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TESIS DOCTORAL

Título
***Staphylococcus aureus* and *Staphylococcus pseudintermedius* in animals: molecular epidemiology, antimicrobial resistance, virulence and zoonotic potential**
Autor/es
Elena Gómez Sanz
Director/es
Carmen Torres Manrique y Mirian Zarazaga Chamorro
Facultad
Facultad de Ciencias, Estudios Agroalimentarios e Informática
Titulación
Departamento
Agricultura y Alimentación
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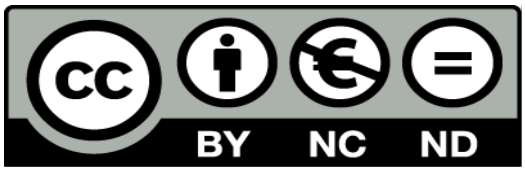

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UNIVERSIDAD DE LA RIOJA
Dpto. Agricultura y Alimentación
Área de Bioquímica y Biología Molecular

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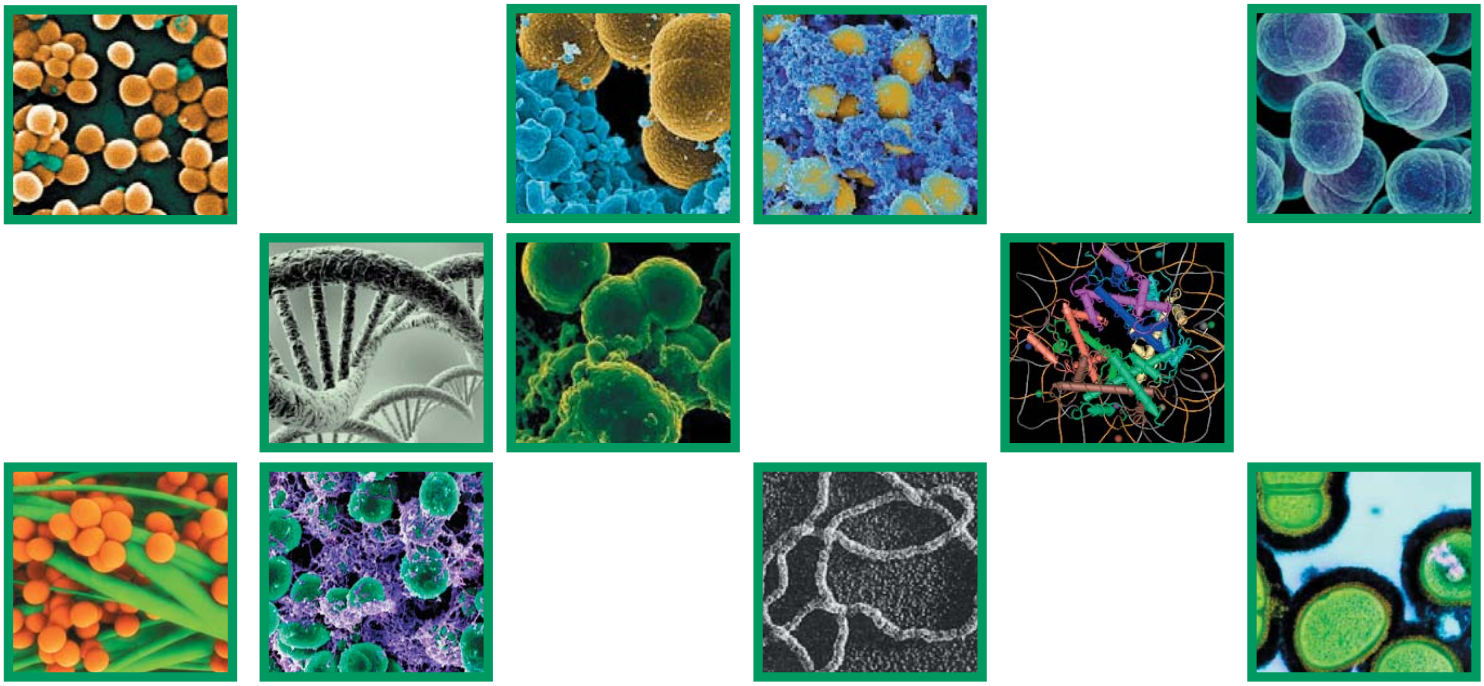

Elena **G**ómez **S**anz

Tesis Doctoral con Mención Internacional

Logroño, 2013

UNIVERSIDAD DE LA RIOJA
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Staphylococcus aureus y *Staphylococcus pseudintermedius* en animales: epidemiología molecular, resistencia a antimicrobianos, virulencia y potencial zoonótico.

Memoria presentada por **ELENA GÓMEZ SANZ** para optar al título de Doctora con la mención internacional por la Universidad de La Rioja.

Logroño, Mayo 2013

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

Dra. **MYRIAM ZARAZAGA CHAMORRO**, Profesora Titular del Área de Bioquímica y Biología Molecular de la Universidad de La Rioja.

Por la presente declaran que,

La memoria titulada **"*Staphylococcus aureus* and *Staphylococcus pseudintermedius* in animals: molecular epidemiology, antimicrobial resistance, virulence and zoonotic potential"**, que presenta Dña. ELENA GÓMEZ SANZ, Licenciada en Biología, 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,

Lo que hace constar en Logroño, a 20 de Mayo de 2013.

Fdo.: Carmen Torres Manrique Fdo.: Myriam Zarazaga Chamorro

"La ciencia es parte de nuestra naturaleza en la vida"

"Die Wissenschaft ist Teil unserer Natur im Leben"

"Science is part of our nature in life"

Charles Darwin (1809-1882)

Al final de un camino, como en la vida, sólo permanecen los buenos recuerdos, y no tengo más que palabras de agradecimiento a tod@s y cada un@ de l@s que, de una manera u otra, habéis formado parte de éste.

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"It doesn't matter if an animal can reason. It matters only that it is capable of suffering and that is why I consider it my neighbor."

Albert Schwitzer (1875-1965)
Premio Novel de la Paz, 1952

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la felicidad es el esfuerzo y la ilusión que pones en conseguir un sueño, el mio; verte sonreir

Txapas

A mis padres,

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DISCUSSION

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Abreviations

agr	accessory gene regulator
AIP	Autoinducing peptide
AMR	Antimicrobial resistance
att	attachment site
bp	base pair
BURP	Based Upon Repeat Pattern
CA-MRSA	Community-acquired methicillin-resistant *S. aureus*
CC	Clonal complex
CHIPs	chemotaxis inhibitor proteins
CoNS	Coagulase negative staphylococci
CoPS	Coagulase positive staphylococci
CWA	Cell wall associated proteins
DHFR	Dihidrofolate reductase
DNA	Deoxyribonucleic acid
dso	Double strand origin of replication
EARSS	European Antimicrobial Resistance Surveillance
EFSA	European Food Safety Authority
EHSG	Extended Host Spectrum Genotype
EMA	European Medicines Agency
ET	Exfoliatin toxins
EUMS	European Union Member States
HA-MRSA	Hospital-acquired methicillin-resistant *S. aureus*
hl	Haemolysin genes
IEC	Immune Evasion Cluster
IR	Inverted repeats
IS	Insertion sequence
Kb	Kilo base
L	Lincosamide resistance phenotype
LA-MRSA	Livestock-associated methicillin-resistant *S. aureus*
LS_A	Lincosamide and Streptogramin A resistance phenotype
Luk	Leukocidin toxins
MDR	Multidrug resistant
MGEs	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MLS_B	Macrolides Lincosamides and Streptogramin B resistance phenotype

MLST	Multilocus Sequence Typing
MRCoNS	Methicillin-resistant coagulase negative staphylococci
MRCoPS	Methicillin-resistant coagulase positive staphylococci
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *S. aureus*
MRSP	Methicillin-resistant *S. pseudintermedius*
MS	Macrolides Streptogramins resistance phenotype
MSSA	Methicillin-susceptible *S. aureus*
MSSP	Methicillin-susceptible *S. pseudintermedius*
PBP	Penicillin Binding Protein
PBP2'/PBP2a	Modified Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PTSAg	Pyrogenic toxin superantigens
PVL	Panton Valentine leukocidin
QRDR	Quinolone Resistance Determining Region
RCR	Rolling Circle Replication
Rep	Replication initiation protein
rRNA	Ribosomal ribonucleic acid
S_A	Streptogramins A resistance phenotype
SAK	staphylokinase
SaPIs	*S.aureus* Pathogenicity Islands
SARM	*S. aureus* resistente a meticilina
SARM-AG	*S. aureus* resistentes a meticilina asociado a ganado
SASM	*S. aureus* sensibles a meticilina
S_B	Streptogramins B resistance phenotype
SCC	Staphylococcal Cassette Chromosome
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SCIN	staphylococcal complement inhibitor
SDM	Sited directed mutagenesis
SE	Staphylococcal enterotoxins
SIG	*S. intermedius* group
SLV	Single Locus Variant
Spa	Staphylococcal protein A gene
spp	species

SPRM	*S. pseudintermedius* resistente a meticilina
sso	Single strand origin of replication
ssoA	Single strand origin of replication type A
SSSS	Staphylococcal scalded skin syndrome
ST	Sequence Type
Tn	Transposon
Tnp	Transposase
tRNA	transference ribonucleic acid
TSST	Toxic shock syndrome toxin
US/USA	United States of America

Summary

ABSTRACT

Staphylococcus aureus is a common colonizer of the skin and mucous of humans and other mammals while dogs are the major host for *Staphylococcus pseudintermedius*. The epidemiology and ecology of these bacterial species in animals has gained interest in the last years, not only for their importance in veterinary medicine, due to the increment of infectious processes caused by these pathogens (especially by methicillin resistant strains and other antibiotics of interest), but for its increasingly evidenced zoonotic potential. Since 2005, we are facing the spread of livetock-associated methicillin-resistant *S. aureus* (LA-MRSA) ST398, especially as colonizer of pigs, and the emergence of multidrug resistant methicillin resistant *S. pseudintermedius* strains (MRSP) in dogs. On the other hand, methicillin-susceptible strains (MSSA and MSSP) play an essential role in the evolution of different genetic lineages.

