In this work, all the PVL-positive isolates were MSSA (28.6% of *S. aureus* detected), contrary to the results obtained from healthy sheep in Tunisia, showing only PVL-positive MRSA (6.8% of *S. aureus* detected) [40]. In a previous study performed on pigs in Senegal, 38.4% of the *S. aureus* isolates harboured the PVL toxin, being 78.6% of them MSSA [13]. Contrarily to the above mentioned studies highlighting the recurrence of PVL-positive *S. aureus* among animals intended for human consumption in Africa, the absence of PVL was noted among *S. aureus* recovered from donkeys for meat consumption in Tunisia [30]. Furthermore, other virulence encoding genes were detected among the isolates (*sea*, *see*, *eta*, *tst*, *hla*, *hlb*, *hld* and *hlg*). The presence of staphylococcal

enterotoxins (SEs) in bovine isolates is worrisome since, as the literature shows, SEs are detected more often in cows with mastitis than in healthy cows [41]. Furthermore, SEA is the enterotoxin most frequently reported in food (encoded by *sea* gene) and the main cause of staphylococcal food poisoning (SFP) in many countries [42]; it is generally detected in meat, poultry and milk, among others. Nevertheless, SEE (encoded by *see* gene) is rarely reported in food and food-producing animals, although it was involved in some cases of SFP outbreaks in France [42]. Interestingly, none of our isolates carried the genes which encode the toxins SEC or SED, described as the most recurrent in bovines [43]. The presence of such virulent factors in *S. aureus* from food-producing animals, especially in African countries like Senegal, is a big concern for public health to the extent that in some cases, the animals are raised in the houses or sold in open markets, where they are in contact with people and retail food products. This easily results in the dissemination of staphylococcal virulence genes in different niches of the community. The lack of *scn* gene in our isolates suggests their being of animal origin, as expected, thus discarding a potential human origin (by handlers during slaughter).

In addition, other species were detected, the most prevalent being *S. sciuri* and *S. simulans*, followed by *S. hyicus*, *S. haemolyticus*, *S. chromogenes* and *S. hominis*. The coagulase-negative species mentioned above seem to be frequent in cattle and poultry samples [36, 44, 45]. Increasingly considered as opportunistic pathogens for humans and animals [44], coagulase-negative staphylococci are thought to be a reservoir for important resistance genes that could be transferred to *S. aureus* isolates [45], hence the importance of their surveillance.

Regarding the antimicrobial resistance, the phenotypes and genotypes observed in this study are frequent among *S. aureus* isolates from food-producing animals and animal-derived food [12, 28, 36, 38]. Similar phenotypes were previously observed among pig isolates of the same country [13]. Resistance to at least one antimicrobial agent was evidenced in 32.5% of the non-*S. aureus* isolates tested (tetracycline, SXT, penicillin, erythromycin and clindamycin), maybe due to the very frequent use of beta-lactams, tetracyclines, lincosamides and sulphonamides in the veterinary sector (food-producing animals and pets) [46].

Conclusion

A relatively low prevalence of *S. aureus* has been observed in nasal samples of food-producing animals (chickens and cows) in Senegal, with *S. aureus* being MSSA in all cases. Nevertheless, all the *S. aureus* isolates detected harboured at least one virulence gene (especially PVL and enterotoxins genes), which could be a concern for food-safety and public health, particularly in a developing country with areas in which access to medical care is difficult and limited.

Methods

Sample collection

From May to July 2017, nasal samples of 149 cows and 199 chickens (348 animals) were taken with aseptic swabs in the General Society of Slaughterhouses of Senegal (SOGAS) and a local market, respectively. In all cases, nasal samples were obtained from dead animals, just after they were sacrificed to human consumption as part of routine work in the slaughterhouse and the market.

Isolation and identification of staphylococci strains

Tubes of 5 ml of Brain Heart Infusion (BHI) broth (+NaCl 6.5%) were inoculated with the nasal swabs and then incubated at 37 °C for 24 h. After growth, bacterial culture was distributed on plates of mannitol-salt-agar (Conda, Madrid/Spain), Baird Parker (Becton Dickinson, Heidelberg/Germany) and oxacillin-resistance-screening-agar-base (Oxoid, Hampshire/England) for *S. aureus* and MRSA recovery. Non-*aureus* staphylococci were also identified and characterised. Up to two colonies/plate with staphylococcal morphology were isolated and subjected to Dnase agar test (Conda, Madrid/Spain) and identification by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker, Massachusetts/USA).

Antibiotic susceptibility and resistance genes detection

For all staphylococci identified, susceptibility to penicillin (10 units), cefoxitin (30 μg), gentamicin (10 μg), tobramycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), clindamycin (2 μg), ciprofloxacin (5 μg), linezolid (30) and SXT (1.25 + 23.75 μg) was analysed by disk-diffusion method [18]. In addition, susceptibility to streptomycin (30 μg) was also tested [19]. Antimicrobial resistance genes were determined by PCR, according to the resistance phenotype of the isolates: penicillin (*blaZ*), tetracycline [*tet*(K), *tet*(L), and *tet*(M)], macrolides [*erm*(A), *erm*(B), *erm*(C), *msr*(A)/*msr*(B)], and trimethoprim (*dfrA*, *dfrD*, *dfrG*, *dfrK*) [20–23] .

Molecular typing and virulence genes study in *S. aureus* isolates

Spa-typing and Multilocus sequence typing (MLST) were performed for *S. aureus* strains by polymerase chain reaction (PCR) and sequencing and the *spa*-type, sequence type (ST), and clonal complex (CC) were determined as previously described [24]. In addition, a specific PCR was performed for the livestock-associated CC398 lineage detection [25]. The presence of the genes encoding the PVL (*lukF/lukS-PV*), exfoliative toxins (*eta*

and *etb*), toxic shock syndrome toxin (*tst*), haemolysins (*hla*, *hlb*, *hld*, *hlg* and *hlgv*) and SEs (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) was screened by PCR [14, 26, 27]. The gene *scn*, was also tested for *S. aureus* isolates [14]. Positive and negative control strains of the University of La Rioja were included in all PCR reactions.

Abbreviations

BHI: Brain Heart Infusion; CA: Community-Associated; CC: Clonal Complex; IEC: Immune Evasion Cluster; MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization Time Of Flight; MLST: Multilocus Sequence Typing; MRSA: Methicillin-Resistant *S. aureus*; MSSA: Methicillin-Susceptible *S. aureus*; PCR: Polymerase Chain Reaction; PVL: Panton-Valentine Leucocidin; SEs : Staphylococcal Enterotoxins; SFP: Staphylococcal Food Poisonning; SSTIs: Skin and Soft Tissues Infections; ST: Sequence Type; SXT: Trimethoprim/Sulfamethoxazole; TSST: Toxic Shock Syndrome Toxin

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Authors' contributions

CT conceived and designed the study. MD, CGMD and BH designed and participate in the sampling procedure. OMM, LRR and MD performed laboratory works. OMM and CT interpreted the results and do the first writing of manuscript. All authors have revised and approved the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

In this study nasal samples were taken from animals during routine processing at the slaughterhouse and the market, once they were dead. No animal was sampled alive or sacrificed for the purpose of this study. Thus, according to the Spanish National law for the protection of animals (RD53/2013) that transcribes the EU directive 2010/63/UE, no ethics committee approval was deemed necessary. Furthermore, ethic committee approval was not deemed necessary based on the Article 7.1 (on the recommendations for animal welfare) and the Article 7.8 (on the use of animals in research and education) of the OIE Terrestrial Animal Health Code followed by Senegal.
At the time of processing the entity responsible for the animals was the slaughterhouse and authorization for sample collection was obtained from the directing board of the slaughterhouse. Also, the chicken sampling was authorized verbally by the owner which is responsible for the animals.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 3: FREQUENCY OF CC398 LINEAGE IN *S. aureus* (MRSA AND MSSA) ISOLATED FROM ANIMAL-DERIVED FOOD IN SPAIN

In Europe, CC398 MRSA is the dominant livestock-associated lineage. It was first described in pigs and then in other production animals and in pets. LA-MRSA-CC398 has been recently associated in human infections. First cases were reported in people with livestock exposure. But later, a transmission from human to human was observed. The human-adapted clone MSSA-CC398 is involved in invasive infections and its presence in animals is unusual. As earlier explained, food of animal origin can be contaminated during slaughter process, improper handling of processed food, and can therefore be a pathway of *Staphylococcus* dissemination. In this chapter, we determined the prevalence of the LA-MRSA-CC398 clone from pig-derived food in Spain, and also checked the presence of the human adapted-clone MSSA-CC398. The molecular characteristics and the antimicrobial resistance and virulence genes of the recovered isolated were studied. *S. aureus* isolates of other lineages detected were analysed as well.

In this work, 101 samples of pig-derived food (chopped meat (n=54); fillet (n=30); and ear/pork snout (n=17)) were collected from 18 butcher retail shops of three market places in La Rioja, Spain, during the period March-October of 2018. *S. aureus* was detected in 33.6% of the samples analysed; the highest rate was found in ear/snout samples (76.5%). CC398 lineage was associated to 64.1% of *S. aureus* isolates recovered (23 MRSA and 2 MSSA). The prevalence of MRSA-CC398 among pig products was 20.8%. MRSA-CC398 isolates were mostly typed as t011. They were all *mecA*-positive, tetracycline resistant and 82.6% were MDR. They were *scn*-negative and 17.4% harboured virulence genes. The MSSA-CC398 isolates detected were *spa*-t5452, *scn*-positive, and resistant to penicillin and erythromycin/clindamycin (inducible). Among the 14 non-CC398 isolates, two were MRSA-ST8, PVL-positive, enterotoxin-positive, *scn*-negative, and two MSSA-CC45, *scn*-positive.

LA-MRSA-CC398 is highly present in pig-derived food and can be transmitted to handlers during manipulation. Otherwise the detection of *scn*-positive isolates suggests contamination of the products by humans. Isolates with virulence genes, especially enterotoxins, were also detected. These results highlighted the fact that food constitute indeed a way of dissemination of MDR, virulent and invasive *S. aureus* isolates, which is a food safety and public health issue.

The background information, the methodology used for the purpose of this study, and deeper analysis of all results obtained are provided in the paper 6 below:

Paper 6: Mama, O.M., Morales, L., Ruiz-Ripa, L., Zarazaga, M., Torres, C., 2019. High prevalence of multidrug resistant *S. aureus*-CC398 and frequent detection of enterotoxin genes among non-CC398 *S. aureus* from pig-derived food in Spain. *Int. J. Food Microbiol*. (in revision)

Paper 6 (in revision)

High prevalence of multidrug resistant *S. aureus*-CC398 and frequent detection of enterotoxin genes among non-CC398 *S. aureus* from pig-derived food in Spain.

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) CC398 is a livestock-associated (LA) lineage, mainly detected in swine. Its dissemination via the food-chain could be a food-safety issue. This work aimed to study the diversity of *S. aureus* lineages in pork-products, to determine the prevalence of MRSA and methicillin-susceptible *S. aureus* (MSSA) of lineage CC398, and to study the antimicrobial resistance phenotype/genotype and the virulence traits of recovered isolates.

One hundred and one samples of pig-derived food were collected in Northern Spain for *S. aureus* isolation. Antibiotic resistance profile was analysed, and associated resistance genes were screened by PCR. Detection of CC398 lineage, *spa*-type, multilocus sequence-type (ST), virulence factors, immune evasion cluster (IEC) genes, and phage ΦSa3 integrase was performed by PCR/sequencing.

The prevalence of *S. aureus* and MRSA among pig-derived food was 33.6% and 21.8%, respectively. Thirty-nine *S. aureus* isolates were recovered and attributed to 19 *spa*-types and 12 STs, ST398 being the predominant lineage (n=25; 64 %). MRSA-CC398 isolates (n=23) were mainly *spa*-t011 (n=16) and 82.6% were multidrug-resistant (MDR). All MRSA-CC398 were tetracycline-resistant and IEC-negative and four hosted either *eta*, *tst* or *sea* gene. The two MSSA-CC398 isolates detected were *spa*-t5452, IEC-positive, and were resistant to penicillin (*blaZ*) and erythromycin/clindamycin (inducible) (*ermT* with/without *ermC*+*msrA*). Among the 14 non-CC398 isolates, only two were MRSA (ST8, PVL-positive, enterotoxin-positive, IEC-negative). The 12 isolates MSSA included two of CC45 IEC-positive.

CC398 lineage is prevalent among *S. aureus* of pig-derived food (both MRSA and MSSA), LA-MRSA-CC398/t011 being the clone most represented. The presence of the IEC-positive MSSA-CC398 and MSSA-CC45 in food products highlights the potential implication of handlers in transmission of foodborne pathogens. Moreover, given the high frequency of MDR isolates and virulence genes detected, hygienic practices should be improved to limit the dissemination risk of *S. aureus* via the food chain.

Keywords: MRSA, MSSA, CC398, multidrug-resistance, enterotoxins, IEC

1. Introduction

Since the early 2000s, methicillin-resistant *S. aureus* (MRSA) isolates of the clonal complex (CC) CC398 have been described as common colonizers of healthy farm animals, especially swine, and considered as livestock-associated MRSA (LA-MRSA). These isolates have also been found colonizing or causing infections in humans with livestock exposure (Benito et al., 2014b; Leibler et al., 2016; Ceballos et al., 2019).

Whole genome analysis established that CC398 isolates cluster into distinct phylogenetic groups: a human-adapted ancestor clade (methicillin and tetracycline susceptible), and a livestock-adapted derived clade (methicillin and tetracycline resistant) (Price et al., 2012). According to Price et al., LA-MRSA-CC398 might have evolved from the human-adapted clade, methicillin-susceptible SA (MSSA) CC398. The jump from humans to animals would have been accompanied by the acquisition of methicillin and tetracycline resistance in one hand, and by the loss of the phage ΦSa3, that carry the immune evasion cluster (IEC) genes on the other hand (Price et al., 2012). The IEC system protects *S. aureus* against the human immune system; there are several IEC-types which all include the *scn* gene (encodes the staphylococcal complement inhibitor), then considered a marker for IEC detection (van Wamel et al., 2006).

S. aureus (SA) is known to be responsible for foodborne diseases and food poisoning. Staphylococcal food poisoning is due to the ingestion of sufficient amounts of staphylococcal enterotoxins (SEs) present in contaminated food (Argudín et al., 2010). Improper handling of food combined with uncontrolled storage conditions, may increase the risk of contamination by SA and the production of SEs which are resistant to heat and low pH (Argudín et al., 2010). MRSA has been isolated with different prevalence from diverse types of food, especially food of animal origin such as dairy products, or raw meat (pork, beef, chicken, turkey etc.) (de Boer et al., 2009; Lozano et al., 2009; Papadopoulos et al., 2019).