In the first chapter of this thesis, the nasal presence and characterization of *S. aureus* and *S. pseudintermedius* strains of dogs, cats and their owners from 43 households were investigated. A total of 42% of owners and 12% of pets were *S. aureus* carriers. The corresponding rates for *S. pseudintermedius* were 22.7 and 4.5 for dogs and humans, respectively. Five cases of direct inter-species transmission (same strain on owner and pet) and 5 cases of possible indirect transmission (*S. aureus* in dogs or *S. pseudintermedius* in humans) were detected. The carriage dynamics of 7 of these residences along one year was investigated and revealed that dogs are normally transient *S. aureus* carriers and that dog owners may be persistently colonized by *S. pseudintermedius*. *S. pseudintermedius* isolates were more clonally diverse and dynamic in time than *S. aureus*. Dogs seem to play a relevant role in the staphylococcal species distribution and colonization of in-contact individuals. The detection of several MSSA ST398 from humans with not contact to livestock supports the hypothesis that LA-MRSA ST398 may have emerged from humans.

In a second chapter, kennel dogs were investigated for the presence and characterization of both bacterial species. High relative MRSP (8%), moderate MSSP (15.3%) and high MSSA (24.5%) nasal carriage rates were revealed. The predominance of multidrug resistant MRSP ST71 among the MRSP lineages detected reflects the success of this clone also in Spain. Distinct phenotypic and genotypic characteristics were revealed among MRSP and MSSP strains. The detection of traditionally human and livestock associated *S. aureus* suggests the ability of dogs to carry common *S. aureus* lineages of in-contact environments. MSSA ST398 was the predominant lineage detected, suspecting a more extended host spectrum of this sub-lineage

than considered. The comparative analysis of the *S. aureus* and *S. pseudintermedius* isolates recovered from household individuals and pound dogs revealed higher levels of antimicrobial resistance and higher virulence gene contend in *S. aureus* isolates from household individuals.

In the third chapter, the presence and characterization of MRSA was investigated in other animal species (pigs at slaughter and hospitalised equines). Half (49%) of tested piglets and 21% of finishing-pigs carried MRSA. High rate of MRSA ST398 (91%) was detected among investigated animals, while a novel ST1379, enclosed within the traditional bovine associated lineage CC97, was detected in 9% of strains. The antimicrobial resistance profile and the clonal distribution of isolates were related to the age of animals investigated. Eight coagulase positive staphylococci from hospitalized equines were additionally characterized. Four strains were multidrug resistant MRSA ST398, revealing its presence in Spain in this animal species. The first detection of a *S. pseudintermedius* ST68 strain in horses was also reported which, regardless of being multidrug resistant and *mec*A positive, presented a clear β-lactams susceptible phenotype.

In the final chapter, several human and porcine *erm*(T) positive LA-MRSA and MSSA ST398 isolates were investigated for the *erm*(T) genetic environment and location in the genome. Four novel distinct plasmids, all carrying a cadmium resistance operon (*cadDX*) were detected. A small plasmid, pUR3912, which was also co-located within the chromosomal DNA of the same strain, most probably IS*431*-mediated, was revealed in MSSA ST398 of human origin. The three other plasmids, from LA-MRSA ST398, carried multiple antimicrobial resistance genes in addition to copper resistance determinants (*copA*, *mco*). The physical linkage of antimicrobial resistance genes and genes that confer resistance to heavy metals may facilitate their persistence and dissemination. In addition, the first description of the *fexA* gene and the transposon Tn*558* in *S. pseudintermedius* is detected. A novel variant (*fexAv*) that only confers chloramphenicol resistance was revealed and evidenced the presence of 2 punctual substitutions that seem to be implicated in the absence of florfenicol resistance.

This thesis studies the population structure of *S. aureus* and *S. pseudintermedius* and the dynamics of colonization in different hosts. Direct contact between animals and humans is a relevant factor to take into account to understand the epidemiology and evolution of both bacterial species. Based on the high capacity of both microorganisms to acquire, maintain and (in *S. aureus*) mobilize antimicrobial resistance genes, further molecular surveillance is warranted to monitor their progress over time.

RESUMEN

Staphylococcus aureus es colonizador común de la piel y mucosas del hombre y otros animales y el perro es el hospedador habitual de *S. pseudintermedius.* Ambas especies son también importantes patógenos oportunistas. La epidemiología y ecología de estas especies bacterianas en animales ha cobrado gran interés en los últimos años no solamente por su importancia en medicina veterinaria, debido al incremento de procesos infecciosos producidos por estos patógenos (especialmente por cepas resistentes a meticilina y otros antibióticos de interés), sino por su cada vez más demostrado potencial zoonótico. Desde el año 2005 asistimos a una gran diseminación de *S. aureus* resistente a meticilina asociado a ganado (SARM-AG) ST398, especialmente como colonizador en cerdos y a la emergencia de cepas *S. pseudintermedius* resistente a meticilina (SPRM) y multirresistente en perros. Por otro lado, las cepas sensibles a meticilina juegan un papel primordial en la evolución de las distintas líneas genéticas.

En el primer capítulo de esta tesis, se investigó la presencia y caracterización de cepas *S. aureus* y *S. pseudintermedius* en fosas nasales de perros, gatos y sus dueños de 43 hogares. El 42% de los dueños y el 12% de las mascotas resultaron ser portadores de *S. aureus*. En el caso de *S. pseudintermedius*, los porcentajes observados fueron 22,7 y 4,5 para perros y humanos respectivamente. Se detectaron 5 casos de transmisión directa inter-especies (misma cepa en dueño y mascota) y 5 casos de posible transmisión indirecta (*S. aureus* en perros o *S. pseudintermedius* en humanos). En 7 de estos hogares se estudió el estado de portador de los individuos a lo largo de un año observando que los perros son normalmente portadores esporádicos de *S. aureus* y que sus dueños pueden estar colonizados de manera persistente por *S. pseudintermedius*. Esta especie bacteriana presentó más diversidad clonal y más dinamismo en el tiempo que *S. aureus*. Los perros parecen jugar un papel relevante en la distribución de especies de estafilococos en los individuos en contacto. La detección de varias cepas SASM ST398 en humanos sin contacto con animales de granja podría apoyar la hipótesis de que SARM-AG ha emergido de humanos.

En un segundo capítulo, se estudió la presencia y caracterización de estas especies de estafilococos en perros de perrera detectándose tasas altas relativas de SPRM (8%), moderadas de SPSM (15.3%) y elevadas de SASM (24.5%). Cabe destacar la detección y predominancia de SPRM ST71. Se observaron características distintivas entre las cepas SPRM y SPSM. La detección de *S. aureus* de linajes tradicionalmente asociados a humanos y a animales de producción

sugiere la habilidad de los perros para ser portadores de *S. aureus* presentes en ambientes en contacto. SASM ST398 fue predominante, lo que hace sospechar de un mayor espectro de hospedador de este sub-linaje de lo esperado. El análisis comparativo de las cepas procedentes de individuos de hogares y las aisladas de perros de perrera reveló mayores tasas de resistencia y de genes de virulencia en *S. aureus* de individuos procedentes de hogares.

En el tercer capítulo, se investigó la presencia y caracterización de SARM en otras especies animales (cerdos de matadero y caballos hospitalizados). Se aisló SARM en el 49% de los lechones y en el 21% de cerdos adultos. Se detecta una elevada prevalencia (91%) de ST398 entre los aislados SARM, mientras que el 9% restante presentó una nueva secuencia tipo, ST1379, perteneciente al linaje tradicionalmente asociado a bovino, CC97. Se observan diferencias en cuanto al perfil de resistencia y a la distribución clonal entre las cepas aisladas de cerdos adultos y de lechones. Se caracterizaron también 8 *Staphylococcus* coagulasa positiva de equinos hospitalizados. Cuatro cepas fueron SARM ST398 mulirresistentes, siendo su primera descripción en España en caballos. Así mismo, se detecta una cepa *S. pseudintermedius* ST68 por primera vez en caballo. Esta cepa, a pesar de presentar el gene *mec*A, exhibía un fenotipo claro de sensibilidad a β-lactámicos.

En el último capítulo se investigó el entorno genético y localización del gen *erm*(T) en varias cepas SARM-AG ST398 y SASM ST398. Se describieron 4 plásmidos nuevos, todos ellos portadores del operón de resistencia cadmio (*cadDX*). Uno de estos plásmidos, pUR3912 de pequeño tamaño, se localizó también integrado en el cromosoma del mimo aislado (SASM ST398 de origen humano). Los otros 3 plásmidos, de cepas SARM-AG ST398, fueron multi-resistentes y contenían también genes de resistencia a cobre (*cop*A, *mco*). La asociación de genes de resistencia a antimicrobianos y a metales en los mismos elementos genéticos puede facilitar su selección, persistencia y diseminación. Se detecta además por primera vez el gen *fexA* y el transposón Tn*558* en *S. pseudintermedius.* Así mismo, se describe por primera vez una variante del *fexA* (*fexAv*) que confiere sólo resistencia cloranfenicol y que presenta dos mutaciones puntuales que parecen estar implicadas en la ausencia de resistencia a florfenicol.

Este trabajo estudia la estructura poblacional de *S. aureus* y *S. pseudintermedius* y su dinámica de colonización en diferentes hospedadores. El contacto directo entre animales y humanos es un factor a tener en cuenta para entender la epidemiología y evolución de ambas especies bacterianas. La elevada capacidad de ambos microorganismos para adquirir, mantener y (en *S. aureus*) movilizar genes de resistencia a antimicrobianos hace esencial continuar con estudios de vigilancia para controlar su evolución en el tiempo.

Introduction

1. STAPHYLOCOCCI

1.1. GENERAL CHARACTERISTICS AND CLASSIFICATION

Staphylococci are Gram-positive bacteria of 0.8-1 mm in diameter, non-motile and non-sporulating, which grow in pairs or clusters similar to grapes (**Fig. 1**). Staphylococci present low guanine-cytosine content. These microorganisms are mesophiles, with optimum growth temperature between 35 and 37° C, aerobic or facultative anaerobic and show a respiratory and fermentative metabolism. The genus *Staphylococcus* belongs to the family Staphylococcaceae, which includes 47 species and 24 subspecies to date (http://www.bacterio.net, last updated in April 2013).

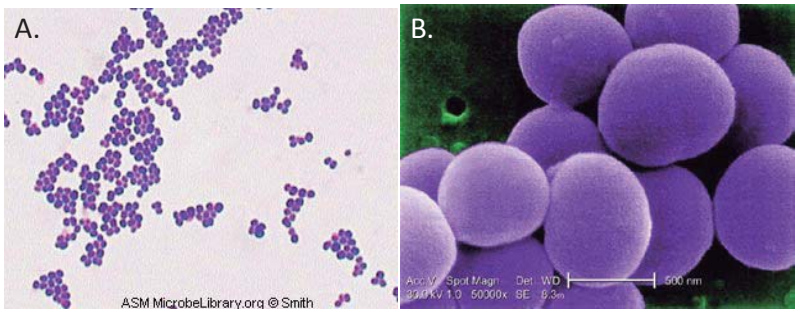


Figure 1. Morphology and clustering of staphylococci. **A.** Gram tincion that shows the clusters of *S. aureus* violet colour the due to the retention of crystal violet primary stain. **B.** Scanning electron micrograph (http://phil.cdc.gov).

Staphylococci are divided in two groups depending on their ability to coagulate citrate serum, for which the enzyme coagulase is necessary. The staphylococcal species that produce this enzyme are termed coagulase-positive staphylococci (**CoPS**), and are as follows: *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Staphylococcus intermedius*, *Staphylococcus delphini*, *Staphylococcus schleiferi* subspecies *coagulans*, *Staphylococcus hyicus* and *Staphylococcus lutrae*. The rest of species of the genus are designated coagulase-negative staphylococci (**CoNS**). CoNS count for a long list of species with marked host specificity, frequently referred to as commensals. However, they are by far the most common cause of bacteremia related to indwelling devices and other nosocomial diseases (Huebner et al., 1999). In addition, CoNS are frequently found in bovine mastitis, especially *Staphylococcus chromogenes* and *Staphylococcus epidermidis*. *S. epidermidis* is the CoNS species with major significance in human medicine.

Staphylococcus aureus

S. aureus is the main species of the genus. It is an opportunistic pathogen commonly found as part of the normal microbiota of humans and many animal species, especially mammals and birds. The most common site of colonization is the anterior of the nasal passages, but it may also colonize the skin, the oropharynx, vagina, axillae, perineum and the intestinal tract (Wertheim et al., 2005).

Staphylococcus intermedius group (SIG)

The *S. intemedius* group (SIG) compile the three closely related coagulase-positive bacterial species: *S. intermedius*, *S. pseudintermedius* and *S. delphini.*

Staphylococcus intermedius was first described by (Hajek et al., 1976) based on isolates from pigeons, dogs, minks and horses. For over 30 years, *S. intermedius* was considered the common cause of skin and soft tissue infections in dogs (Bannoehr et al., 2012). However, recent work has demonstrated that isolates phenotypically identified as *S. intermedius* are indeed differentiated into three different but related species *S. intermedius*, *S. pseudintermedius* and *S delphini*, which are together refer to as SIG (Sasaki et al., 2007). This data has clarified that *S. pseudintermedius* and not *S. intermedius* is the SIG species which colonises and causes infections in dogs and cats (Perreten et al., 2010).

Recent genome sequencing approaches have revealed a great degree of similarity of the three species (core genome average identity of >93.6%), being close to the threshold for species delineation (95%) (Ben Zakour et al., 2012). It is difficult therefore to differentiate *S. intermedius* from *S. pseudintermedius* during routine diagnostic procedures, but the vast majority of canine isolates are *S. pseudintermedius*. Hence, it has been proposed to report all strains belonging to the SIG from dogs as S*. pseudintermedius*, unless genomic investigations prove that the strain belongs to another related species (EMA, 2010a).

S. pseudintermedius is now recognised as the major colonizer and skin pathogen of dogs, which predominates in the normal cutaneous microbiota of skin, hair follicles/coat and mucocutaneous sites, such as the nose, mouth and anus (Bannoerh et al., 2012). *S. intermedius* has been isolated only from pigeons to date and *S. delphini* has been recovered from a number of animal species, including horses, mink and pigeons (Ben Zakour et al., 2012).

1.2. CARRIAGE STAGES, CARRIAGE RATES AND INTERSPECIES TRANSMISSION

Several different carriage stages are distinguished. Animals and humans can be contaminated, be carriers or infected by the different staphylococci. **Contamination** occurs in humans, animals, food, the environment, etc, where the bacteria are present due to the exposure from another site (i.e. an infected or colonized host or the environment such as dust). Animals and humans can be contaminated at external surfaces (skin, hair, fur, etc), and there is no multiplication the bacteria and no clinical symptoms (EFSA, 2009a). The terms **carriage** and **colonization** are in many occasions indistinctively used when humans and animals present staphylococci that multiply in the nares, throat or other superficial sites but without causing disease (EFSA, 2009a). **Infection** is a condition whereby staphylococci have invaded the body site, is multiplying in tissue, and is causing clinical manifestations of disease (EMA, 2010a).

However, the term carriage is more correctly used in cross-sectional studies, where there is no notion whether there is real colonization or mere contamination. Henceforth in this thesis, "carriage" will apply to the presence of staphylococci in a cross-sectional study, while colonization will only be employed in a longitudinal approach. Longitudinal studies distinguish at least three carriage patterns in healthy individuals: **persistent**, **intermittent carriage**, and **non-carriage** (Wertheim et al., 2005). Therefore, an individual classified as a carrier in cross-sectional studies could either be a persistent (colonized) or an intermittent carrier. This distinction is important because persistent carriers have higher *S. aureus* loads and a higher risk of acquiring *S. aureus* infections. On the other hand, the definition of persistent carriage varies from study to study and there is no general consensus on how many cultures should be taken and how many cultures should be positive to define persistence (Werheim et al., 2005).

Longitudinal studies show that about 20% of individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers and about 50% are non-carriers (Wertheim et al., 2005). Persistent carriers are often colonized by a single strain of *S. aureus* over long time periods, whereas intermittent carriers may carry different strains over time (Wertheim et al., 2005).

In contrast to *S. aureus*, there are no accurate figures on the *S. pseudintermedius* carriage patterns in the dog population, since only four studies to date have focused on the longitudinal carriage of *S. pseudintermedius* (Bannoehr et al., 2012). These studies reported higher persistent carriage values than those for *S. aureus* in humans (>50%) (Cox et al., 1988;

Hartmann et al., 2005; Paul et al., 2012; Saijonmaa-Koulumies et al., 2002), but further longitudinal approaches are needed for accurate estimations. Also, *S. pseudintermedius* seems to be more common in dogs than *S. aureus* in humans, with average carriage rates of 40% among healthy dogs (Bannoehr et al., 2012). However, highly variable values have been observed depending on the body site tested and the methodology employed.