In the light of the above, we wondered whether the food chain could be a way of transmission of LA-MRSA-CC398 to humans, whether this specific clone could be a potential agent of food poisoning, and finally what the prevalence would be in pig-derived food in our region, La Rioja/Spain. A few years ago, our research group performed two studies about the prevalence of SA and MRSA in food samples of animal origin including pork, chicken, beef, lamb, and turkey, among others (Lozano et al., 2009; Benito et al., 2014a). These works revealed a low prevalence of 2.5% of MRSA-CC398 among pork samples and the absence of MSSA-CC398, but the number of samples

analysed was low. The present work focussed on pig, as pig-derived products constitute an important sector of the region's food industry. The prevalence of CC398 (both MRSA and MSSA) and SA of other lineages was determined, and the antimicrobial resistance phenotypes/genotypes and the virulence gene profiles were studied.

2. Material and methods

2.1. S. aureus isolation and identification

A total of 101 samples of pig-derived food [chopped meat (n=54); fillet (n=30); and ear/pork snout (n=17)] were collected from 18 butcher retail shops of three market places in La Rioja, Spain, during the period March-October of 2018. Nine visits were performed to the butcher retail shops, and one or two samples were collected per visit (not all butcher retail shops were tested in each visit). All types of pig products included in this study are usually eaten in Spain.

Samples were enriched in 5 ml of Brain Hearth Infusion broth (BHI, +NaCl 6.5%) (Conda, Madrid, Spain), and incubated at 37ºC during 24h. Aliquots were then inoculated into mannitol-salt-agar (Conda, Madrid/Spain) and oxacillin-resistance-screening-agar-base (Oxoid, Hampshire/England) for SA and MRSA recovery, respectively. Up to two colonies/plate, with different staphylococcal morphologies were chosen and further identified. The identification was performed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker, Massachusetts/USA), and SA isolates were characterized.

2.2. Antimicrobial resistance phenotype and resistance genes detection

For all the SA identified, susceptibility to penicillin, cefoxitin, gentamicin, tobramycin, tetracycline, chloramphenicol, erythromycin, clindamycin and linezolid was analysed by disk-diffusion method (CLSI, 2018). Cefoxitin resistance was considered the marker of methicillin resistance phenotype. The detection of the following antimicrobial resistance genes was performed by PCR, in accordance with resistance phenotype: beta-lactams (*mecA*, *blaZ*), aminoglycosides [*aac*(6')-Ie–*aph*(2'')-Ia, *ant*(4')-Ia], tetracycline [*tetK*, *tetL*, and *tetM*], macrolides-lincosamides [*ermA*, *ermB*, *ermC*, *ermT*, *msrA*, *lnuA*, *lnuB*, *vgaA*], and chloramphenicol (cat_{pC194}, cat_{pC221}, cat_{pC223}, *cfr*, *fexA* and *fexB*) (Kehrenberg and Schwarz, 2006; Gómez-Sanz et al., 2010; Benito et al., 2014b; Ruiz-Ripa et al., 2019).

2.3. Molecular typing and virulence genes study

For all the SA isolates, *spa*-typing was performed as previously described (Ruiz-Ripa et al., 2019). Multilocus sequence typing (MLST) was carried out for 19 representative strains (one of each *spa*-type detected, and all strains with a new *spa*-type). For this

purpose, PCR and sequencing of seven housekeeping genes (www.pubmlst.org) were performed to determine the sequence type (ST) and the clonal complex (CC) (Ruiz-Ripa et al., 2019). In addition, a specific PCR was carried out for CC398 lineage determination (Stegger et al., 2011). Furthermore, the presence of the genes encoding the Panton Valentine Leukocidin (PVL) (*lukF/lukS-PV*), the exfoliative toxins (*eta* and *etb*), the toxic shock syndrome toxin (*tst*), the haemolysins (*hla*, *hlb*, *hld*, *hlg* and *hlgv*) and the staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) was screened by PCR (Benito et al., 2014a). The presence of *scn* gene was studied and the IEC type was determined for *scn*-positive isolates (van Wamel et al., 2006). Moreover the presence of the Phage ΦSa3 integrase (Sa3int) was studied for the IEC-positive isolates, as previously described (Goerke et al., 2009). Positive and negative control strains of the University of La Rioja were included in all PCR reactions.

3. Results

3.1. Prevalence of S. aureus and MRSA in pig-derived food

Staphylococcus aureus was detected in 33.6% of the samples analysed (34/101). Considering the different types of samples, the prevalence was 24.1%, 26.6%, and 76.5% in chopped meat, fillet and ear/snout samples, respectively. The prevalence of MRSA was 21.8% (22/101) with different rates among food types: 3.3% (fillet), 14.8% (chopped meat) and 76.5% (ear/snout).

A total of 39 isolates were recovered (25 MRSA and 14 MSSA) (Table I), corresponding to one isolate/positive-sample, except for five samples which harboured two SA isolates with different *spa*-types and/or antimicrobial resistance phenotypes (included in Table II). The antimicrobial resistance rates and genes detected among all the isolates recovered in this study are shown in Table III. The isolates were assigned to 19 different *spa*-types and 12 STs. The CC398 was the predominant lineage, being assigned to 25 isolates, all of them of sequence type ST398 (64.1%).

3.2. S. aureus-CC398 and characteristics of recovered isolates

The 25 isolates of lineage CC398 were recovered from 21 samples (overall prevalence of 20.8%), with the presence in four samples of two distinct CC398 isolates (Table II): a) one chopped meat sample carrying both MSSA-CC398 and MRSA-CC398; b) three snout/ear samples with two MRSA-CC398 isolates (with different *spa*-type or antimicrobial resistance profile). The 25 CC398 isolates were divided into two groups: MRSA-CC398 (n=23; detected in 20 samples) and MSSA-CC398 (n=2; detected in 2 samples).

The 23 MRSA-CC398 isolates belonged to six *spa*-types (t011, t1451, t1606, t4030, t108 and t779) and most of them were t011 (n=16) (Table I). They all carried the *blaZ* gene and showed resistance to tetracycline (always mediated by *tetM* and *tetK* genes but combined with *tetL* in nine isolates). The high resistance to macrolides and lincosamides among MRSA-CC398 strains was also noted, with the following phenotypes/genotypes: erythromycin+clindamycin (n=11; *ermB*, *ermC*, *ermT*, *msrA*, and/or *vgaA*), erythromycin (n=1; *msrA*) and clindamycin (n=5; *lnuA* and/or *vgaA*) (Table I). Moreover, 82.6% of the isolates showed a multidrug resistance (MDR) phenotype, and resistance to beta-lactams, tetracycline, macrolides and/or lincosamides was the most common profile.

Regarding the virulence, two MRSA-CC398 isolates hosted the *eta* gene, one harboured the *sea* gene and another one the *tst* gene. All MRSA-CC398 isolates were IEC-negative. Concerning the haemolysin encoding genes, *hla* was present in all these isolates. The genes *hld* and *hlg* were found in all but three isolates, while the gene *hlb* was undetected in six. All the isolates lacked the *hlgv* gene.

On the other hand, the MSSA-CC398 isolates were assigned to the *spa*-type t5452. They were resistant to penicillin (*blaZ*), and both showed an erythromycin/clindamycin-inducible resistance phenotype mediated by the *ermT* gene, alone or associated with *ermC* and *msrA* genes (Table I). One isolate was additionally resistant to gentamicin. Both isolates were typed as IEC-C and carried the phage integrase Sa3int. It is also notable that both MSSA-CC398 isolates showed an MDR phenotype, and had the same haemolysin genes profile (*hla*, *hld* and *hlg*).

3.3. S. aureus of non-CC398 lineages and characteristics of recovered isolates

Among the 14 non-CC398 isolates, two were MRSA (ST8/CC8-t8151) and 12 MSSA (10 ST/CC and 11 different *spa*-types detected, including two new ones: t18358 and t18461) (Table I). The MRSA isolates showed only resistance for beta-lactams, carrying the *mecA* and *blaZ* genes. Both harboured the PVL encoding genes, as well as one SE gene (*see* or *seq*); moreover, these MRSA isolates were *scn*-negative, and consequently, IEC negative. Additionally, they carried the same haemolysin genes (*hla*, *hlb*, *hld* and *hlgv*).

The MSSA isolates were mainly resistant to penicillin (n=7; *blaZ*), tetracycline (n=4; *tetM*, *tetK*, *tetL*) and clindamycin (n=4; *vgaA*), although resistance to antibiotics of the macrolide and aminoglycoside families was also observed (Table I). Eight of the 12 MSSA non-CC398 isolates (of lineages ST45, ST9, ST1, ST22, ST133 and ST581) harboured enterotoxin genes, being five of them carriers of the *egc*-like cluster (Table I).

Furthermore, the *tst* gene was detected in one isolate (ST133). All the isolates were *scn*-negative, except the two ST45 (typed as IEC-B and containing the phage integrase Sa3int). Moreover, they all carried the genes *hla* and *hld,* and only one lacked the gene *hlb*. Interestingly, all the isolates carried the *hlgv* gene, except three which were positive for *hlg*. None of the isolates hosted both *hlg* and *hlgv*; most of the isolates CC398 were *hlg*-positive while the non-CC398 were mostly *hlgv*-positive.

4. Discussion

Livestock, especially swine, constitutes a reservoir for SA and MRSA belonging to different clonal lineages, including mostly CC398. The contamination of animal-derived food by staphylococcal isolates would be a concern for human health and transmission could occur through the food chain by eating or manipulating.

The present work revealed a prevalence of SA among pig products of 33.6%. Previous studies found the presence of SA in pig-derived food with a higher prevalence ranging from 45 to 60% (Jackson et al., 2013; Tang et al., 2017). The prevalence of MRSA isolates in pig-derived food in this work is high (21.8%) compared to results obtained few years ago in different countries, including Spain (2-10%) (Lozano et al., 2009; O'Brien et al., 2012; Benito et al., 2014a). Comparing the different types of food analysed in our study, it appears that samples with skin (ears and snout: 76.5%) carry more frequently MRSA than those without skin (chopped meat and fillet: 10.7%), which could explain the high rate observed in our study. Previous studies also found higher rates of SA and/or MRSA in samples with skin (snout, nares, carcasses, etc.) compared to samples without skin (minced meat, bacon, etc.) (Verhegghe et al., 2016). This was expected since this bacterium is described as commensal of animals and humans' skin and nares.

Our SA isolates belonged to a great diversity of genetic lineages with predominance of CC398 (64.1%). Many studies consider the MRSA-CC398 clone as the most frequent among livestock in Europe, particularly in pig and pig-derived food (Lozano et al., 2011; Sharma et al., 2016). It is worth noting that t011 was the *spa*-type mostly associated with cases of LA-MRSA-CC398, as was the case in our study. Moreover, t011 was the predominant *spa*-type detected among MRSA-CC398 of Spanish hospitals located in areas with high pig density (Ceballos et al., 2019). This highlights the easy ability of adaptation of MRSA-CC398, especially t011 to distinct ecosystems, its potential ways of dissemination and the important role of swine as reservoir.

MSSA-CC398 is considered of human-adapted origin and the ancestor of the LA-MRSA-CC398 of specific *spa*-types (Price et al., 2012). MSSA-CC398 has been increasingly reported in human infection cases mainly in Europe, and the *spa*-type t571 was associated with cases of bacteremia in French patients, living in animal free environments (Bonnet et al., 2018). Both MSSA-CC398 (t5452) isolated in our study were susceptible to tetracycline and interestingly, were typed as IEC-C. The presence of IEC in these MSSA-CC398 isolates suggests an human origin (Cuny et al., 2019). To the best of our knowledge, the detection of MSSA-CC398/IEC-positive, out of the human niche, is scarce. However, MSSA-CC398 isolates (t571, IEC-C, tetracycline-susceptible, erythromycin-resistant) were detected from white storks nestlings exposed to human residues in Spain (Gómez et al., 2016). These results highlight the spread of MSSA-CC398 of human clade in animals and animal-derived food.

Comparing the MRSA-CC398 and MSSA-CC398 isolates of this study, it appears that resistance to tetracycline is a constant phenotype among MRSA-CC398 isolates, while absent among MSSA-CC398 isolates. Resistance to macrolides and lincosamides was also frequently observed among both groups, but the phenotype erythromycin-clindamycin-inducible was only notable among MSSA isolates. Based on these results, it should be further investigated the erythromycin-clindamycin inducible resistance phenotype as a potential marker for MSSA-CC398 detection. The gene *ermT*, present in both MSSA-CC398, has been previously suggested as marker of the MSSA-CC398 human clade (Bonnet et al., 2018). The carriage of both MRSA and MSSA-CC398 by the same sample highlights the coexistence of isolates of different phylogenetic clades, and the presence of IEC genes in MSSA-CC398 suggests a human contamination.

The detection of toxin encoding genes (*sea*, *eta* or *tst*) among MRSA-CC398, though infrequent, is worrisome especially in food products (Argudín et al., 2010). Generally, it was observed a high frequency of MDR among CC398 isolates and the presence of relevant virulence genes, which is a concern for public health, knowing the ease of adaptation of this lineage to distinct environments.

A great diversity of genetic lineages was observed among the non-CC398 isolates. MRSA isolates were ascribed to CC8, while MSSA were assigned to ten other CCs being especially relevant CC45 and CC1. Both CC8 isolates harbored the PVL encoding genes, which is a marker of Community-associated (CA) MRSA (Planet et al., 2015), but interestingly lacked the *scn* gene, suggesting an animal origin. Three MRSA-CC8/PVL-positive/*scn*-positive isolates were previously detected in pork samples in Denmark

(Tang et al., 2017), and two MRSA-CC8-USA300/*scn*-positive isolates in lamb and goat samples in Spain (Mama et al., 2019b). Consequently, the CA-MRSA-CC8 should be considered a potential contaminant of animal-derived food, and its dissemination and adaptation in livestock should be further investigated. MSSA-ST45 has been found in chicken meat samples (Benito et al., 2014a) and in calve nasal samples in Spain (Mama et al., 2019b). In both cases, the isolates carried the *egc*-like cluster genes, and were thought to be of human origin (IEC-B), as noted in the present study. MRSA-CC1/t127 is well-known as an important human pathogen, although it is increasingly being recognized as an LA-MRSA lineage (Elstrøm et al., 2019). The high presence of pyrogenic toxin superantigens in our isolates is worrisome, knowing their role in food poisoning (Argudín et al., 2010).

Overall, transmission of SA CC398 and of other genetic lineages from food of animal origin to the handlers could occur during manipulations at the butchery level, and vice-versa (Verhegghe et al., 2016). Hence the importance to respect the good hygiene and Food processing practices and to institute surveillance measures in pork production chain.

5. Conclusion

Pig-derived food frequently contain the LA-MRSA-CC398 clone, which is characterized by a tetracycline resistance phenotype. Moreover, the detection of CC398-MSSA isolates carrying the IEC system highlights the risk of meat contamination by isolates of possible human origin through manipulation. Finally, high rates of MDR CC398 isolates and high frequency of toxigenic isolates among no-CC398 isolates, including those of community-associated lineages (ST8 and ST45) in food products, is worrisome for human health. Therefore, hygienic practices along the food production chain should be improved in order to limit the presence of food-borne pathogens, as well as their resistance and virulence genes.