Fig. 2 shows the different carriage sites and rates of *S. aureus* in humans *and S. pseudintermedius* in dogs. While the anterior nares are the primary site for *S. aureus*, the oral mucosa and the perineum are the primary sites for *S. pseudintermedius* (**Fig. 2**). No data is to date available on the possible preferential body site and the carriage rates for *S. aureus* in dogs or *S. pseudintermedius* in humans.

Although humans and dogs are the primary natural hosts for *S. aureus* and *S. pseudintermedius*, respectively, interspecies transmission of such bacteria can occur between humans and in-contact dogs with a reverse direction. Transmission of *S. pseudintermedius* from the canine to the human host is named **(direct) zoonotic interspecies transmission** while the transference of *S. aureus* from the human to the canine host is termed **(direct) anthropozoonotic interspecies transmission** (or reverse zoonosis) (**Fig. 2**). In addition, indirect evidence of interspecies transmission occurs when *S. pseudintermedius* is detected in humans and *S. aureus* in dogs but not in the other host. In these cases, the terms **indirect zoonotic** (dog→human) and **indirect anthropozoonotic** (human→dog) transmission will be used in this thesis.

Both bacterial species are able to survive in the environment for long periods, including soil and air, which has an important role in transmission and dissemination (Fessler et al., 2012; Hasman et al., 2010; Laarhoven et al., 2011; Verhegghe et al., 2012;).

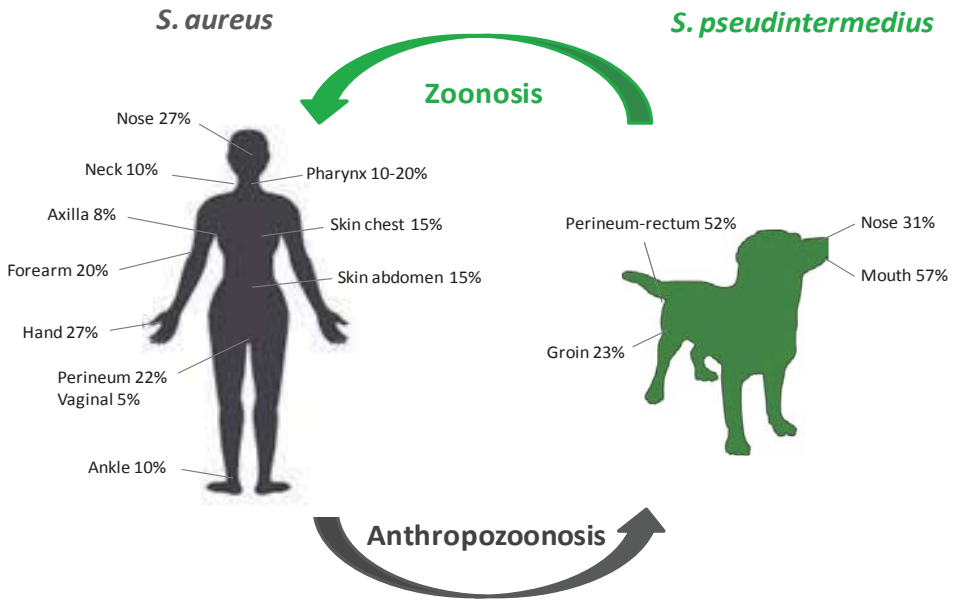


Figure 2. *S. aureus* and *S. pseudintermedius* carriage rates per body site in humans and dogs, respectively (adapted from Bannoehr et al., 2012 and Wertheim et al., 2005).

1.3. VIRULENCE FACTORS AND INFECTION

Staphylococci, in special CoPS, are some of the most important opportunistic pathogens for mammalian species. In particular, *S. aureus* is the most invasive species and an etiological agent of diverse human an animal maladies, including skin infections, abscesses, food poisoning, toxic shock syndrome septicaemia, endocarditis, and pneumonia. *S. aureus* is one of the most prominent causes of nosocomial- and community acquired bacterial infections worldwide (Weigelt, 2008). But *S. pseudintermedius* is also an opportunistic pathogen, major cause of canine pyoderma, and is primarily associated with skin and ear infections in these animals, and it can be involved in nearly any type of community- and veterinary hospital-acquired infections (Bannoehr et al., 2012).

The pathogenicity of *S. aureus* is characterized by the production of certain **enzymes** (coagulase, hyaluronidase, catalase, thermonuclease, etc.) and the production of certain **toxins**. Moreover, **cell wall adhesion** (CWA) components (mucoid polysaccharide capsule, adhesins, protein A, teichoic acid, etc) of the bacteria are also involved in virulence. The expression of these virulence factors help the bacteria adapt to hostile environments, facilitating their survival and promoting infection due to cell invasion, the degradation of the immune system cells and tissues, facilitate the multiplication of the bacteria and are involved in the onset of clinical symptoms (Gordon et al., 2008).

Similar to *S. aureus*, *S. pseudintermedius* produces a variety of virulence factors, including **enzymes** (coagulase, thermonuclease proteases, etc), **toxins** (cytotoxins, exfoliative toxin and enterotoxins) and **surface proteins** (clumping factor and protein A, etc) (Futagawa-Saito et al., 2004a; Futagawa-Saito et al., 2004b; Futagawa-Saito et al., 2006; Hajek et al., 1976; Raus et al., 1983; Terauchi et al., 2003; Wladyka et al, 2008). In addition, both bacterial species have been shown to form biofilm (Futagawa-Saito et al., 2006). However, our knowledge about the pathogenesis of *S. pseudintermedius* is still very limited, and most virulence factors have not been characterized in detail. Moreover, epidemiological studies on the distribution of the different toxins of *S. pseudintermedius* are virtually absent.

The toxins described to date in both bacterial species can be classified into three main groups: **cytotoxins** (leukocidins, haemolysins), **exfoliatins** and **pyrogenic toxin superantigens** (PTSAgs). This latter group includes the toxic shock syndrome toxin (TSST) and enterotoxins.

Leukocidins: One of the most virulent toxins is the Panton-Valentine leukocidin (**PVL**) (a β-pore-forming toxin) encoded by the genes *lukS-PV* and *lukF-PV*. This cytotoxin is composed of two protein subunits, LukS-PV and LukF-PV, which act together assembling in the membrane of host defense cells, in particular, white blood cells, monocytes, and macrophages, inducing the formation of pores, altering the permeability and thus destroying the cell. PVL produce leukocyte destruction causing necrotising pneumonia – an aggressive condition that often kill patients - and skin and shoft tissue infections (SSTIs) (Gillet et al., 2002; Lina et al., 1999). Only 2-3% of *S. aureus* strains produce this toxin. Rarely, PVL is responsible for other *S. aureus* infections such as osteomyelitis, septicemia or endocarditis. The leukotoxin **LukE/LukD** (*luKE/D* genes) produces dermonecrosis in rabbits but has a weak leukotoxic activity and no hemolytic activity. Ruminant polymorphonuclear leukocytes are highly sensitive to the leukotoxin **LukM/LukF-PV** (encoded by *lukM*), and its presence has been associated with cases of bovine mastitis (Kaneco et al., 2004) (**Fig. 3**).

Similar to the PVL, *S. pseudintermedius* produces a bicomponent leukotoxin **Luk-I** (**Fig. 3**), which is encoded by two cotranscribed genes (*lukS-I* and *lukF-I*), and has shown to be leukotoxic for polymorphonuclear cells but only slightly haemolytic for rabbit red blood cells (Futagawa-Saito et al., 2004a; Prevost et al., 1995).

Haemolysins: Five hemolysins are described to date alfa (α-haemolysin, encoded by *hla*), beta (β-haemolysin, *hlb*), delta (δ-haemolysin, *hld*), gamma (γ-haemolysin, *hlg*) and gamma-variant (*hlgv* o *hlg*-2) (**Fig. 3**). The vast majority of *S. aureus* cells produce any of these

haemolysins. Its broad distribution is due, in part, to their location in very stable regions of the chromosomal DNA (Kaneco et al., 2004). The best studied haemolysin is **α-haemolysin**; it has dermonecrotic and neurotoxic activity (Dinges et al., 2000), being lethal for a variety of eucariotic cells of different animal species. Susceptibility to this toxin depends on the animal species (rabbit cells are 1000 times more susceptible than human cells) and the cell type (erythrocytes are more susceptible than fibroblasts) (Menestrina et al., 2001; Murray et al., 2009). The **β-haemolysin** has sphingomyelinase activity with a high affinity for sphingomyelin. It is then active against a variety of cells including erythrocytes, leukocytes, fibroblasts and macrophages (Murray et al., 2009). The **δ-haemolysin** acts as a surfactant that alters the cell membranes by a detergent-like action. It has a broad spectrum of cytolytic activity, affecting erythrocytes and other mammalian cells, and also to various subcellular structures protoplasts, spheroplasts and lysosomes (Verdon et al., 2009). The **γ-hemolysin** lyses red blood cells and other mammalian cells, and is also a bi-component toxin (Kaneco et al., 2004).

S. pseudintermedius produces **α-haemolysin** and **β-haemolysin** and causes haemolysis of rabbit erythrocytes and hot-cold haemolysis of sheep erythrocytes (Bannoerh et al., 2012).

Exfoliatins: There are three exfoliative toxins or epidermolytic toxins first detected in humans, **EtA** (*eta* gene), **EtB** (*etb*) and **EtD** (*etd*) (Yamaguchi et al., 2002) and other of animal origin **EtC** (*etc*), first described in horses but whose activity has not yet been determined (Sato et al., 1994) (**Fig. 3**). The exfoliatins are proteases which act by cutting peptide bonds between the extracellular domains of desmoglein 1 (Nishifuji et al., 2008), a transmembrane protein that forms part of desmosomes that bind the epithelial cells. These toxins are responsible for the staphylococcal scalded skin syndrome (SSSS) disease that usually affects children and is characterized by loss of superficial layers of skin, dehydration and secondary infections by other microorganisms.

In *S. pseudintermedius*, the exfoliatins **SIET**, **ExpA** and **ExpB** (**Fig. 3**), which have been shown to cause erythema, exfoliation, crusting and/or superficial pyoderma in dogs, have been described to date (Futagawa-Saito et al., 2009; Iyori et al., 2010; Iyori et al., 2011; Lautz et al., 2006).

Pyrogenic toxin super antigens (PTSAgs): The bacterial PTSAgs are exocellular proteins with the ability to stimulate non specifically a large number of T cells in the host, resulting in cytokine production to toxic levels (Fraser at al., 2008; Ortega et al., 2010). PTSAgs enclose the

Toxic Shock Syndrome Toxin (TSST) and 18 staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SEU) (**Fig. 3**).

The **TSST**, causative agent of the Toxic Shock Syndrome (TSS), produces high fever, headache, disorientation, vomiting, diarrhea and rashes. In the 80s, an increased number of studies reported more cases of TSS which were soon associated with tampon use in young women. It was observed that those patients showed intravaginal colonization of *S. aureus* producing TSST (Torres et al., 2001). New animal variants were described subsequently, the ovine TSST and bovine TSST (Lee et al., 1992). Eighteen staphylococcal enterotoxins have been described to date in addition to some variants of SEC, SEG, SEH and SEI. These toxins are heat stable and resistant to digestive enzymes being responsible for food poisoning caused by *S. aureus*. Other diseases that have been associated with this type of PTSAgs include arthritis, atopic dermatitis, inflammatory bowel disease, collagen vascular disease, Kawasaki disease and autoimmune disease (Larkin et al., 2009; Thomas et al.; 2007). In *S. aureus*, a putative nursery of enterotoxin genes, named *egc*, has been detected and observed to be highly prevalent (Jarraud et al., 2001). This cluter carries the SEG, SEI, SEM, SEN, SEO, and variants with SEU have been observed (Argudín, 2011).

The *S. pseudintermedius* enterotoxin genes **SEC$_{canine}$** and **SE-INT** have been described to cause pyoderma, and se-int additional chronic otitis (Edwards et al., 1997; Futagawa-Saito et al., 2004b) (**Fig. 3**). The presence of the aforementioned *S. aureus* enterotoxins genes in *S. pseudintermedius* isolates is rare.

In addition, in *S. aureus*, a so far *S. aureus*-specific gene cluster that serve the bacteria to survive in the human host via evasion of the innate immune system has been described (Van Wamel et al., 2006). It is named **Immune Evasion Cluster** (IEC) and compiles the immune modulators staphylokinase (SAK, *sak* gene), staphylococcal complement inhibitor (SCIN, *scn* gene) and chemotaxis inhibitor (CHIPs, *chp* gene), which cluster on the conserved 3′ end of β-haemolysin (*hlb*)-converting bacteriophages (βC-ϕs) (Van Wamel et al., 2006) (**Fig. 3**). These human-specific innate immune modulators SCIN, CHIPS, and SAK form an IEC that is easily transferred among *S. aureus* strains by a diverse group of β-haemolysin-converting bacteriophages. The IEC is gaining interest in the last years due to its specificity for human-adapted *S. aureus* strains and it has been suggested that can be a good predictor of the human or animal origin of a given isolate (McCarthy et al., 2012; Price et al., 2012; Vincze et al., 2012).

Whether this cluster serves the bacteria to survive on other mammal species has not yet been elucidated.

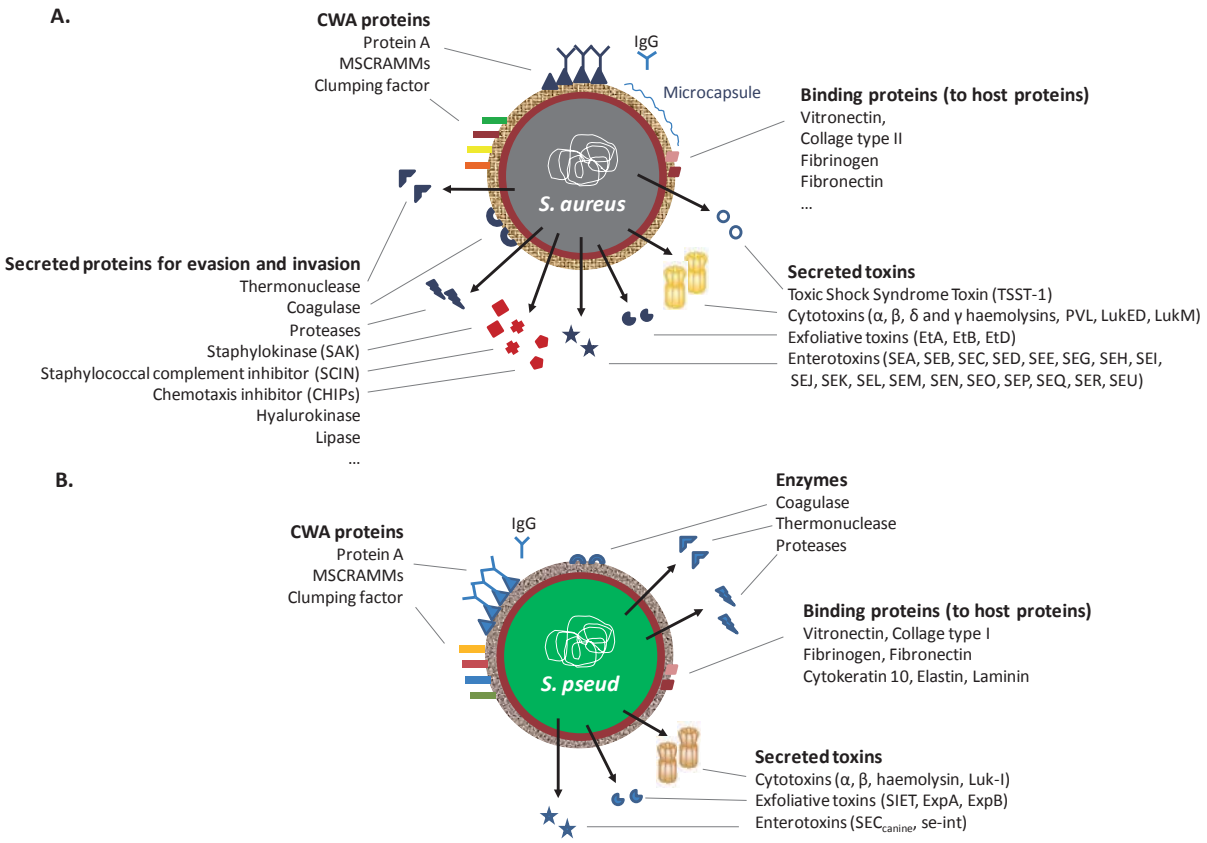


Figure 3. Simplified schematic diagram of selected CWA and secreted virulence factors of **(A)** *S. aureus* and **(B)** *S. pseudintermedius*.

2. MOLECULAR TYPING IN *S. aureus* AND *S. pseudintermedius*

The differences observed between isolates of *S. aureus*, both methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) showed the need to establish a subdivision or intra-species differentiation, for which different typing techniques were developed. These methodologies applied to epidemiological studies have allowed the establishment of prevalent and endemic lineages versus sporadic and emerging *S. aureus* clones in a given community, country or geographic region. More recently, in parallel to the emergence of methicillin-resistant *S. pseudintermedius* (MRSP), similar methodologies to those for *S. aureus* have been likewise developed for *S. pseudintermedius*.

Different molecular techniques are to date available to type *S. aureus* and *S. pseudintermedius* isolates; however, in the absence of a consensus on the optimal typing method today, several of these techniques are simultaneously employed. The most commonly used molecular typing methods for *S. aureus* and *S. pseudintermedius* isolates are described.