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Table VI: Molecular characteristics, antimicrobial resistance phenotype/genotype and virulence factors of MRSA and MSSA isolated from 101 samples of pig-derived food

ST/CC (Nº isolates)	***spa*-type** (Nº isolates)	**Antimicrobial resistance phenotype**[a] (Nº isolates) **Beta lactams / Others**	**Antimicrobial resistance genes** (Nº isolates)	**Virulence genes** (Nº isolates)	**IEC type** (Nº isolates)
ST398/CC398 (25)	t011 (16)	PEN, **FOX** / TET, ERY, CLI (6)	*blaZ*(6), *mecA*(6), *tetM*(6), *tetK*(6), *tetL*(5), *ermB*(2), *ermC*(4), *ermT*(4), *msrA*(1), *vgaA*(1)	***eta***(1), *hla*(6), *hlb*(5), *hld*(5), *hlg*(4)	-
		PEN, **FOX** / TET, CLI (4)	*blaZ*(4), *mecA*(4), *tetM*(4), *tetK*(4), *tetL*(1), *lnuA*(1), *vgaA*(2)	***sea***(1), *hla*(4), *hlb*(4), *hld*(2), *hlg*(4)	-
		PEN, **FOX** / TET (3)	*blaZ*(3), *mecA*(3) *tetM*(3), *tetK*(3), *tetL*(1)	*hla*(3), *hlb*(2), *hld*(3), *hlg*(3)	-
		PEN, **FOX** / TET, GEN, TOB, CLI (1)	*blaZ, mecA, tetM, tetK, aac(6')-Ie-aph(2")-Ia, vgaA*	*hla, hlb, hld, hlg*	-
		PEN, **FOX** / TET, ERY, CLI, CHL (1)	*blaZ, mecA, tetM, tetK, ermT, vgaA, fexA*	*hla, hlb, hld, hlg*	-
		PEN, **FOX** / TET, CHL (1)	*blaZ, mecA, tetM, tetK*	***eta**, hla, hlb, hld, hlg*	-
	t1451 (2)	PEN, **FOX** / TET, ERY, CLI (1)	*blaZ, mecA, tetM, tetK, ermC, msrA*	***tst**, hla, hlb, hld, hlg*	-
		PEN, **FOX** / TET (1)	*blaZ ,mecA, tetM, tetK*	*hla, hld, hlg*	-
	t1606 (2)	PEN, **FOX** / TET, ERY, CLI, TOB, CHL (1)	*blaZ, mecA, tetM, tetK, tetL, ermC, msrA, ant(4')-Ia, fexA*	*hla, hld, hlg*	-
		PEN, **FOX** / TET, ERY, CLI, CHL (1)	*blaZ, mecA, tetM, tetK, ermC,ermT, msrA, fexA*	*hla, hld, hlg*	-
	t4030 (1)	PEN, **FOX** / TET, ERY, CLI	*blaZ, mecA, tetM, tetK, tetL, ermC, msrA, vgaA*	*hla, hlb, hld, hlg*	-
	t108 (1)	PEN, **FOX** / TET, ERY, CHL	*blaZ, mecA, tetM, tetK, msrA, fexA*	*hla, hlb, hld, hlg*	-
	t779 (1)	PEN, **FOX** / TET, GEN, TOB	*blaZ, mecA, tetM, tetK, aac(6')-Ie-aph(2")-Ia*	*hla, hld*	-
	t5452 (2)	PEN / ERY, CLI[c] (1)	*blaZ, ermT, ermC, msrA*	*hla, hld, hlg*	**C**
		PEN / ERY, CLI[c], GEN (1)	*blaZ, ermT, aac(6')-Ie-aph(2")-Ia*	*hla, hld, hlg*	**C**
Other lineages (14)	t8151(2)	PEN, **FOX** (2)	*blaZ*(2), *mecA*(2)	***lukF/lukS-PV***(2), ***seq***(1), ***see***(1), *hla*(2), *hlb*(2), *hld*(2), *hlgv*(2)	-
ST8/CC8 (2)	t230 (2)	PEN (2)	*blaZ*(2)	***egc*-like**[d](2), ***sec***(2), *hla*(2), *hlb*(2), *hld*(2), *hlg*(2)	**B** (2)
ST45/CC45 (2)	t1939 (1)	PEN, TET, ERY, CLI	*blaZ, tetM, tetK, ermB, vgaA*	***egc*-like**[d], *hla, hlb, hld, hlgv*	- -

	t4644 (1)	PEN	*blaZ*	***egc*-like**[d], *hla, hlb, hld, hlgv*	-
ST9/CC9 (2)	t127	ERY, CLI	*ermA*	***seh***, *hla, hlb, hld, hlgv*	-
	t701	PEN, TET	*blaZ*	*hla, hlb, hld, hlgv*	-
ST1/CC1(1)	t091	Susceptible	-	*hla, hlb, hld, hlgv*	-
ST6/CC6 (1)	t156	Susceptible	-	*hla, hlb, hld, hlgv*	-
ST7/CC7 (1)	t1862	Susceptible	-	***egc*-like**[d], *hla, hlb, hld, hlg*	-
ST12/CC12 (1)	t18461[b]	Susceptible	-	***tst***, ***sec***, *hla, hlb, hld, hlgv*	-
ST22/CC22 (1)	t18358 [b]	PEN, TET, ERY, CLI	*blaZ, tetM, ermB, msrA*	***see***, *hla, hlb, hld, hlgv*	-
ST133/CC133 (1)	t2247	PEN, TET, CLI, GEN, TOB	*blaZ, tetL, aac(6')-Ie-aph(2")-Ia, ant(4')-Ia*	*hla, hld, hlgv*	
ST581 (1)					
ST692 (1)					

[a] PEN: penicillin; FOX: cefoxitin; TET: tetracycline, ERY: erythromycin, CLI: clindamycin; GEN: gentamycin; TOB: tobramycin; CHL: chloramphenicol

[b] new *spa*-type

[c] inducible resistance phenotype

[d] *egc*-like cluster (*seg*, *sei*, *sem*, *sen*, *seo* and *seu*)

Resistance to cefoxitin (FOX), and *tst*, *eta*, enterotoxins, and PVL encoding genes are marked in bold

Table II: Pattern of coexistence of S. aureus isolates in a same pig-derived food sample

Sample	Origin	MRSA/MSSA	Isolate	*spa*-type	ST[a]	PCR CC398	IEC-type	Antimicrobial resistance phenotype[b]
1	Ear	MRSA	X74	t1451	(ST398)	+	-	PEN, FOX, TET, ERY, CLI
		MRSA	X42	t1606	(ST398)	+	-	PEN, FOX, TET, ERY, CLI, CHL
30	Ear	MRSA	X358	t011	(ST398)	+	-	PEN, FOX, TET
		MRSA	X352	t011	(ST398)	+	-	PEN, FOX, TET, CHL
6	Snout	MRSA	X65	t011	(ST398)	+	-	PEN, FOX, TET, CLI
		MRSA	X48	t4030	ST398	+	-	PEN, FOX, TET, ERY, CLI
CP17	Chopped meat	MSSA	X504	t5452	ST398	+	C	PEN, ERY, CLI[c], GEN
		MRSA	X488	t011	(ST398)	+	-	PEN, FOX, TET, CLI
F15	Fillet	MSSA	X699	t127	(ST1/CC1)	-	-	ERY, CLI
		MRSA	X664	t011	(ST398)	+	-	PEN, FOX, TET, ERY, CLI

[a] the STs in brackets are assumed according to the *spa*-type detected. The MLST has been determined by PCR/sequencing for the STs without brackets.

[b] PEN: penicillin; FOX: cefoxitin; TET: tetracycline, ERY: erythromycin, CLI: clindamycin; GEN: gentamycin; TOB: tobramycin; CHL: chloramphenicol

[c] inducible resistance phenotype

Table VIII: Antibiotic resistance rate and resistance genes detected among 39 S. aureus isolates obtained from 101 samples of pig-derived food

Antibiotics tested	N° of resistant isolates (%)	Resistance genes detected (N° of positive isolates)
Penicillin	35 (89.7%)	*blaZ* (33)
Cefoxitin	25 (64.1%)	*mecA* (25)
Tetracycline	27 (69.2%)	*tet*(M) (26), *tet*(K) (25), *tet*(L) (9)
Clindamycin	23 (58.9%)	*lnu*(A) (1), *vgaA* (7)
Erythromycin	17 (43.6%)	*erm*(A) (1), *erm*(B) (4), *erm*(C) (10), *erm*(T) (9), *msrA* (9)
Tobramycin	5 (12.8%)	*ant*(4')-*Ia* (2)
Gentamicin	4 (10.3%)	*aac(6')-Ie-aph(2")-Ia* (4)
Tobramycin + gentamicin	3 (7.7%)	*aac(6')-Ie-aph(2")-Ia* (3), *ant*(4')-*Ia* (1)
Chloramphenicol	4 (10.3%)	*fexA* (4)
Linezolid	0	-

CHAPTER 4: FREQUENCY AND CHARACTERISTICS OF ISOLATES OF LINEAGE CC398 AMONG INVASIVE MSSA AND MRSA ISOLATES IN SPANISH HOSPITALS

S. aureus of lineage CC398 is causing humans infections. While LA-MRSA-CC398 occurs mostly in patients with livestock exposure, no link was found between MSSA-CC398 infections and farming activity. A recent multicentre study of our research group evidenced that highest detection rates of MRSA-CC398 in Spanish hospitals were obtained in zones with high pig density (HPD) (Ceballos et al., 2019). MSSA-CC398, especially of *spa*-type t571, is a human adapted-clone responsible for invasive infections. Cases were reported in two countries geographically close to Spain (France and Portugal), making us to speculate on possible cases of MSSA-CC398 invasive infections in our country. The work of Ceballos et al., 2019 focussed on MRSA of skin and soft tissue and respiratory tract infections, and invasive isolates were scarcely represented. In this chapter, we analysed the frequency of MSSA-CC398 and MRSA-CC398 among *S. aureus* isolates recovered from blood cultures of patients in Spanish hospitals located in regions with different pig densities. Isolates of the lineage CC398 were then characterised (molecular typing, antibiotic pheno/genotype, virulence and host-adaptation genes).

A preliminary study was performed in one hospital of Zaragoza (Aragón). Of 84 blood culture *S. aureus* isolates recovered during 30 months in the hospital Royo Vilanova, 77 could be analysed for this study (50 MSSA and 27 MRSA). The prevalence of clonal complex CC398 among isolates studied was 5.2%. All CC398 isolates were MSSA (8% of MSSA) and were ascribed to *spa*-types t571 and t1451 (ST398). The CC398 isolates all showed erythromycin and clindamycin-inducible resistance phenotype, mediated by the *erm*(T) gene. Although this resistance phenotype was observed in a few non-CC398 isolates, none of them harboured the *erm*(T) gene. None of MRSA isolates was tetracycline-resistant (Tet^R) nor CC398-positive.

To check the consistency of these observations, a multicentre study was performed, including 17 Spanish hospitals located in different geographical regions. In this case, of 951 *S. aureus* isolates recovered from blood culture during 3, 9 or 12 months (during 2018-2019), the MSSA (n=706) and Tet^R-MRSA (n=18) were analysed. The prevalence of CC398 isolates among *S. aureus* was 4.3%. MSSA-CC398 represented 90.2% of total CC398 isolates, corresponding to 5.2% of MSSA and 3.9% of *S. aureus*. Six *spa*-types were

detected among MSSA-CC398 isolates, with predominance of t571 (43.2%) and t1451 (35.1%). Resistance to erythromycin and clindamycin-inducible mediated by *erm*(T) gene was observed in 72.9% of MSSA-CC398 isolates. MDR isolates were found among MSSA-CC398 isolates. All but three isolates MSSA-CC398 were *scn*-positive typed as either IEC-B or IEC-C.

Of the TET^R-MRSA isolates, 22.2% belonged to CC398 (0.4% of total *S. aureus*). MRSA-CC398 were typed as t011 and t034. They were MDR, and tetracycline resistance was mediated by *tet*(M) +/- *tet*(K) genes. Among TET^R-MRSA isolates, another lineage of interest was found, CC1, associated to t127. They were also MDR but in this case, tetracycline resistance was mediated by only *tet*(K) gene. Resistance to erythromycin and clindamycin was observed in MRSA-CC398 and -CC1 isolates, but the phenotype inducible was detected in only one isolate CC1. However, none of the MRSA isolates harboured the *erm*(T) gene. The *scn* gene was not detected among MRSA-CC398 isolates but was present in two MRSA-CC1 isolates, one of them hosting the PVL encoding genes.

Based on the multicentre study, the prevalence of MSSA-CC398 among blood culture *S. aureus* isolates in Spanish hospitals is 3.9%. Both works showed that *spa*-types t571 and t1451 were prevalent among MSSA-CC398 invasive isolates. Moreover, they revealed that resistance to erythromycin and clindamycin-inducible, mediated by *erm*(T) gene is a characteristic only observed on MSSA-CC398 isolates. Furthermore, MRSA-CC398 isolates are very unusual in blood culture.

The corresponding papers manuscripts submitted to scientific journals or in preparation are below enclosed.

Paper 7: Letter submitted to the journal "*Enfermedades Infecciosas y Microbiología Clinica*". Title: Importancia del complejo clonal CC398 en las bacteriemias por *Staphylococcus aureus* en un hospital secundario de Aragón. _ Relevance of clonal complex CC398 in bacteraemia caused by *Staphylococcus aureus* in a secondary hospital of Aragon, Spain.

Paper 8: Manuscript in preparation for the journal "*Journal of Antimicrobial Chemotherapy*". Title: Lineage CC398 among methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* isolates of blood cultures. A multicentre study in Spanish Hospitals.

PAPER 7 (in revision)

Importancia del complejo clonal CC398 en las bacteriemias por *Staphylococcus aureus* en un hospital secundario de Aragón.

Relevance of clonal complex CC398 in bacteremia caused by *Staphylococcus aureus* in a secondary hospital of Aragon, Spain.

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Palabras clave: *bacteriemia, Staphylococcus aureus,* t571, CC398, ***erm*(T)**

Keywords: *bacteremia, Staphylococcus aureus,* t571, CC398**, *erm*(T)**

La bacteriemia por *Staphylococcus aureus* es una entidad de especial importancia por su frecuencia y su gravedad, con una incidencia en constante aumento. La epidemiología de las cepas que más comúnmente causan esas infecciones invasivas, tanto en cepas sensibles (SASM) como resistentes a meticilina (SARM), es variable según las zonas geográficas en que nos situemos[1].

En nuestra área geográfica se detectan con frecuencia cepas de SARM del complejo clonal 398, que suelen estar relacionadas epidemiológicamente con ganado porcino[2]. Sin embargo, los datos sobre la variante SASM-CC398 son escasos hasta la fecha, pero se plantean como una entidad emergente en países como Francia[3,4, 5] o Portugal[6] lo que, dada su proximidad geográfica a nuestra región, parece razón suficiente para especular con una epidemiología similar.

Para conocer la importancia de estas cepas en infecciones invasivas en nuestro entorno, se estudiaron los aislados de *S. aureus* (primer aislado por paciente) obtenidos de hemocultivos en el Hospital Royo Villanova (HRV) de Zaragoza, durante un periodo de 30 meses (01/06/2015–31/12/2017). En este periodo se obtuvieron 84 aislados *S. aureus* (30 SARM y 54 SASM), de los que se pudieron recuperar 77 (27 SARM, todos ellos *mec*A-positivos y 50 SASM), que fueron incluidos en este estudio.

Se determinó la sensibilidad a antimicrobianos de los 77 aislados de *S. aureus* (paneles Combo 31, Microscan ®, Beckman, y difusión en agar), y se estudió por PCR si las cepas pertenecían al complejo clonal CC398[7]. En los aislados del linaje CC398 se analizó la presencia de genes de resistencia a antibióticos, en función del fenotipo de resistencia detectado (*blaZ*, *erm*(A), *erm*(B), *erm*(C), *erm*(T) y *msr*(A))[8]. Por otro lado, se analizó la presencia de genes de resistencia a macrólidos en los aislados *S. aureus* de linajes diferentes al CC398 para compararlos con los de CC398.

Se detectaron cuatro aislados del linaje CC398, todos ellos SASM, correspondiendo a un 8% de las cepas SASM y un 5,2% del total de los aislados *S. aureus*. Fueron adscritos a 2 tipos-*spa* diferentes: t571 y t1451, y asociados a la secuencia tipo (ST) 398 (Tabla 1). Ninguno de los aislados SARM se asoció al complejo CC398 (todos sensibles a tetraciclina). Los cuatro aislados SASM-CC398 carecían del fenotipo tetraciclina-resistente (marcador de SARM-CC398) y todos ellos fueron resistentes a eritromicina y a clindamicina (inducible), mediada dicha resistencia por el gen inusual *erm*(T), asociado al gen *msr*(A) en tres aislados. De los aislados no-CC398, 12 (16,4%) fueron resistentes a

eritromicina y clindamicina (inducible), mediada dicha resistencia por el gen *erm*(A) o *erm*(B), asociado al gen *msr*(A) en 9 casos. El gen *erm*(T) no fue detectado entre los aislados no-CC398. Por lo tanto, la resistencia a eritromicina-clindamicina (inducible), mediada por el gen *erm*(T), puede ser un marcador fenotípico de la variante sensible a meticilina del CC398 en *S. aureus* y coincidente con otras series[3,9], a diferencia de la resistencia a la tetraciclina, característica de SARM-CC398. Los cuatro aislados CC398 fueron sensibles al resto de antibióticos testados, con la única excepción de la penicilina, mientras que los aislados no-CC398 fueron además resistentes a ciprofloxacina (n=32), aminoglucósidos (n=16), mupirocina (n=14) y/o trimetoprim-sulfametoxazol (n=1). Las características de los aislados CC398 puede consultarse en Tabla 1.

Destacamos que CC398 es un linaje emergente entre las cepas SASM invasivas en nuestro medio, representando un porcentaje del 8% en nuestro hospital. En Francia se ha detectado con una frecuencia variable pero creciente en los últimos años[3], con cifras que pueden llegar a ser tan elevadas como el 20% en una serie de 2017[4]. Además de su presencia en bacteriemias, se han descrito en este país como causante de otras infecciones graves como neumonías, endocarditis o infecciones articulares[9], lo que concuerda con nuestra pequeña serie. Entre los *spa*-tipos detectados en nuestro estudio está el t571, que se asocia a infecciones de origen nosocomial o asociadas a cuidados sanitarios y sin relación con ganado, y que está ganando protagonismo en países vecinos como Francia y Portugal[3,5,6]. Más controvertida es la epidemiología de t1451, y aunque en uno de los dos casos presentados se encontró relación profesional anterior con ganado, son necesarios más estudios para extraer conclusiones válidas.

La dispersión de estas cepas parece preocupante, tanto por su frecuencia ascendente en entornos geográficos próximos como porque están asociadas con mayor virulencia que otros linajes y relacionadas con infecciones graves, como infecciones articulares, neumonías, bacteriemias o endocarditis, entre otras [3-5, 9,10]. Por otro lado, su especial resistencia a los antimicrobianos se centra especialmente en la familia de macrólidos-lincosamidas, lo que podría tener interés en casos vinculados a infecciones de piel y partes blandas o respiratorios, pero menos en bacteriemias (en las que apenas se utilizan esta familia de antimicrobianos). Éstas podrían beneficiarse de las terapias de primera elección como cloxacilina o cefalosporinas de primera generación, por no esperarse resistencias que la compliquen.

El estudio, pese a su reducido número de casos, parece indicar la presencia del complejo clonal CC398 entre los aislados invasivos de SASM y su ausencia en cepas de SARM. Sería de interés ampliar el estudio a otros hospitales y a otros entornos epidemiológicos a fin de completar el conocimiento sobre este linaje emergente entre aislados SASM.

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Tabla 1: ***Características de los aislados SASM-CC398 aislados de hemocultivos del hospital Royo Villanova***

Paciente	**Aislado SASM**	**tipo-*spa***	**PCR CC398**	**[a]Fenotipo de resistencia**	**Genotipo de resistencia**	**Relación con ganado**	**Cuadro clínico/ Características**
P1	X732	t571	+	ERI, CLI[b], PEN	*erm*(T), *msr*(A)	*No*	*Endocarditis infecciosa*
P2	X746	t571	+	ERI, CLI[b]	*erm*(T), *msr*(A)	*No*	*Artritis séptica*
P3	X753	t1451	+	ERI, CLI[b], PEN	*erm*(T), *msr*(A)	*No*	*Bacteriemia asociada a catéter*
P4	X729	t1451	+	ERI, CLI[b], PEN	*erm*(T)	*Si (ganadero de vacas hace años). Contacto ocasional con otros animales (gallinas, perros, cerdos)*	*Neumonía nosocomial*

[a]Antibióticos testados (EUCAST): penicilina (PEN), cloxacilina, oxacilina, cefoxitina, eritromicina (ERI), clindamicina (CLI), gentamicina, tobramicina, amikacina, ciprofloxacina, fosfomicina, levofloxacina, vancomicina, teicoplanina, cotrimoxazol, ácido fusídico, mupirocina, daptomicina y linezolid.
[b] Fenotipo de resistencia inducible

Anexo paper 7:

Fenotipo y genotipo de resistencia a antibióticos de los 73 aislados no-CC398 de hemocultivos del Hospital Royo Villanova

	Fenotipo de resistencia [a] **(N.º aislados)**	**Genes detectados (N.º aislados)**
SASM (n=46)	SENSIBLE (6)	-
	PEN (19)	*blaZ* (19)
	PEN, ERI (5)	*blaZ*, *msr*(A) (4)
		blaZ, *msr*(A), *erm*(B) (1)
	PEN, ERI, CLI [b] (4)	*blaZ*, *erm*(A) (2)
		blaZ, *erm*(A), *msr*(A) (1)
		blaZ, *msr*(A), *erm*(A) (1)
	PEN, MUP (2)	*blaZ* (2)
	CIP (1)	-
	PEN, CIP (1)	*blaZ*
	ERI, CLI [b] (1)	*erm*(A), *msr*(A)
	PEN, ERI, TOB (1)	*blaZ*, *msr*(A), *ant*(4')-*Ia*
	PEN, TOB, MUP (1)	*blaZ*, *ant*(4')-*Ia*
	PEN, GEN, TOB (1)	*blaZ*, *aac(6')-Ie-aph(2")-Ia*, *ant*(4')-*Ia*
	PEN, ERI, CIP, MUP (1)	*blaZ*, *msr*(A)
	ERI, CIP, MUP (1)	*msr*(A)
	ERI, TOB, CIP, MUP (1)	*msr*(A), *ant*(4')-*Ia*
	PEN, ERI, CLI [b], CIP, MUP (1)	*blaZ*, *erm*(A), *msr*(A)
SARM (n=27)	PEN, FOX, ERI, CIP (10)	*blaZ*, *mecA*, *msr*(A) (9)
	PEN, FOX, TOB, CIP (3)	*blaZ*, *mecA*, *ant*(4')-*Ia* (3)
	PEN, FOX, CIP (2)	*blaZ*, *mecA* (2)
	PEN, FOX, ERI, CLI [b], CIP (2)	*blaZ*, *mecA*, *erm*(A), *msr*(A) (1)
		blaZ, *mecA*, *erm*(C) (1)
	PEN, FOX, GEN, TOB, CIP, MUP (2)	*blaZ*, *mecA*, *aac(6')-Ie-aph(2")-Ia*, *ant*(4')-*Ia* (2)
	PEN, FOX, ERI, CLI [b], TOB, CIP, MUP (2)	*blaZ*, *mecA*, *msr*(A), *ant*(4')-*Ia* (1)
		blaZ, *mecA*, *erm*(A), *msr*(A) (1)
	PEN, FOX (1)	*blaZ*, *mecA*
	PEN, FOX, ERI, GEN, CIP (1)	*blaZ*, *mecA*, *msr*(A), *aac(6')-Ie-aph(2")-Ia*
	PEN, FOX, TOB, CIP, MUP (1)	*blaZ*, *mecA*, *ant*(4')-*Ia*
	PEN, FOX, ERI, CLI [b], GEN, TOB, CIP (1)	*blaZ*, *mecA*, *erm*(B), *msr*(A), *aac(6')-Ie-aph(2")-Ia*, *ant*(4')-*Ia*
	PEN, FOX, ERI, CLI [b], GEN, TOB, CIP, MUP (1)	*blaZ*, *mecA*, *erm*(A), *msr*(A), *aac(6')-Ie-aph(2")-Ia*, *ant*(4')-*Ia*
	PEN, FOX, ERI, GEN, TOB, CIP, MUP, SXT (1)	*blaZ*, *mecA*, *msr*(A), *aac(6')-Ie-aph(2")-Ia*, *ant*(4')-*Ia*

[a] Antibióticos testados (EUCAST): penicilina (PEN), cefoxitina (FOX), eritromicina (ERI), clindamicina (CLI), gentamicina (GEN), tobramicina (TOB), ciprofloxacina (CIP), trimetroprim sulfametoxazol (SXT), mupirocina (MUP) y linezolid.

[b] Fenotipo de resistencia inducible

PAPER 8 (in preparation to be submitted to a journal)

Lineage CC398 among methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* isolates of blood cultures. A multicentre study in Spanish Hospitals

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Abstract

Background: Prevalence of Livestock-Associated (LA) MRSA-CC398 at hospital level is closely related in Spain to pig-farming density and tetracycline resistance (TET^R) is a phenotypic marker. MSSA-CC398 is an emerging and leading cause of invasive infections in some European countries, but data and epidemiological characteristics are still scarce.

Objectives: To determine the prevalence of MSSA- and LA-MRSA-CC398 among blood culture *S. aureus* (SA) isolates recovered from patients in 17 Spanish hospitals located in regions with different pig-farming density and characterize the recovered isolates.

Methods: MSSA (n=706) and TET^R-MRSA (n=18) isolates recovered from blood cultures during 3-12 months (2018-2019) in 17 Spanish hospitals were analysed. CC398 identification, detection of *spa*-types, antibiotic resistance genes, human immune evasion cluster (IEC) genes, *eta*, *etb* and Panton-Valentine leucocidin (PVL) encoding genes were performed by PCR/sequencing.

Results: MSSA represented 74.2% of total SA recovered. MSSA-CC398 prevalence was 3.9% of SA (n=37; 5.2% of MSSA) and MRSA-CC398 prevalence was 0.4% of SA (n=4; 22.2% of TET^R-MRSA). Among MSSA-CC398, six *spa*-types were recorded (t571: 43.2% and t1451: 35.1% being the predominant) and all, but three strains, were IEC-positive. Resistance to erythromycin and clindamycin inducible (with *erm*(T) gene) was detected in 72.9% of MSSA-CC398. Three strains harboured the *eta* gene. All MRSA-CC398 isolates were *spa*-t011 or t034, IEC-negative, and were detected in high pig density (HPD) regions.

Conclusions: LA-MRSA-CC398 is infrequent in blood culture samples but is related to HPD regions, contrarily to MSSA-CC398, which seems to be a human-adapted clone. Presence of *erm*(T) gene associated with erythromycin-clindamycin inducible resistance could be a marker for MSSA-CC398 detection.

Keywords: MSSA, LA-MRSA, CC398, blood culture, Spanish hospitals

Introduction

S. aureus (SA) is an important opportunistic pathogen that can cause infections in animals and humans. Livestock-associated (LA) methicillin-resistant SA (MRSA) of

clonal complex (CC) 398 have gained much attention during the past decade because, apart from colonizing farm-animals, they have become an emerging cause of skin, soft tissues (SST) and invasive infections, mainly in humans in contact with livestock[1–4] and to a less extent in patients with no livestock exposure.[5] More recently, MSSA-CC398 have been increasingly reported as a cause of invasive infections in patients without livestock contact, mainly in France.[6–8] Moreover, this clone is easily transmissible among humans and has a genome that is well adapted to the human host.[9]

Based on whole genome sequences, some authors suggest that LA-MRSA-CC398 have evolved from human-adapted methicillin susceptible *SA* (MSSA) CC398. They would have acquired methicillin and tetracycline resistance and lost the phage ΦSa3, that carries the immune evasion cluster (IEC) genes; IEC is a set of genes which protects *S. aureus* against the human immune system.[10] The gene *scn* is present in all types of IEC, and is therefore considered a marker of this cluster.

As most of LA-MRSA-CC398 show tetracycline-resistance (TET^R), maybe due to the high use of this antimicrobial in food animals, TET^R is considered a marker for LA-MRSA-CC398 detection among epidemiological or clinical strains.[3,4,11] Based on this observation, a recent multicentric study performed by our research group established a strong correlation between LA-MRSA-CC398 prevalence in Spanish hospitals and their location pig density.[11] This study mainly included MRSA isolates of SST and respiratory tract infections samples and found a prevalence of MRSA-CC398/SA of 1.2%. A recent study focused on blood cultures of one Spanish hospital revealed a prevalence of MSSA-CC398/SA of 5.2% (and of MSSA-CC398/MSSA of 8%), with absence of MRSA-CC398.[12]

In the light of the above and knowing that SA represents the second most common cause of bloodstream infections in developed countries,[6] this study aimed to determine the epidemiology of CC398 among blood culture SA isolates in hospitals of different Spanish regions, and to give more information on MSSA-CC398 molecular and epidemiological characteristics.