2.1. TYPING OF THE POLYMORPHIC REGION OF *SPA* GENE

Spa typing was developed in 1996 to type *S. aureus* isolates (both MSSA and MRSA) (Frénay et al., 1996) and is based on the amplification and subsequent sequencing of a polymorphic region of the gene encoding the surface protein A (*spa*). The sequence variation in the variable region X consists of duplications or deletions of whole repeats fragments (normally of 20 bp) or point mutations within repeats. Thus, analyzing the number of repetitions and their combination and variability the different types (*spa*-types) are assigned (Harmsen et al., 2003) using Ridom Staph-Type software (Ridom GmbH). Grouping of related *spa*-types can be performed using the Based Upon Repeat Pattern (BURP) using the same software. This technique is of great interest because of its quickness and simplicity. Furthermore, a good correlation has been observed in many cases with the results obtained using MLST (see below) (Oliveira et al., 2002).

Recently, a species-specific *spa* typing protocol was developed following the discovery of a staphylococcal protein A homolog in the genome of *S. pseudintermedius* (Moodley et al., 2009).The *spa* gene in *S. pseudintermedius* ED99 is smaller and has 68% nucleotide and 55% predicted amino acid identity to the homologous gene in the *S. aureus* reference strain 8325-4 where protein A was originally described. The principle is the same but repetitions are normally of 30 bp. The predicted protein contained a number of functional regions and conserved domains previously described in *S. aureus* (**Fig. 4**). This method yield positive results for MRSP; however, this is not an effective method for typing MSSP isolates as over 50% are not typeable (either primers do not amplify or yield multiple unspecific bands) (Moodley et al., 2009).

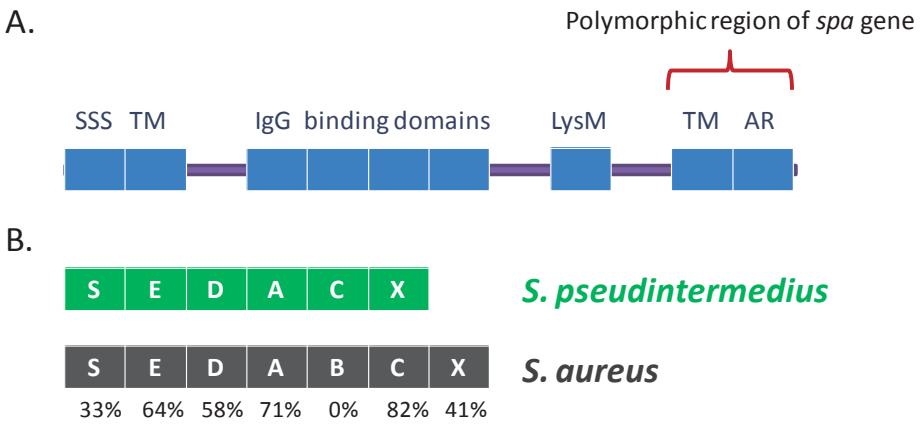


Figure 4. Fig. 1. Schematic representation of protein A in *S. pseudintermedius* based on nucleotide and predicted amino acid sequence analysis. **A.** Conserved domain structure of protein A in the MSSP strain ED99. SSS, start signal sequence; TM, transmembrane domain; four IgG-binding domains; LysM, lysin motif; AR, anchor region. **B.** Predicted amino acid similarity of protein A between *S. pseudintermedius* ED99 and *S. aureus* 8325-4 in the segments coding for the signal sequence (S), the immunoglobulin G-binding regions (A–E) and hypervariable region X (adapted from Moodley *et al.,* 2009).

2.2. MULTILOCUS SEQUENCE TYPING (MLST)

MLST is a technique developed in 1998 (Maiden et al., 1998) which is based on the nucleotide sequence analysis of internal fragments (about 500 bp) of certain housekeeping genes coding for essential functions. Normally, this technique includes 7-8 loci, depending on the microorganism. This analysis allows the detection of neutral variations that define relatively stable clonal lineages (Dominguez et al., 2005). MLST is considered the valuable method for long-term and global epidemiology.

In the case of *S. aureus*, this technique was developed in 2000 targeting the following housekeeping genes: *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) and *yqiL* (acetyl coenzyme A acetyltransferase) (Enright et al., 2000). The sequences of these genes are compared with already known allele sequences that are accessible on the website www.mlst.net. The allelic profile obtained is used to deduce the sequence type (ST) of the isolate. In addition, the algorithm eBURST is used to define different clonal complexes (CCs). CC is defined as a group of STs in which every genotype shares at least 6 loci in common with at least one other member of the group. Each CC takes the name of the predicted founder of the group, as of the ST with a greater number of single locus variants (SLV) (STs that differ in a single allele).

It is important to clarify that CC5 represents to date the greatest group formed with eBURST v3 (as of 04.03.2013). Recently, some traditionally stable CCs, such as CC1, CC8, CC9, CC15 and CC97, have clustered together within the CC5, as a result of the increasing number of lineages being described in this bacterial species (**Fig. 5**). Since the results from this algorithm are dynamic, STs within traditionally independent CCs are now enclosed within CC5, and shows the rapid dynamism of connections among different *S. aureus* lineages. In this way, new connections between STs are established and previous unrelated groups are being put together. Along this thesis, the traditional clones CC1, CC5, CC8, CC9, CC15 and CC97 have been maintained for discussion.

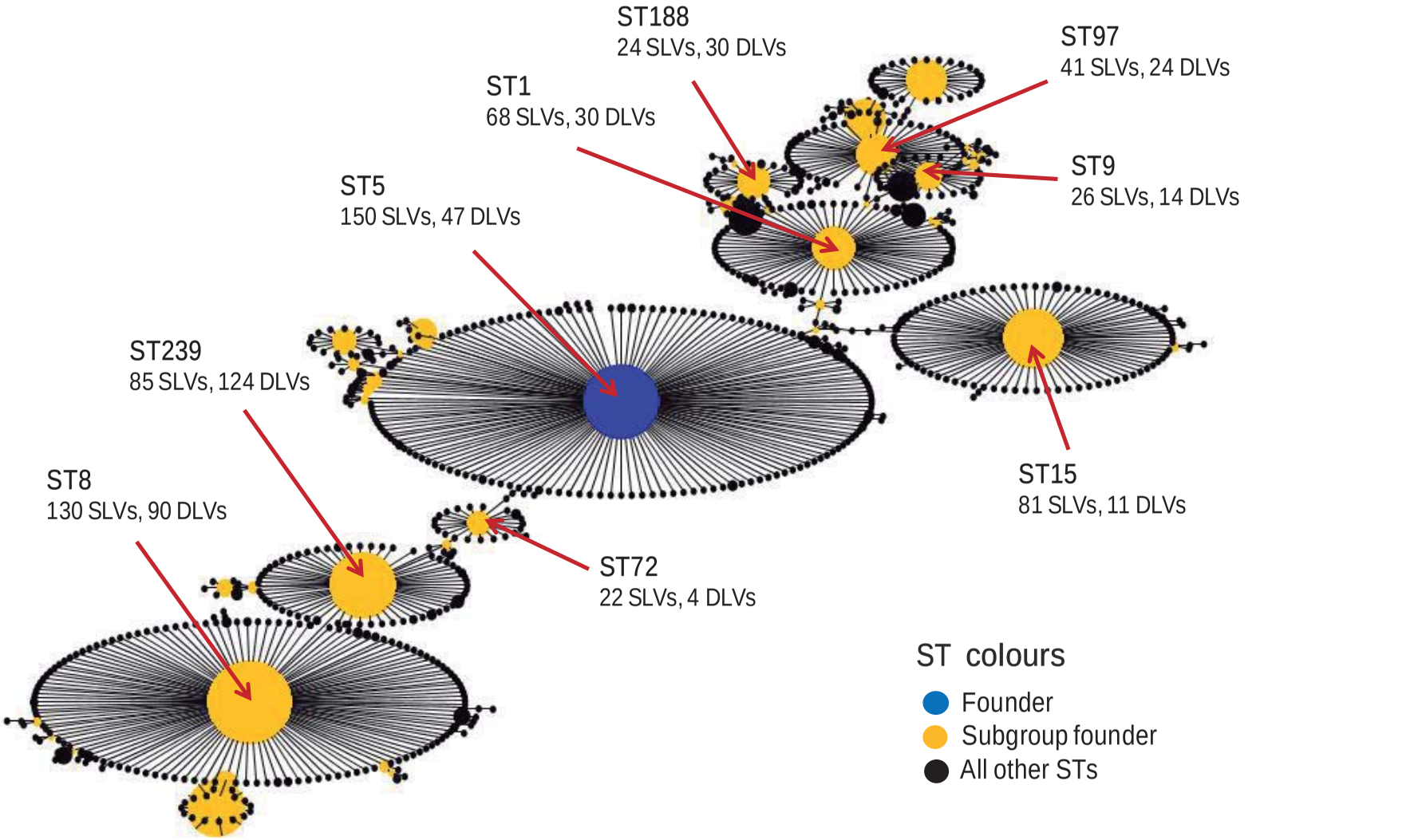


Figure 5. Alignment of the different STs that belong to the current CC5 (as of 04.03.13) using the algorithm eBURST. The area of the circle is proportional to the number of isolates of a given ST/CC (www.mlst.net)

In *S. pseudintermedius*, two related recent schemes have been developed. A foundation sequencing approach based on five housekeeping genes was developed by (Bannoerh et al., 2007). The 16S rRNA, *cpn60* (chaperonin 60), *tuf* (elongation factor Tu), *pta* (phosphate acetyltransferase), and *agrD* (autoinducing peptide, AIP) genes, were selected for this approach. This approach has enable researchers to analyse the distribution of MRSP clones, leading to the identification of two major genetic lineages, ST71 in Europe and ST68 in North America (Bannoerh et al., 2007).