Methods

Strains collection

A total of 951 SA isolates of blood cultures (1 isolate/patient) were collected in 17 Spanish hospitals during the whole year 2018 (10 hospitals), or during the first 9 months (3 hospitals) or 3 months of 2019 (4 hospitals) (Table 1). Hospitals were located in regions

with different pig farming densities (Ministerio de Agricultura, Pesca y Alimentación, on Pigs in 2018 Spain), and they were classified as follows: Low Pig Density (LPD): 0-10*10^3 pigs/km^2; Medium Pig Density (MPD): 11-60*10^3 pigs/km^2; High Pig Density (HPD): >160*10^3 pigs/km^2.

Antibiotic resistance phenotype was determined for all isolates by Combo 31 panels, MicroScan®, Beckman, and agar diffusion.

Molecular characterization

All MSSA (n=706) and all TET^R-MRSA (n=18) of this collection were included in our study. Identification of the CC398 lineage was carried out by specific PCR.[13] *Spa*-type was determined by PCR and sequencing for all MSSA-CC398 and TET^R-MRSA isolates.[3] Additional characterization was performed by PCR as previously described:[3] 1) antibiotic resistance genes detection according to their resistance phenotype ; 2) *scn* gene detection and IEC-typing for *scn*-positive strains; and 3) *eta*, *etb*, *tst* and *lukF/S-PV* gene screening.

Results and Discussion

Prevalence of CC398 isolates

In the hospitals studied, MSSA represented 74.2% of total SA among blood culture isolates. The lineage CC398 was detected in this multicentre study in 4.3% of total SA isolates of blood cultures, similar to the rate detected by our research group in a Spanish hospital (5.1%).[12] Our results showed an intermediate prevalence compared to data reported by different studies in France, one of the countries with most publications on CC398 invasive isolates: 2007-2010 (2.3%),[7] 2010-2014 (8.7%),[8] and 2010-2017 (12.7%).[14] In these mentioned studies, most CC398 isolates were MSSA, as is the case in ours. We detected 37 isolates MSSA-CC398, corresponding to 3.9% of SA (5.2% of MSSA). MSSA-CC398 distribution among the hospitals was heterogeneous, with higher rates (MSSA-CC398/MSSA) observed in areas with different pig density levels, suggesting that this factor doesn´t impact on MSSA-CC398 incidence.

Four out of 18 TET^R-MRSA (22.2%) were ascribed to CC398 lineage (prevalence of 0.4% respect to all SA). MRSA-CC398 isolates were recovered from three hospitals located in zones with HPD (Aragón and Cataluña). These results support a previous study which demonstrated that increased pig population density leads to an increase in MRSA-CC398 cases among hospitals.[11] Moreover, they show that MRSA-CC398 are less

frequent in isolates of invasive infections than in those of SST and respiratory tract infections.[11]

Genetic characterization of CC398 isolates

MRSA-CC398 isolates were typed as t011 and t034, *spa*-types strongly related to livestock[10] and associated to LA-MRSA-CC398 in Spanish hospitals.[3,4,11] They were all MDR, and resistance to tetracycline was mediated by *tet*(M) +/- *tet*(K) genes, while methicillin-resistance was due to *mecA* gene. The lack of *scn* confirms their animal origin. No other virulence gene was detected.

The two *spa*-types less frequently related with livestock[7] were predominant among our MSSA-CC398 isolates (t571 (n=16) and t1451 (n=13). Of note, t571 is the *spa*-type most commonly associated with MSSA-CC398 bloodstream infections in Europe.[6,7,15] In our study, thirty-one (83.8%) of MSSA-CC398 were resistant to at least one antibiotic, including seven multidrug-resistant isolates (MDR)[16] (Table 2). It is worth noting that 25.8% of MDR-MSSA-CC398 isolates were typed as t1451. Indeed, t1451 (both MRSA and MSSA) is usually associated with a MDR phenotype.[3,4,15] Moreover, resistance to erythromycin and clindamycin inducible, mediated by *erm*(T) alone or combined with other genes is a recurrent characteristic among MSSA-CC398, yet reported[6,12,17] and here reiterated (n=27; corresponding to 72.9% of MSSA-CC398). Therefore, this phenotype as well as the presence of *erm*(T) gene may be a marker for MSSA-CC398 detection.

All MSSA-CC398, but three isolates (2 *spa*-t011, and 1 *spa*-t1451), harboured the *scn* gene and were typed as either IEC-C (n= 20) or IEC-B (n= 9). Interestingly, one of the MSSA-CC398/t011/*scn*-negative isolates was recovered in a hospital located in an HPD region, and the patient was a pig farmer. The other t011 *scn*-negative isolate was recovered in a hospital located in an LPD area. The absence of IEC and the absence of the TET^R phenotype in both t011 MSSA-CC398 isolates (*spa*-type usually pig-related), might indicate an intermediary evolutive step of this microorganism in the human-animal interface. In addition, the *eta* gene was detected in three isolates: t571 (n=1) and t1451 (n=2), but neither *etb* nor *lukF/S-PV* were found.

Other genetic lineages of interest

CC1 is a community-associated clone detected in humans, but the t127/CC1 is widely spread in livestock.[3,4,10] Of the 18 TET^R-MRSA, four were typed as t127/CC1. Two were *scn*-negative, suggesting an animal origin and can be considered as LA-MRSA-CC1.

Interestingly, they were detected in hospitals located in HPD areas. One of the two isolates *scn*-positive harboured the PVL genes, which is a marker of CA-MRSA. Resistance to tetracycline seems to be a marker for MRSA-CC1. Resistance to methicillin and tetracycline were mediated by *mecA* and *tet*(K) genes, respectively. The four isolates were MDR and resistant to erythromycin and clindamycin, mediated by *erm*(C) alone or combined with *msr*(A) (Table 2).

Conclusion

LA-MRSA (CC398 and CC1) were infrequently detected among blood culture isolates and those detected were found in HPD regions, associated to isolates of potential animal origin. The distribution of MSSA-CC398 within the hospitals was variable (not clearly associated to pig farming density) and the prevalence was 3.9% of SA and 5.2% of MSSA. Resistance to erythromycin and clindamycin inducible and presence of the *erm*(T) gene could be a good marker for MSSA-CC398 detection. The characteristics of MRSA and MSSA of CC398 should be studied more deeply in order to understand better their clinical and epidemiological burden in Spanish regions.

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Transparency declarations

None to declare

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Table 8: prevalence of MSSA, MSSA-CC398 and LA-MRSA-CC398+CC1, in hemoculture samples from 17 hospitals in Spain

Hospital Code [a]	Regions	Pig density (10^3 pigs /km^2)	Number of strains					Rate (%)					
			Isolation Time (in months)	*S. aureus*	MSSA	MSSA-CC398	MRSA CC398+CC1	MSSA/ *S. aureus*	MSSA-CC398/ *S. aureus*	MSSA-CC398/ MSSA	MRSA/ *S. aureus*	MRSA-CC398/ *S. aureus*	MRSA-CC1/ *S. aureus*
HUAV***	Lleida	358.4	9[d]	53	37	4	2+0	69.8	7.5	10.8	30.2	3.7	0.0
HSJ***	Huesca	258.8	3[c]	5	2	0	0+0	40.0	0.0	0.0	60.0	0.0	0.0
HB***	Huesca	258.8	3[c]	3	2	0	0+0	66.7	0.0	0.0	33.3	0.0	0.0
HUV***	Barcelona	256.0	3[c]	7	6	0	0+0	85.7	0.0	0.0	14.3	0.0	0.0
HUMS***	Zaragoza	166.8	12[b]	104	77	5	1+0	74.0	4.8	6.5	26.0	1.0	0.0
HULB***	Zaragoza	166.8	12[b]	81	63	3	1+1	77.8	3.7	4.8	22.2	1.2	1.2
HRV***	Zaragoza	166.8	12[b]	38	18	2	0+1	47.4	5.3	11.1	52.6	0.0	2.6
CHN**	Navarra	58.8	12[b]	56	41	3	0+0	73.2	5.4	7.3	26.8	0.0	0.0
CUN**	Navarra	58.8	12[b]	11	10	0	0+0	90.9	0.0	0.0	9.1	0.0	0.0
HVM**	Sevilla	47.8	12[b]	109	90	5	0+1	82.6	4.6	5.6	17.4	0.0	0.9
HUB**	Burgos	33.3	9[d]	48	32	1	0+0	66.7	2.1	3.1	33.3	0.0	0.0
HSP**	La Rioja	24.3	12[b]	71	50	2	0+0	70.4	2.8	4.0	29.6	0.0	0.0
HG*	País Vasco	5.0	12[b]	48	35	5	0+0	72.9	10.4	14.3	27.1	0.0	0.0
HUA*	País Vasco	5.0	9[d]	49	41	2	0+1	83.7	4.1	4.9	16.3	0.0	2.0
HUD*	País Vasco	5.0	3[c]	31	27	0	0+0	87.1	0.0	0.0	12.9	0.0	0.0
HUGM*	Madrid	2.2	12[b]	143	108	3	0+0	75.5	2.1	2.8	24.5	0.0	0.0
HMV*	Cantabria	0.3	12[b]	94	67	2	0+0	71.3	2.1	3.0	28.7	0.0	0.0
Total				**951**	**706**	**37**	**4+4**	**74.2**	**3.9**	**5.2**	**25.8**	**0.4**	**0.4**

[a] Code for the hospitals (H.). HUGM, H. universitario Gregorio Marañon; HUMS, H. universitario Miguel Servet; HVM, H. Virgen Macarena; HMV, H. Marques Valdecilla; HULB, H. Universitario Lozano Blesa; HSP, H. San Pedro; CHN, Complejo Hospitalario de Navarra; HG, H. de Galdakao; HRV, H. Royo Villanova; CUN, Clínica Universitaria de Navarra; HUD, H. Universitario de Donostia; HUAV, H. Universitario Arnau Vilanova; HUB, H. Universitario de Burgos; HUA, H. Universitario de Álava, HUV, H. Universitari de Vic; HSJ, H. San Jorge; HB, H. Barbastro.

*Hospitals located in a low pig density area: 0-10*10^3 pigs/km^2; ** Hospitals located in a medium pig density area: 11-60*10^3 pigs/km^2; *** Hospitals located in a high pig density area: >160*10^3 pigs/km^2. Data were taken from the report of the Ministerio de Agricultura, Pesca y Alimentación, on Pigs in 2018, Spain.

[b] Isolation Time. 12 months: January-December 2018.

[c] Isolation Time. 3 months: January-March 2019.

[d] Isolation Time. 9 months: April-September 2019

Table 9: Characteristics of 37 strains MSSA-CC398, and 8 strains LA-MRSA-CC398+CC1 recovered from hemoculture samples from 17 hospitals in Spain (during the year 2018 or 3month in 2019)

			Antimicrobial resistance				
	***spa*-type** [nº strains]	**Hospital** [a] [nº strains]	**Phenotype** [b] [nº strains]	**Genotype** [nº strains]	**Virulence genes** [nº strains]	***scn*** [nº trains]	**IEC-type** [nº strains]
MSSA-CC398 n=37	**t571** [16]	HG [4], HUGM [2], HUMS [2], HSP [2], HVM [2], HUAV [2], CHN [1], HUA [1]	ERY, CLI [c] [10]	*erm*(T) [8]	*eta* [1]	+ [8]	C [7], B [1]
				erm(T), *erm*(A), *msr*(A) [1]	-	+	C
				erm(T), *erm*(C), *lnu*(A), *Vga*(A) [1]	-	+	B
			PEN, ERY, CLI [c] [1]	*blaZ*, *erm*(T), *erm*(A), *erm*(C), *Vga*(A)[1]	-	+	C
			PEN [1]	*blaZ*	-	+	B
			SUSCEPTIBLE [4]	-	-	+ [4]	B [4]
	t1451 [13]	HUMS [3], HULB [2], HVM [2], HRV [1], HMV [1], CHN [1], HG [1], HUGM [1], HUB [1]	ERY, CLI [c] [5]	*erm*(T) [3]	*eta* [1]	+ [3]	C [3]
				erm(T), *erm*(A), *msr*(A) [1]	-	+	B
				erm(T), *erm*(A), *lnu*(A) [1]	-	+	C
			ERY, CLI [c], PEN [6]	*erm*(T), *erm*(A), *blaZ* [2]	+	+ [2]	C [2]
				erm(T), *erm*(A), *msr*(A), *blaZ* [1]	-	+	C
				erm(T), *erm*(A), *msr*(A), *lnu*(A) *blaZ* [1]	*eta*	+	B
				erm(T), *msr*(A), *blaZ* [1]	-	+	C
				erm(T), *blaZ* [1]	-	+	C
			ERY, CLI [c], PEN, TOB [1]	*erm*(T), *erm*(C), *msr*(A), *blaZ*, *ant(4´)-la*	-	+	B
			ERY, CLI [c], PEN, GEN, TOB [1]	*erm*(T), *blaZ*, *aac(6´)-Ie-aph(2″)-Ia*	-	-	-
	t011 [2]	HUAV [1] HMV [1]	PEN [1]	*blaZ*	-	-	-
			SUSCEPTIBLE [1]	-	-	-	-
	t4030 [2]	HRV [1] CHN [1]	ERY, CLI [c] [1]	*erm*(T), *erm*(A)	-	+	C
			SUSCEPTIBLE [1]	-	-	+	C
	t899 [1]	HULB	PEN, TET	*blaZ*, *tet*(M)	-	+	B
	t7880 [1]	HVM	ERY, CLI [c]	*erm*(T), *erm*(A)	-	+	C
	N.D [d] [2]	HUAV [1] HUA [1]	PEN [1]	*blaZ*	-	+	C
			ERY, CLI [c] [1]	*erm*(T)	-	+	C

MRSA-CC398 n=4	**t011** [3]	HULB [1], HUMS [1]	FOX, PEN, TET, ERI, CLI, GEN, TOB, CIP [2]	*mecA*, *tet*(M), *tet*(K), *erm*(B), *aac(6´)-Ie-aph(2″)-Ia* [2]	-	-	-
		HUAV [1]	FOX, PEN, TET, ERI, CLI, CIP [1]	*mecA*, *tet*(M), *tet*(K), *erm*(C)	-	-	-
	t034 [1]	HUAV [1]	FOX, PEN, TET, CLI, SXT [1]	*mecA*, *tet*(M), *dfrA*, *dfrG*	-	-	-
MRSA-CC1 n=4	**t127** [4]	HVM [1]	PEN, FOX, TET, ERI, CLI [c] [1]	*mecA*, *tet*(K), *erm*(C)	-	+	-
		HUA [1]	PEN, FOX, TET, ERI, CLI [1]	*mecA*, *tet*(K), *erm*(C)	*lukF/S-PV*	+	E
		HULB [1]	PEN, FOX, TET, ERI, CLI, CIP [1]	*mecA*, *tet*(K), *erm*(C)			
		HRV [1]	PEN, FOX, TET, ERI, CLI, TOB [1]	*mecA*, *tet*(K), *erm*(C), *msr*(A), *ant(4´)-la*	-	-	-

[a] Code for the hospitals (H.). HUGM, H. universitario Gregorio Marañon; HUMS, H. universitario Miguel Servet; HVM, H. Virgen Macarena; HMV, H. Marques Valdecilla; HULB, H. universitario Lozano Blesa; HSP, H. San Pedro; CHN, Complejo hospitalario de Navarra; HG, H. de Galdakao; HRV, H. Royo Villanova; HUB, H. Universitario de Burgos; HUAV, H. universitario Arnau Vilanova; HUA, H. universitario de Álaba.[b] PEN, penicillin; TET, FOX, cefoxitin; tetracycline; ERY, erythromycin; CLI, clindamycin; GEN, gentamycin; TOB, tobramycin; CIP, ciprofloxacin; FUS, fusidic acid; MUP, mupirocin.[c] Inducible resistance phenotype.

[a] N.D, *spa*-type not determined, but PCR CC398 positive.

DISCUSSION

CHAPTER 1

The first chapter dealt with molecular epidemiology of *Staphylococcus* spp. in non-conventional food animals such as wild boar and horses. A great diversity of species was observed in both animal species.

In wild boars, 19 staphylococcal species were identified with predominance of *S. sciuri* and *S. aureus* (respectively 28 and 22% of total isolates recovered). As previously reported, wild animals (wild rodents, wild birds, deer, etc.) are natural hosts of CoPS, especially *S. aureus* (Gómez et al., 2014; Monecke et al., 2016; Bengtsson et al., 2017; Mrochen et al., 2018; Ruiz-Ripa et al., 2019). The prevalence of *S. aureus* in wild boar in Spain was lower than in other European countries such as Portugal and Germany (Porrero et al., 2014; Seinige et al., 2017; Sousa et al., 2017). *S. pseudintermedius* is infrequent in wild boar, but it is usually found in wild Felidae (Guardabassi et al., 2012; Nowakiewicz et al., 2016). In our study, CoNS were more represented than CoPS species (17/4). Unfortunately, the other studies performed on wild boar couldn't confirm our results since they all focussed on CoPS. However, CoNS (*S. sciuri*, *S. epidermidis*, *S. saprophyticus*, among others) were highly detected in other wild animals such as Cope´s gray tree-frogs or birds of prey (Slaughter et al., 2001; Sousa et al., 2014). We can though affirm that wild animals, in our case wild boar, are a reservoir for both CoPS and CoNS.

Concerning *S. aureus* genetic lineages, a large variety was observed among wild boar. The predominant t3750-ST2328/CC133 lineage found in this study is apparently the most frequent in wild boar in Spain (Porrero et al., 2014). Interestingly, this lineage was not found in Germany, but was present in Portugal, maybe because of its geographical proximity with Spain (Seinige et al., 2017; Sousa et al., 2017). Furthermore, the livestock-associated lineage MRSA-CC398 was detected in these two neighbour countries. In our case, the isolate typed as t011 was *scn*-negative (suggesting an animal origin), while the Portuguese isolate t899 was *scn*-positive, what suggest human adaptation (Sousa et al., 2017). LA-MRSA-CC398 are generally associated to pig, and interestingly, the wild boar harbouring the latter clone was hunted in a region with many pig farms. LA-MRSA-CC398 was detected in wild boar meat in Germany, which suggests a possible contamination by the animal during manipulation. This shows that farm lineages are spreading to humans and wildlife and that the different ecosystems are interconnected. LA-MRSA-CC398 are responsible for infections in humans, therefore, their presence in hunting animals is worrisome. According to Ruiz-Fons, interactions between humans

and wild boar leading to pathogen transmission could come from different ways. However, Direct transfer is mainly restricted to exposure of hunters and game professionals to infected animals or carcasses or by consumption of raw, smoked or undercooked meat from wild boar (Ruiz-Fons, 2017). Therefore, hunting activity should be strictly regulated and controlled to limit zoonotic transmission risks.

Regarding antimicrobial resistance, both CoPS and CoNS were highly susceptible to the antimicrobials tested (74.5% and 77.6%, respectively), probably because wild boar live in an environment with low antibiotic selective pressure. However, many resistance genes were detected among the resistant isolates. Resistance to penicillin (*blaZ*) and streptomycin (*str*) were the most observed in CoPS. The most frequent resistance genes detected in CoNS were those related to tetracycline (*tet*(K)), lincosamides (*lnu*(A)) and cefoxitin/methicillin resistance (*mecA*), although resistance to other agents were also found: chloramphenicol (*fexA* and *cat*), trimethoprim (*dfrG*) or macrolides-lincosamides (*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(43), *mphC* and *msr*(A)/*msr*(B)). Most of these resistance genes were detected among CoNS from birds of prey, all isolates being MDR (Aires-de-Sousa, 2017). CoNS of both animal and human origins are thought to represent an important reservoir of genetic elements leading to resistance to beta lactam antibiotics and other antibiotic classes (Becker et al., 2014b). The resistance genes can be though transferred to other species potentially pathogen and be interchanged between different ecosystems. The presence of such a panoply of resistance genes in CoNS from hunting wild animals is worrisome.

The second animal species studied, showed less diversity species than wild boar, with 12 staphylococcal species identified. The predominant species in horses were *S. aureus*, *S. sciuri* and *S. delphini*. *S. aureus* occurrence in our study was much higher than reported in Germany and Denmark (Islam et al., 2017; Kaspar et al., 2019). *S. delphini* was highly represented among staphylococcal isolates from horses in Canada (Stull et al., 2014) and from donkeys in Tunisia (Gharsa et al., 2015). In our study, many other CoNS species were identified namely, *S. fleurettii*, *S. saprophyticus*, *S. lentus*, *S. haemolyticus*, *S. xylosus*, *S. schleiferi*, *S. vitulinus*, *S. simulans* and *S. hyicus*. Indeed, horses have been frequently reported as reservoir of a great diversity of CoNS species (Busscher et al., 2006; Corrente et al., 2009; Karakulska et al., 2012; Nemeghaire et al., 2014). The genetic lineages most frequent in our *S. aureus* isolates were ST1640/t2559 (n = 21) and ST1 (n = 8). The ST1 is common among horses (Agabou et al., 2017; Islam et al., 2017; Kaspar et al., 2019), but

the ST1640 is here found for the first time among this animal species. Even though the human and equine origin was discarded (absence of *scn* and *scn-eq* genes), its origin remains unclear. We could only find a case of colonization of t2559 but associated to CC5/CC30 in a patient with no infection signs in the Netherlands (Donker et al., 2009).

A high susceptibility to antimicrobials was also detected in CoPS from horses (88.6%) but not in CoNS (46%). Apparently, *S. aureus* from horses are mostly MSSA and highly susceptible to other antibiotic classes (Agabou et al., 2017; Islam et al., 2017; Kaspar et al., 2019). On the contrary, resistance to methicillin and MDR is frequently observed among CoNS, especially in *S. sciuri* group (Corrente et al., 2009; Karakulska et al., 2012; Mallardo et al., 2013). All isolates except those of the lineage ST1640 were well-adapted to the equine host (*scn-eq*- positive) and harboured the equine virulent gene *lukPQ*. The latter leukocidin can destroy equine neutrophils, and at high concentrations, human neutrophils (Koop et al., 2017). The presence of such virulent *S. aureus* isolates and MDR CoNS in animals destined for human consumption may be of food-safety concern, particularly in horses which are animals very close to humans.

Overall, this chapter highlights that wildlife animals harbour a great diversity of staphylococcal species subjected to a low antimicrobial selective pressure. However, human contact with wild animals, particularly those which enter in the food chain, increases risks of zoonotic transmission and the easy spread of some staphylococcal lineages in the human ecosystem. In the same context, including horses in human diet, widens the transmission ways of equine pathogens to humans. Moreover, horses are very close to humans, therefore, they can be involved in the dissemination of human staphylococcal clones. Based on these findings, strict regulations and high hygiene and practice measures should be implemented and applied during processing of non-conventional animal-derived food due to their high risk of zoonotic pathogen transmission.

CHAPTER 2

In this second chapter, staphylococcal carriage of conventional food-producing animals in Senegal and Spain were analysed. Among the animal species studied, staphylococci were more frequently detected in goat and lamb (50-54%), then in cows (21-26.8%) and finally in chickens (3%). Similar rates were observed in cows in both countries. Among CoPS, *S. aureus* was the predominant species but seems to be infrequent in bovine compared to ovine and caprine, as previously reported in a Grecian work (Papadopoulos et al., 2018). *S. aureus* genetic lineages differed according to the countries and the animal species. In Spain, the most frequent lineage was CC133, previously described as particularly frequent in small ruminants in a European country (Eriksson et al., 2013), though also present in Africa (Ben Said et al., 2017). The lineage CC133 was indeed detected in lamb samples in our study. In Senegal, the prevalent lineage ST291 (a double locus variant of ST398) was the major lineage in cattle and buffalos with mastitis and derived milk in Egypt and Iran (El-ashker et al., 2015; Panahi and Saei, 2019). The presence of this lineage in healthy animals destined for human consumption is therefore worrisome. Six species were identified among CoNS in each country, with prevalence of *S. sciuri* and *S. simulans*. According to previous reports, the species detected in our works are frequent in cattle and poultry (Becker et al., 2014b; Boamah et al., 2017).

Concerning antimicrobial resistance profile, CoPS isolates were mostly pan susceptible in Spain even though two were MRSA, and 47% of CoNS were resistant to at least one antibiotic tested (including methicillin). CoPS isolates from Senegal were all MSSA but mostly resistant to penicillin, and 32.5% of CoNS showed resistance to at least one antibiotic tested. Generally, resistance to penicillin, tetracycline and SXT was observed in Senegal isolates, which is in accordance with a similar study performed in pigs in the same country (Fall et al., 2012). However, in Spain in addition to those antimicrobials, resistance to methicillin, streptomycin, clindamycin, erythromycin, chloramphenicol and tobramycin was detected. According to Prestinaci and collaborators, 2015, beta lactams, lincosamides and sulphonamides are the antibiotic classes most used in veterinary medicine (Prestinaci et al., 2015). The difference of antibioresistance profiles reflects the antibiotics most used in each country.

Important virulence genes were detected in both studies. PVL genes were found in the two MRSA isolates of Spain, which belonged to the clone ST8-USA300. This clone

has become a major cause of SSTIs in the USA in 2004 and can also cause other life-threating diseases, such as community-acquired pneumonia, osteomyelitis, and bloodstream infections (Planet, 2017). The presence of this clone in animals is the result of human contamination. Moreover, as these animals are destined for human consumption, manipulation may increase the dissemination risk of such a pathogenic clone in derived food and handlers. In Senegal isolates, PVL genes were also highly detected. Enterotoxin, exfoliative and TSST genes were found in isolates of both countries. The spread of virulent isolates in animals or food might lead to important health issues, particularly in the context of a developing country with limited access to healthcare in some regions.

Overall, the results of this chapter should be considered as a call for sensitization of the public on the importance of hygienic and practice measures in food sector from slaughter to process steps in any country. The presence and spread of virulent human-adapted pathogens in food could be then avoided or limited. In addition, although antimicrobial resistance rates and profiles determined among Senegal isolates were not alarming, the national antimicrobial resistance control plan should be revitalised in order to inform the population better on the burdens of this global public health issue.

CHAPTER 3

In this chapter, the prevalence of lineage CC398 among *S. aureus* from pig-derived products was determined, and isolates detected were characterised. *S. aureus* was detected in 33.6% of samples analysed, the samples with skin (ear/snout) having the highest rates. A similar study, performed a few years ago in Belgium, also concluded that pig-derived food with skin showed higher rates of *S. aureus* contamination than product without skin (Verhegghe et al., 2016). This is related to the fact that *S. aureus* are normal colonizers of animals' skins. A great diversity of genetic lineages was detected among the isolates. The predominant lineage CC398 (64.1% of *S. aureus*), was associated in 92% of cases to MRSA isolates (n=23/25). Indeed, as said earlier, MRSA-CC398 is the most common clone colonizing livestock, especially pigs (Guardabassi et al., 2007; Lozano et al., 2011; Crombé et al., 2013; Sharma et al., 2016). Though its presence in pig products is not surprising and is often described. Previous works of our research group of 2009 and 2014 detected LA-MRSA-CC398 in pork with respective prevalence of 2% and 1% of analysed samples (Lozano et al., 2009; Benito et al., 2014b), way lower than our present result (20.8%). This shows a considerable increase of LA-MRSA-CC398 spread among pig-derived products over the years, certainly related to higher colonization rates of pigs. According to Price and collaborators hypothesis, LA-MRSA-CC398 would have evolved from the human clone MSSA-CC398, which would have acquire resistance to methicillin and tetracycline after its jump to pig host (Price et al., 2012). If we follow this hypothesis, a high selective pressure of antibiotics used in animals could be an important factor leading to the increase of MRSA-CC398 colonization rate in pig and pig-derived food (Crombé et al., 2013). Two MSSA-CC398 isolates were detected among the samples and were *scn*-positive, contrarily to the MRSA-CC398 isolates, which was expected. Different evolution steps of CC398 lineage in the pig environment could explain the detection of these variants, although we cannot discard a human origin of MSSA-CC398-*scn*-positive isolates.

Spa-typing revealed that most LA-MRSA-CC398 isolates were typed as t011. Moreover, they were all resistant to tetracycline. These results are in accordance with previous works which considered t011 as the predominant *spa*-type among LA-MRSA-CC398 and tetracycline resistance as a marker for this clone detection (Lozano et al., 2012b; Benito et al., 2014b; Ceballos et al., 2019). It is worth noting that most isolates were MDR and four harboured virulent genes. This is a big issue for public health, as LA-MRSA-CC398 is involved in several clinical cases in pigs and in human infections

(Crombé et al., 2013; Smith and Wardyn, 2015; Becker et al., 2017; Ceballos et al., 2019). The MSSA-CC398 isolates were t5452. They both showed resistance to erythromycin and inducible resistance to clindamycin and harboured *erm*(T) gene. This gene has been previously suggested as marker of the human clone MSSA-CC398 (Bonnet et al., 2018), but we cannot lead to this conclusion since the number of isolates is low. More isolates MSSA-CC398 of the human subclade should be characterised in order to confirm their common characteristics.

On another hand, other genetic lineages of interest were detected including community- and livestock-associated lineages such as CC1, CC8, CC45 inter alia. Although antimicrobial resistance rates were low, relevant virulent genes were found: PVL encoding genes, *tst* and enterotoxin genes mostly. It is particularly alarming to find isolates with food poisoning potential (Argudín et al., 2010).

Overall, this chapter reveals that LA-MRSA-CC398 (t011) is the main contaminant of pig-derived food, when referring to *S. aureus* clones. The characteristics previously detected are here confirmed, so that tetracycline resistance can be used as marker for this clone detection. However, finding LA-MRSA-CC398 with MDR resistance phenotype and/or virulent genes in food samples, as well as MSSA-CC398 isolates is a big threat for human health considering their implication in human infections. Moreover, detection of human-adapted isolates in food increases their dissemination as said in the previous chapter. The frequent detection of enterotoxins in isolates indicates a high risk of food poisoning occurrence if good hygiene, food processing practices and proper preservation are not respected. Pig-derived food constitute a reservoir for the CC398 lineage and a potential vehicle of dissemination.