Very recently, (Solyman et al., 2012) have developed an expanded MLTS approach that increases the discriminatory power of the method by including 7 loci. Three housekeeping genes of the previous approach, *tuf*, *cpn60*, and *pta*, have been maintained. The novel loci included are: *purA* (adenylosuccinate synthetase), *fdh* (formate dehydrogenase), *ack* (acetate kinase), and *sar* (sodium sulfate symporter). It is of interest that ST71 could not be further distinguished using the new scheme, while ST68, has been further divided into three STs, indicating that this lineage is not as clonal as revealed by the previous approach.

2.3. ALLOTYPING OF THE *AGR* OPERON

The accessory gene regulator (*agr*) quorum-sensing system was first described in *S. aureus* but is a global regulatory system of the genus *Staphylococcus* and has been found in the majority of staphylococcal species examined (Bannoerh et al., 2007; Dufour et al., 2004). This system is thought to have diversified along lines of speciation, giving rise to a number of subspecies groups that have the capacity for intra- and interspecies inhibition of virulence (Robinson et al., 2005; Wright *et al*., 2005). It is an operon that includes a system of two-signal transduction components responsible for controlling the expression of many virulence factors and pathogenicity. This operon is self-induced by a peptide named AIP (for auto inducing peptide), located in the same locus. AIP diffuses into the target cell and acts as a receptor of the signal when the cell density is sufficient. This is a population density sensor or *quorum sensing* and is the only *quorum-sensing* system in the staphylococcal genome (Traber et al., 2008).

The locus *agr* consists of several genes: ***agrB*** encodes a protein responsible for membrane translocation and modification of AgrD; ***agrD*** encodes an octapeptide precursor of AIP; ***agrC*** encodes an AIP receptor signal membrane protein and ***agrA*** encodes a response regulator of the signal (binds to the promoter and activates transcription when it is phosphorylated) (**Fig. 6**).

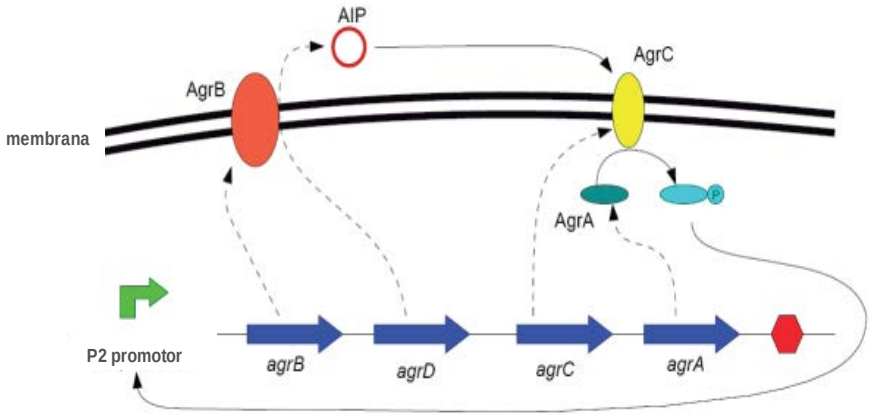


Figure 6. Graphical representation of the *agr* operon and its functional activity.

S. aureus strains can be classified according to the intra-variability of different regions of the *agr* operon. There are four types for this species. It has been observed that strains belonging to the same group can activate the *agr* response in other strains of the same group and inhibit those belonging to another group, representing a form of interference between bacteria (Shopsin et al., 2003). It has been suggested that these *agr* groups can influence in the capacity of *S. aureus* to colonize the host by competing with other bacteria, including other staphylococcal species. This also affects the transmission ability of these bacteria between hosts and the epidemiology of the different *S. aureus* clones (Shopsin et al., 2003). Additionally, the expression of the *agr* appears to be important during the early stages of *S. aureus* infection (Traber et al., 2008).

In *S. pseudintermedius*, the *agr* type is based on the variability of the motif KYPTSTGFF of the AIP (*agrD* gene) (Bannoerh et al., 2007). Interestingly, sequence analysis of the allelic variation of *agrD* in *S. pseudintermedius*, *S. intermedius* and *S. delphini* (SIG), revealed four AIP variants encoded by *S. pseudintermedius* isolates, and identical AIP variants shared among the three species, suggesting that a common *quorum-sensing* capacity has been conserved in spite of species differentiation in largely distinct ecological niches (Bannoerh et al., 2007).

2.4. PULSED FIELD GEL ELECTROPHORESIS (PFGE)

PFGE technique is based on the generation of macrorestriction fragments by digesting the genomic DNA of a bacterium with endonuclease enzymes that cut at low frequences. The PFGE patterns obtained for the different strains can be compared to

establish genetic relationships between them. This technique was first used in *S. aureus* in 1995 (Bannerman et al., 1995) and has since become one of the most used typing methods. PFGE is generally regarded as one of the most discriminatory methods for bacterial typing and therefore particularly indicated for surveillance and outbreak investigations.

A standardized PFGE protocol for *S. aureus* was developed in 2003 to allow comparison between laboratories, and it was so called the "Harmony PFGE protocol" (Murchan et al., 2003). The enzyme used for the digestion of the chromosomal DNA in this protocol is *SmaI*. Once the electrophoretic profiles of each isolate are obtained, they are visually analysed based on Tenover's criteria (Tenover et al., 1995), although software packages (such as GelCompar) are also usually employed to allow comparison of multiple DNA sequences. However, a new clonal lineage that is resistant to the *SmaI* digestion is emerging in recent years. This clonal lineage belongs to the MLST 398 and is PFGE non-typeable using the *SmaI* enzyme (Voss et al., 2005). This is due to methylation at adenine and cytosine residues that prevent *SmaI* cleavage. Numerous studies have tested other enzymes, such as *XmaI*, *ApaI*, *BstZI*, *SacII* or *Cfr9I* in order to use this technique in *S. aureus* ST398, with variable results (Bosch et al., 2010; Kadlec et al., 2009a; Rasschaert et al., 2009).

To date, however, a standardized PFGE protocol for *S. pseudintermedius* does not exist. The protocol originally developed for *S. aureus* by (Murchan et al., 2003) has been adapted in some *S. pseudintermedius* reports, but other methods using *SmaI* have been implement in several studies (Bannoerh et al., 2012). This situation does not facilitate comparison of results between studies and communication of PFGE data between laboratories. The development of a standardized protocol for this species would be extremely useful.

2.5. SCC*MEC* TYPING

MRSA and MRSP can be additionally typed by PCR mapping of staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element which contains the *mec*A gene, conferring meticillin resistance (see next section). This technique is based in the polymorphism of intern regions of SCC*mec*. A summary of the composition and function of the different elements that compose the SCC*mec* cassette are described below:

The *mec*A gene encodes PBP2a, whose expression is regulated by two genes, *mec*I and *mec*R1. The first gene codes for a repressor protein of the *mec*A transcription and the

other functions as a transcriptional regulator of the gene. In absence of β-lactams, *mec*A gene expression is inhibited by the action of MecI (linkage to the operator of *mec*A); however, in presence of β-lactams, the protein encoded by mecR1 (an integral membrane protein) recognises the antimicrobial at its receptor domain and induces an autocatalytic protease that inactivates the repressor, hence allowing *mec*A transcription (Zhang et al., 2001). Different types of *mec* complexes have been distinguished, which comprises the *mec* operon (*mec*A, *mec*I, *mec*R1) and the insertion sequences IS*431* and/or IS*1272* (**Table 1**).

Table 1. Types of *mec* complexes in *S. aureus* (Deurenberg *et al*., 2008).

Class of *mec* complex	Structure of the *mec* operon and ISs
A	IS*431*-***mecA***-***mecR1***-***mecI***
B	IS*431*–***mecA***-**Δ*mecR1***-IS*1272*
C1	IS*431*[a]–***mecA***–**Δ*mecR1***–IS*431*[a]
C2	IS*431*[b]–***mecA***–**Δ*mecR1***–IS*431*[b]
D	IS*431*–***mecA*** **–Δ*mecR1***
E	*blaZ*-***mecA*** $_{LGA251}$[c]-***mecR1*** $_{LGA251}$-***mecI*** $_{LGA251}$

[a]In the same orientation; [b]In different orientation; [c] also known as *mecC*.