CHAPTER 4

S. aureus is a leading cause of bloodstream infections in Europe. In humans, ST398 *S. aureus* infections can range from minor, localized disease to more severe invasive illnesses. Several severe infections caused by ST398-MSSA strains, mostly acquired in the absence of animal contact in young healthy people were yet reported (Kashif et al., 2019). MSSA-CC398 has emerged as an invasive clone particularly in France, associated to *spa*-t571. France is one of the countries with most publications on this regard and is geographically very close to Spain. Increasing prevalence were reported over the years ranging from 2.3% in 2011 to 12.7% in 2019 (Valentin-Domelier et al., 2011; Bouiller et al., 2016; Sauget et al., 2019). The rate detected in Spain in our work (3.9%) is low compared to the most recent data from France. However, a national surveillance of this clone incidence should be done over the years to evaluate its evolution tendency. The prevalence of MRSA-CC398 is much lower than MSSA-CC398's in blood culture, as in the previous studies above mentioned. Interestingly, MRSA-CC398 were detected in regions with high pig density (HPD), namely Cataluña and Aragón, confirming results of Ceballos and collaborators which demonstrated that high pig density leads to a significant increase in MRSA-CC398 in hospitals in Spain (Ceballos et al., 2019). By contrast, there is no obvious correlation between MSSA-CC398 occurrence and pig density since the isolates were detected in several regions with different pig density degrees. These results are in accordance with the fact that MSSA-CC398 (mostly) are *scn*-positive, though from humans, and that MRSA-CC398 are *scn*-negative belonging to the animal subclade.

This chapter revealed that the *spa*-types t571 and t1451 are the most involved in MSSA-CC398 invasive infections. According to previous reports, t571 was the *spa*-type most associated to invasive infections (Valentin-Domelier et al., 2011; Mediavilla et al., 2012; Bonnet et al., 2018). However, the *spa*-type t1451, was more generally associated to human colonization although it was scarcely reported in invasive infections (Valentin-Domelier et al., 2011). Indeed MSSA-t1451 was detected in healthy dog-owning household members in Spain, Australian pig veterinarians or homeless in Portugal (Gómez-Sanz et al., 2013a; Groves et al., 2016; Conceição et al., 2019). Its presence in bloodstream infections may be usual but, the lack of molecular typing could explain the few reports on this regard. Of note is the fact that more than 60% of MSSA-t1451 were MDR, and 15% harboured *eta* gene. Consequently, surveillance measures of MSSA-CC398 isolates especially of *spa*-types t571 and t1451 should be established in healthcare

institutions to control their dissemination. Concerning MRSA-CC398, t011 and t034 were the *spa*-types detected, corresponding to the most frequently associated to livestock (Guardabassi et al., 2007; Molla et al., 2012; Heikinheimo et al., 2016; Islam et al., 2017; Zarazaga et al., 2018).

On another hand, most of MSSA-CC398 isolates (72.9%) shared characteristics absent in the other clones detected, namely erythromycin and clindamycin-inducible resistance phenotype mediated by *erm*(T) gene, yet observed in the previous chapter and reported by Bonnet and collaborators (Bonnet et al., 2018). These properties may be established as a marker of MSSA-CC398 isolates.

LA-MRSA, especially of CC398 have been frequently related to any types of human infections (mastitis, SSTIS, etc) but in few cases to invasive infections (Crombé et al., 2013; Smith and Wardyn, 2015; Ceballos et al., 2019). Another lineage with clones in different evolutionary stages was identified among our *S. aureus* isolates. Indeed, MRSA-CC1 is a CA clone responsible for human infections, but it is increasingly spreading in livestock, especially in Spain (Lozano et al., 2012b; Benito et al., 2014b; Alba et al., 2015; Zarazaga et al., 2018; Elstrøm et al., 2019). Our two isolates MRSA-CC1 *scn*-positive, are thought to be human clones while the *scn*-negative are likely to be of animal origin. Furthermore, one of the MRSA-CC1, *scn*-positive was PVL-positive, which is a marker of CA isolates (Planet et al., 2015). Both isolates MRSA-CC1, *scn*-negative were detected in hospitals of HPD areas, contrarily to the others.

S. aureus of lineage CC398 is indeed an etiological agent of invasive infections in Spain, although its prevalence is low. MSSA-CC398 frequency of detection was not related to the pig density degree. *Spa*-types to t571 and t1451 were the most frequent among human-adapted MSSA-CC398 isolates. The detection rates of LA-MRSA-CC398 and LA-MRSA-CC1 was low and was correlated with high pig density (especially MRSA-CC398). The presence of MDR phenotypes and virulence factors like exfoliative toxins and PVL genes among invasive isolates is worrisome. Therefore, surveillance measures should be implemented in the healthcare institutions to limit the dissemination of such pathogens. Also, the phenotype erythromycin and clindamycin-inducible and the presence of *erm*(T) gene could be a marker for MSSA-CC398 detection.

CONCLUSIONS /CONCLUSIONES

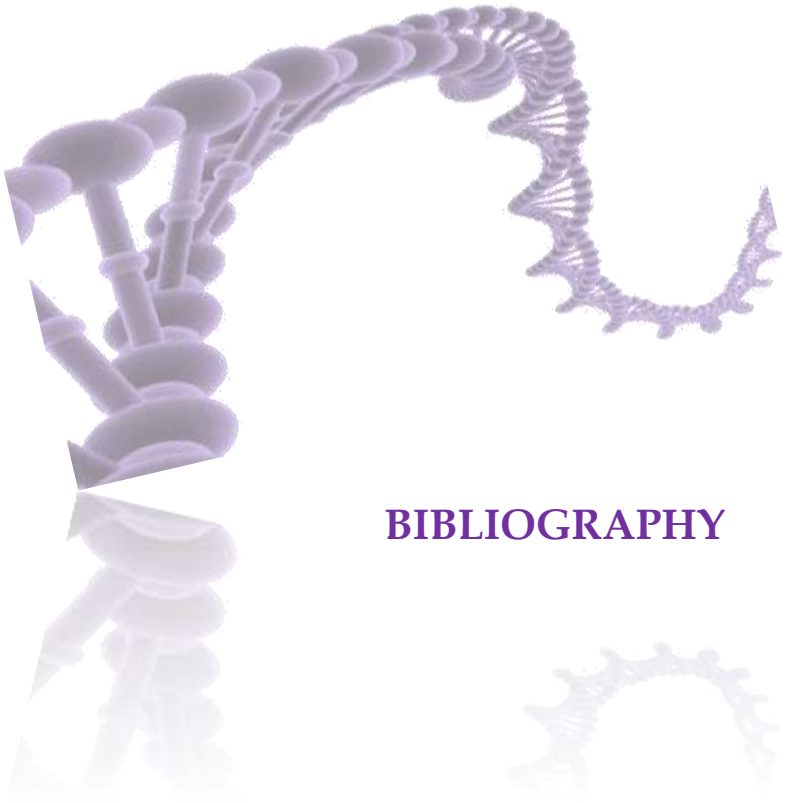
1. Based on our results, wild boars could be considered a reservoir of a large diversity of staphylococcal species, *S. aureus* (of lineage t3750-ST2328/CC133) and S. sciuri being the predominant species.
2. Wild boar staphylococcal isolates were mostly pan susceptible (CoPS (73,1%); CoNS (77,6%)), although MDR phenotypes were observed among CoNS.
3. Four *S. aureus* genetic lineages (ST1, ST816, ST1640 and ST1660) were detected from horse isolates, ST1640 being the predominant one.
4. All *S. aureus* isolates from horses, except those of lineage ST1640, carried the genes encoding the equid-adapted leukocidin (LukPQ) and the blocker of equine complement system activation (eqSCIN).
5. Hunting animals and horses intended for human consumption were frequently carriers of staphylococci with relevant antimicrobial resistance and virulence genes, which could have public health implications.
6. Food-producing animals studied in Spain and Senegal were frequently staphylococci nasal carriers; being *S. aureus* the predominant species (with genes encoding TSST, enterotoxins and PVL), although CoNS (mostly *S. simulans* and *S. sciuri*) were also detected.
7. The detection of the clone MRSA-USA300 (ST8, PVL-positive and *scn*-positive) in food-producing animals should be considered a public health concern.
8. CoNS species from wild, domestic and production animals could be an important source of antimicrobial resistant genes and potential ways of dissemination.
9. Pig-derived food were frequently contaminated by LA-MRSA-CC398 (mostly in products with skin), associated to *spa*-type t011. Moreover, virulent and/or invasive *S. aureus* MSSA-CC398, MRSA-CC8 and MSSA-ST45 were also found.
10. All LA-MRSA-CC398 isolates from pig-derived food were tetracycline resistant and *scn*-negative, and an MDR phenotype was detected in 82.6% of the isolates.
11. Regarding clinical isolates, MSSA-CC398 seems to be an emerging etiological agent in invasive infections among Spanish hospitals included in the multicentre study, representing 3.9% of *S. aureus* and 5.2% of MSSA. MRSA-CC398 was also detected, but with lower frequency (0.4% of *S. aureus*).
12. MSSA-CC398 isolates from invasive infections were mostly associated to *spa*-types t571 and t1451 (*scn*-positive), while LA-MRSA-CC398 were typed as t011 and t034 (*scn*-negative).

13. The resistance phenotype erythromycin-clindamycin-inducible mediated by *erm*(T) gene, was only detected among MSSA-CC398 isolates (72.9%), and may be a good marker for its detection.
14. There was no obvious correlation between MSSA-CC398 occurrence at hospital level and the pig density of the region where hospitals were located; on the contrary, MRSA-CC398 were only detected in hospitals of HPD regions. This fact suggests the human origin of SASM-CC398 and corroborates the animal origin of SARM-CC398.
15. The detection of *scn*-negative LA-MRSA-CC398 isolates in wild boar, pig-derived food and humans (invasive infections) reflects the capacity of spread and adaptation of this animal-related clone in different niches.

1. Los jabalíes pueden ser considerados un reservorio de diversas especies estafilocócicas, siendo *S. aureus* (del linaje t3750-ST2328/CC133) y *S. sciuri* las especies predominantes.
2. La mayoría de los aislados de *Staphylococcus* spp. de jabalíes fueron pan sensibles (SCoP (73,1%); SCoN (77,6%), aunque se detectaron fenotipos de multirresistencia entre los SCoN.
3. Se detectaron cuatro líneas genéticas entre los aislados de caballos (ST1, ST816, ST1640 y ST1660), siendo ST1640 la predominante.
4. Todos los aislados *S. aureus* de caballos, excepto los de la línea genética ST1640, portaban los genes codificantes de la leucocidina especifica de équidos (LukPQ) y del bloqueador de la activación del sistema complementario equino (eqSCIN).
5. Los animales de caza y los caballos destinados al consumo humano portaban frecuentemente estafilococos con genes de resistencia a antibióticos y de virulencia de especial relevancia, lo cual puede tener implicación en salud pública.
6. Los animales de producción estudiados en España y Senegal portaban con frecuencia cepas de estafilococo, siendo *S. aureus* (con relevantes genes de virulencia) la especie predominante. También se detectaron SCoN, destacando las especies *S. simulans* y *S. sciuri*.
7. La detección del clon SARM-USA300 (ST8, PVL-positivo y *scn*-positivo) en muestras nasales de animales de producción debe ser considerado como un riesgo de salud pública.
8. Los SCoN aislados de animales salvajes, animales domésticos y de producción pueden ser considerados importantes reservorios de genes de resistencia a antibióticos, pudiendo ser potenciales vías de diseminación de los mismos.
9. Los productos cárnicos derivados de cerdo estuvieron con frecuencia contaminados por SARM-CC398, principalmente del *spa*-tipo t011, y en menor medida por clones virulentos y/o invasivos SASM-CC398, SARM-CC8 y SASM-ST45.
10. Todos los aislados SARM-CC398 de productos derivados de cerdo fueron resistentes a tetraciclina y *scn*-negativos, y un 82,6% de dichos aislados presentó un fenotipo de multirresistencia.
11. En cuanto a los aislados clínicos, el clon SASM-CC398 parece ser agente etiológico emergente en infecciones invasivas en los hospitales españoles del

estudio multicéntrico; representando un 3,9% y un 5,2% de los aislados *S. aureus* y SASM, respectivamente. MRSA-CC398 fue también detectado, aunque con menor frecuencia (0,4% de *S. aureus*).

12. Los aislados SASM-CC398 de infecciones invasivas fueron mayoritariamente asociados a los *spa*-tipos t571 y t1451 (*scn*-positivos), mientras que los aislados SARM-CC398 fueron tipados como t011 y t034 (*scn*-negativos).
13. El fenotipo de resistencia a eritromicina y clindamicina-inducible mediada por el gen *erm*(T) fue detectado únicamente en los aislados SASM-CC398 (72.9%), y podría ser un buen marcador para la detección de ese clon.
14. No se observó una correlación entre la prevalencia de SASM-CC398 en hospitales y el nivel de densidad porcina de las provincias correspondientes; por el contrario, SARM-CC398 fue detectado exclusivamente en hospitales de zonas de alta densidad porcina. Esto podría indicar el posible origen humano de SASM-CC398 y corroborar el origen animal de SARM-CC398.
15. La detección del clon asociado al ganado SARM-CC398 en animales salvajes, productos cárnicos de origen porcino y en humanos refleja una gran capacidad de diseminación y de adaptación en diferentes nichos.



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ANNEX

ANNEX

Annex: primers and PCR conditions used in this thesis

Primers Name and sequence (5´→3´)	anplicon size(bp)	Reference
Molecular typing		
spa *(S. aureus)* F: AGACGATCCTTCGGTGAGC R: GCTTTTGCAATGTCATTTACTG	Variable	Shopsin et al., 1999
spa *(S. pseudintermedius)* F : AACCTGCGCCAAGTTTCGATGAAG R : CGTGGTTTGCTTTAGCTTCTTGGC	Variable	Moodley et al., 2009
CC398 F: AGGGTTTGAAGGCGAATGGG R: CAGTATAAAGAGGCGAATGGG	296	Stegger et al., 2011
MLST *(S. aureus)*		
arcC F: TTGATTCACCAGCGCGTATTGTC R: AGGTATCTGCTTCAATCAGCG	456	Enright at al., 2000
aroE F: ATCGGAAATCCTATTTCACATTC R: GGTGTTGTATTAATAACGATATC	456	Enright at al., 2000
glpF F: CTAGGAACTGCAATCTTAATCC R: TGGTAAAATCGCATGTCCAATTC	465	Enright at al., 2000
gmk F: ATCGTTTTATCGGGACCATC R: TCATTAACTACAACGTAATCGTA	429	Enright at al., 2000
pta F: GTTAAAATCGTATTACCTGAAGG R: GACCCTTTTGTTGAAAAGCTTAA	474	Enright at al., 2000
tpi F: TCGTTCATTCTGAACGTCGTGAA R: TTTGCACCTTCTAACAATTGTAC	402	Enright at al., 2000
yqil F: CAGCATACAGGACACCTATTGGC R: CGTTGAGGAATCGATACTGGAAC	516	Enright at al., 2000
MLST *(S. pseudintermedius)*		
tuf F: CAATGCCACAAACTCG R: GCTTCAGCGTAGTCTA	500	Bannoehr et al., 2007
Cpn60 F: GCGACTGTACTTGCACAAGCA R: AACTGCAACCGCTGTAAATG	552	Bannoehr et al., 2007

pta F: GTGCGTATCGTATTACCAGAAGG R: GCAGAACCTTTTGTTGAGAAGC	570	Bannoehr et al., 2007
purA F: GATTACTTCCAAGGTATGTTT R: TCGATAGAGTTAATAGATAAGTC	490	Solyman et al., 2013
fdh F: TGCGATAACAGGATGTGCTT R: CTTCTCATGATTCACCGGC	408	Solyman et al., 2013
ack F: CACCACTTCACAACCCAGCAAACT R: AACCTTCTAATACACGCGCACGCA	680	Solyman et al., 2013
agr		
***agr* I** F: GTCACAAGTACTATAAGCTGCGAT R: ATGCACATGGTGCACATGC	440	Shopsin et al., 2003
***agr* II** F: GTATTACTAATTGAAAAGTGCCATAGC R: ATGCACATGGTGCACATGC	572	Shopsin et al., 2003
***agr* III** F: CTGTTGAAAAAGTCAACTAAAAGCTC R: ATGCACATGGTGCACATGC	406	Shopsin et al., 2003
***agr* IV** F: CGATAATGCCGTAATACCCG R: ATGCACATGGTGCACATGC	656	Shopsin et al., 2003
nuc (*S. delphini* group A) F: TGAAGGCATATTGTAGAACAA R: CGRTACTTTTCGTTAGGTCG	661	Sasaki et al., 2010
nuc (*S. delphini* group B) F: GGAAGRTTCGTTTTTCCTAGAC R: TATGCGATTCAAGAACTGA	1135	Sasaki et al., 2010

Primers Name and sequence (5´→3´)	anplicon size(bp)	Reference
Resistance genes		
mecA F: GGGATCATAGCGTCATTATTC R: AACGATTGTGACACGATAGCC	527	CRL-AR, 2009
blaZ F: CAGTTCACATGCCAAAGAG R: TACACTCTTGGCGGTTTC	772	Schnellmann et al., 2006
Tetracycline		
***tet*(K)** F: TTAGGTGAAGGGTTAGGTCC R: GCAAACTCATTCCAGAAGCA	697	Aarestrup et al., 2000
***tet*(M)** F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	576	Aarestrup et al., 2000
***tet*(L)** F: CATTTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCGG	456	Aarestrup et al., 2000
Macrolides/ lincosamides		
***erm*(A)** F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	645	Sutcliffe et al., 1996
***erm*(B)** F: GAAAAGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTA	639	Sutcliffe et al., 1996
***erm*(C)** F: TCAAAACATAATATAGATAAA R: GCTAATATTGTTTAAATCGTCAAT	642	Sutcliffe et al., 1996
***erm*(F)** F: CGGGTCAGCACTTTACTATTG R: GGACCTACCTCATAGACAAG	466	Chung et al., 1999
***erm*(T)** F: CCGCCATTGAAATAGATCCT R: TTCTGTAGCTGTGCTTTCAAAAA	200	Gómez-Sanz et al., 2010
***erm*(Y)** F : AGGCCCCTTTTAAAGACGAAGGCA R : GGCGCGATTGTTCATTTTAAGGCCC	320	Gómez-Sanz et al., 2010
***erm*(43)** F: TACAGC AGATGATAACATTG R: TGTTGTTTCGATATTTTA TTTAAG	609	Schwendener and Perreten, 2012

***msr*(A)/*msr*(B)** F : GCAAATGGTGTAGGTAAGACAACT R : ATCATGTGATGTAAACAAAAT	399	Wondrack et al., 1996
***mph*(C)** F: ATGACTCGACATAATGAAAT R: CTACTCTTTCATACCTAACTC	900	Schnellmann et al., 2006
***lnu*(A/A')** F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGATC	322	Lina et al., 1999
***lnu*(B)** F: CCTACCTATTGTTTGTGGAA R: ATAACGTTACTCTCCTATTC	944	Bozdogan et al., 1999
***vga*(A)** F: AGTGGTGGTGAAGTAACACG R: GGTTCAATACTCAATCGACTGAG	1264	Lozano et al., 2012
Aminoglycosides		
***aac*(6´)-Ie-*aph*(2´´)-Ia** F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	220	Van de Klundert et al., 1993
***ant*(4´)-Ia** F: GCAAGGACCGACAACATTTC R: TGGCACAGATGGTCATAACC	165	Van de Klundert et al., 1993
ant(6)-Ia F: ACT GGC TTA ATC AAT TTG GG R: GCC TTT CCG CCA CCT CAC CG	597	Clark et al., 199
str F: TATTGCTCTCGAGGGTTC R: CTTTCTATATCCATTCATCTC	646	Schnellmann et al., 2006
Sulfamethoxazole trimethoprim		
***dfr*A** F: CCTTGGCACTTACCAAATG R: CTGAAGATTCGACTTCCC	374	Schnellmann et al., 2006
dfrD F: TTCTTTAATTGTTGCGATGG R: TTAACGAATTCTCTCATATATATG	582	Schnellmann et al., 2006
dfrK F: GAGAATCCCAGAGGATTGGG R: CAAGAAGCTTTTCGCTCATAAA	423	Gómez-Sanz et al., 2010
dfrG TCGGAAGAGCCTTACCTGACAGAA CCCTTTTTGGGCAAATACCTCATTCCA	323	Gómez-Sanz et al., 2010
mupA F: CCCATGGCTTACCAGTTGA R: CCATGGAGCACTATCCGAA	419	Udo et al., 2003

Phenicol/ oxazolidinone		
fexA F: GTACTTGTAGGTGCAATTACGGCTGA R: CGCATCTGAGTAGGACATAGCGTC	1272	Kehrenberg et al., 2005
fexB F: TTCCCACTATTGGTGAAAGGAT R: GCAATTCCCTTTTATGGACGTT	816	Liu et al., 2012
cat_{pC194} F: CGACTTTTAGTATAACCACAGA R: GCCAGTCATTAGGCCTAT	570	Schnellmann et al., 2006
cat_{pC221} F: ATTTATGCAATTATGGAAGTTG R: TGAAGCATGGTAACCATCAC	434	Schnellmann et al., 2006
cat_{pC223} F: GAATCAAATGCTAGTTTTAACTC R: ACATGGTAACCATCACATAC	283	Schnellmann et al., 2006
optrA F: AGGTGGTCAGCGAACTAA R: ATCA ACTGTTCCCATTCA	1395	Wang et al., 2015

Primers Name and sequence (5´→3´)	anplicon size(bp)	Reference
Virulence genes		
S. aureus		
***lukF*-PV/*lukS*-PV** F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAAGTGTATTGGATAGCAAAAGC	443	Lina *et al.*, 1999
lukPQ F: CCTGATGGTGAACTGTCAGCGCAT R: TTGTGTGCCTCGACACCCCAAC	939	Koop et al., 2017
hla F: CTGATTACTATCCAAGAAATTCGATTG R: CTTTCCAGCCTACTTTTTTATCAGT	443	Jarraud *et al.*, 2002
hlb F: GTGCACTTACTGACAATAGTGC R: GTTGATGAGTAGCTACCTTCAGT	309	Jarraud *et al.*, 2002
hld F: AAGAATTTTTATCTTAATTAAGGAAGGAGTG R: TTAGTGAATTTGTTCACTGTGTCGA	111	Jarraud *et al.*, 2002
hlg F: GTCAYAGAGTCCATAATGCATTTAA R: CACCAAATGTATAGCCTAAAGTG	535	Jarraud *et al.*, 2002
***hlg*$_v$** F: GACATAGAGTCCATAATGCATTYGT R: ATAGTCATTAGGATTAGGTTTCACAAAG	390	Jarraud *et al.*, 2002
eta F: ACTGTAGGAGCTAGTGCATTTGT R: TGGATACTTTTGTCTATCTTTTTCATCAAC	190	Jarraud *et al.*, 2002
etb F: CAGATAAAGAGCTTTATACACACATTAC R: AGTGAACTTATCTTTCTATTGAAAAACACTC	612	Jarraud *et al.*, 2002
tst F: TTCACTATTTGTAAAAGTGTCAGACCCACT R: TACTAATGAATTTTTTTATCGTAAGCCCTT	180	Jarraud *et al.*, 2002
Enterotoxins		
sea F : ATGGTTATCAATGTGCGGGTGIIIIICCAAACAAAAC R : TGAATACTGTCCTTGAGCACCAIIIIIATCGTAATTAAC	344	Hwang et al., 2007
seb F : TGGTATGACATGATGCCTGCACIIIIIGATAAATTTGAC R : AGGTACTCTATAAGTGCCTGCCTIIIIIACTAACTCTT	196	Hwang et al., 2007
sec F : GATGAAGTAGTTGATGTGTATGGATCIIIIIACTATGTAAAC R : AGATTGGTCAAACTTATCGCCTGGIIIIIGCATCATATC	399	Hwang et al., 2007

sed		
F : CTGAATTAAGTAGTACCGCGCTIIIIIATATGAAAC	451	Hwang et al., 2007
R : TCCTTTTGCAAATAGCGCCTTGIIIIIGCATCTAATTC		
see		
F : CGGGGGTGTAACATTACATGATIIIIICCGATTGACC	286	Hwang et al., 2007
R : CCCTTGAGCATCAAACAAATCATAAIIIIICGTGGACCCTTC		
seg		
F : ATAGACTGAATAAGTTAGAGGAGGTIIIIIGAAGAAATTATC	594	Hwang et al., 2007
R : TTAGTGAGCCAGTGTCTTGCIIIIIAATCTAGTTC		
seh		
F : CATTCACATCATATGCGAAAGCAGIIIIITTACACG	218	Hwang et al., 2007
R : CTTCTGAGCTAAATCAGCAGTTGCIIIIITTACTCTC		
sei		
F : AGGCGTCACAGATAAAAACCTACCIIIIICAAATCAACTC	154	Hwang et al., 2007
R : ACAAGGACCATTATAATCAATGCCIIIIITATCCAGTTTC		
sej		
F : TGTATGGTGGAGTAACACTGCATGIIIIIAATCAACTTTATG	102	Hwang et al., 2007
R : CTAGCGGAACAACAGTTCTGATGCIIIIIATCCATAAAT		
sek		
F : GTGTCTCTAATAATGCCAGCGCTIIIIICGATATAGG	282	Hwang et al., 2007
R : CGTTAGTAGCTGTGACTCCACCIIIIITGTATTTAG		
sel		
F : ATTCACCAGAATCACACCGCTIIIIITACTCGTA	469	Hwang et al., 2007
R : GTGTAAAATAAATCATACGAGIIIIIAGAACCATCATTC		
sem		
F : CGCAACCGCTGATGTCGGIIIIITGAATCTTAGG	572	Hwang et al., 2007
R : CAGCTTGTCCTGTTCCAGTATCIIIIIAGTCATAAG		
sen		
F : CATGCTTATACGGAGGAGTTACGIIIIITGATGGAAATC	103	Hwang et al., 2007
R : AACCTTCTTGTTGGACACCATCIIIIIATACATTAACGC		
seo		
F : GTGGAATTTAGCTCATCAGCGATTTCIIIIIAATTTCTAGG	116	Hwang et al., 2007
R : GTACAGGCAGTATCCACTTGATGCIIIIIATGACAATGTGC		
sep		
F : ATCATAACCAACCGAATCACCAGIIIIIGGGTGAAACTC	574	Hwang et al., 2007
R : GTCTGAATTGCAGGGAACTGCIIIIIGCAATCTTAG		
seq		
F : GGTGGAATTACGTTGGCGAATCAIIIIITAGATAAACC	330	Hwang et al., 2007
R : CTCTGCTTGACCAGTTCCGGTGIIIIICAAATCGTATG		
ser		
F : TTCAGTAAGTGCTAAACCAGATCCIIIIICTGGAGAATTG	368	Hwang et al., 2007
R : CTGTGGAGTGCATTGTAACGCCIIIIIATATGCAAACTCC		
seu		
F : ATGGCTCTAAAATTGATGGTTCTAIIIIITTAAAAACAG	410	Hwang et al., 2007
R : GCCAGACTCATAAGGCGAACTAIIIIITTCATATAAA		

femA F : ACAGCTAAAGAGTTTGGTGCCTIIIIIGATAGCATGC R : TTCATCAAAGTTGATATACGCTAAAGGTIIIIICACACGGTC	723	Hwang et al., 2007
Immune Evasion Cluster (IEC)		
scn F: AGCACAAGCTTGCCAACATCG R: TTAATATTTACTTTTTAGTGC	257	Van Wamel et al., 2006
chp F: TTTACTTTTGAACCGTTTCCTAC R: CGTCCTGAATTCTTAGTATGCATATTCATTAG	366	Van Wamel et al., 2006
sak F: AAGGCGATGACGCGAGTTAT R: GCGCTTGGATCTAATTCAAC	223	Van Wamel et al., 2006
sea F: AGATCATTCGTGGTATAACG R: TTAACCGAAGGTTCTGTAGA	344	Van Wamel et al., 2006
sep F: AATCATAACCAACCGAATCA R: TCATAATGGAAGTGCTATAA	196	Van Wamel et al., 2006
Integrase		
Sa3int F: GAAAAACAAACGGTGC TA R: TTATTGACTCTACAGGCTGA	475	Goerke et al., 2009
S. pseudintermedius		
lukF-I F: CCTGTCTATGCCGCTAATCAA R: AGGTCATGGAAGCTATCTCGA	572	Futagawa et al., 2004a
lukS-I F: TGTAAGCAGCAGAAAATGGGG R: GCCCGATAGGACTTCTTACAA	503	Futagawa et al., 2004a
***sec*$_{canine}$** F: GGGAAGCTITGTAATITTTGATATTCGCACT R: CCCGGATCCTATCAAAATCGGATTAACA	425	Futagawa et al., 1997
se-int F: GCAAGCATATCATTACATTTG R: ACTTGATATACCCTGTTTCGT	147	Futagawa et al., 2004b
expA F: GTKTTAATTGGWAAAAATACA R: ATNCCWGAKCCTGAATTWCC	413	Futagawa et al., 2009
expB F: GGGCATGCACATATGATGAAGCC R: CCAGATCTATCTTCTGATTCAGC	740	Ivori et al., 2010
siet F: ATGGAAAATTTAGCGGCATCTGG R: CCATTACTTTTCGCTTGTTGTGC	359	Lautz et al., 2006

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