SCC*mec* is integrated within the *orfX* chromosomal DNA by two recombinase genes, *ccrA* and *ccrB*, that mediate excision and integration of the whole element. The origin of this element is to date unknown but it is believed to have originated from *Staphylococcus sciuri* (Wu *et al*., 2001) or other CoNS, due to the high diversity of SCC*mec* types detected in this group.

To date, eleven SCC*mec* types (I–XI) have been identified based on the allotype of recombinase gene (*ccr*) and the class of *mec* gene complex, plus a variety of subtypes depending on variations in the three joining regions (J1–J3) (**Fig. 7, Table 2**) (Ito *et al*., 2001; (http://www.sccmec.org/Pages/SCC_TypesEN.html). For routine analysis, several sets of multiplex PCRs have been developed to identify the different SCC*mec* elements and subtypes (Kondo et al., 2005; Kondo et al., 2007; Oliveira et al., 2006; Zhang et al., 2005).

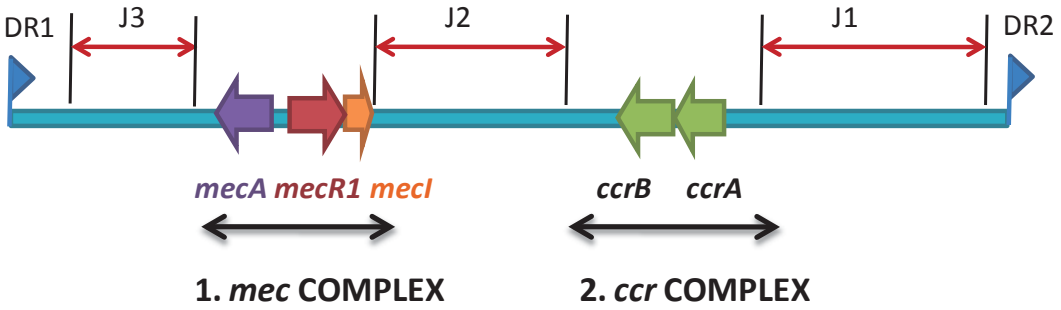


Figure 7. Graphical representation of the basic elements of a standarized SCC*mec*: *mec* complex, *ccr* and junkyard regions (J1-3). DR, direct repeat generated at the integration site within *orfX*.

Table 2. SCC*mec* types detected to date in *S. aureus*.

SCC*mec* type[1,2]	*ccr* type	*mec* complex
I	1 (*ccrA1ccrB1*)	B
II	2 (*ccrA2ccrB2*)	A
III	3 (*ccrA3ccrB3*)	A
IV	2 (*ccrA2ccrB2*)	B
V	5 (*ccrC*)	C2
VI	4 (*ccrA4ccrB4*)	B
VII	5 (*ccrC*)	C1
VIII	4 (*ccrA4ccrB4*)	A
IX	1 (*ccrA1ccrB1*)	C2
X	7 (*ccrA1ccrB6*)	C1
XI	8 (*ccrA1ccrB3*)	E

The staphylococcal genome is in constant evolution, acquiring genes and mobile genetic elements (MGEs) that allow the bacteria develop resistance to the agents employed. Thus, additional non β-lactam antimicrobial resistance genes carried in MGEs have been found within the SCC*mec* element, such as aminoglycosides, erythromycin, clindamycin, rifampicin, tetracycline or trimethoprim (**Table 3**) (Hiramatsu *et al*., 1999; Li *et al*., 2011; Turlej *et al*., 2011).

Table 3. Mobile genetic elements **(**MGEs) located within SCC*mec* elements.

MGE	SCC*mec*	Gene/s	Resistance
pUB110	I, II, IVa	*ble/aadD*	Bleomycin/tobramycin
Tn554	II, VIII	*erm*(A)/*spc*	Streptomycin/spectinomycin
pT181	III, V	*tet*(K)	Tetracyclines
ψTn554	III	*cad*	Cadmium
Tn4001	IV (IVa y $IV_{2B\&5}$)	*aacA-aphD*	Aminoglycosides

This constant change is also reflected in the presence of metal resistance genes within the SCC*mec* element. Remarkably, the last three SSC*mec* elements (IX, X, XI) and the last SCC*mec* subtype described (5C2&5) to date all harbour heavy metal resistance determinants: (i) **SCC*mec* IX** and **X** exhibit resistance determinants to cadmium (*cadDX* operon), arsenate (*arsRBC* or *arsDARBC* operons) and copper (*copB-mco* genes) (Li et al., 2011), (ii) the novel **SCC*mec* XI** carries arsenate resistance determinants (*arsRBC*) (Shore et al., 2011) and (iii) the **SCC*mec* V (5C2&5)** carries the cadmium and zinc resistance gene *czrC* (Cavaco et al., 2010). This association is also evident in **SCC*mec* III**, a composite SCC element that carries SCC_{Hg}, wich presents mercury resistance determinants (*mer* operon) (Chongtrakool et al., 2006).

Importantly, the last SCC*mec* described to date, SCC*mec* XI, carries a novel *mec* gene (70% similarity to *mec*A) that cannot be amplified using the normal primers employed for the *mec*A gene. This novel element appears to be associated to MRSA strains of bovine origin, although it has been also detected among human isolates (García-Álvarez *et al*., 2011; Shore *et al*., 2011).

These multiplex PCR approaches originally developed for SCC*mec* typing of MRSA have been used for characterization of MRSP isolated from dogs. Some SCC*mec* types observed in MRSP correspond to types previously found in MRSA (e.g. SCC*mec* V in ST68), whereas others are not found in MRSA (SCC*mec* II–III in ST71) or are untypeable using the SCC*mec* typing scheme developed for this species (Bannoerh et al., 2012).

Two additional so far species-specific SCC*mec* cassettes have been described in MRSP isolates: (i) SCC*mec* II-II (*mec* complex A + *ccrA3/ccrB3*) is a combination of the SCC*mec* II of *S. epidermidis* and the SCC*mec* III of *S. aureus* but lacks the cadmium resistance operon *cadBC* (**Fig. 8**), and (ii) SCC*mec* VII (*mec* complex A + *ccrA5/ccrB5*) (Descloux et al., 2008).

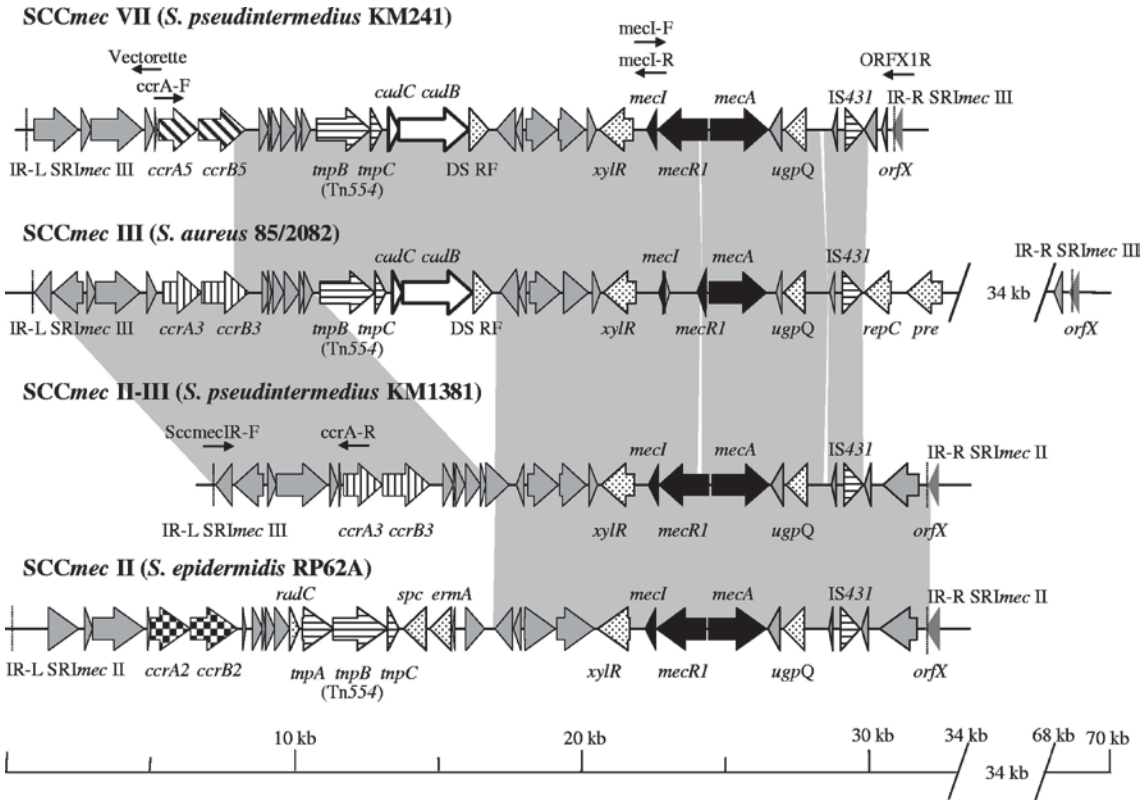


Figure 8. Novel SCC*mec* elements detected in MRSP and its possible ancestors (taken from Descloux et al., 2008).

In any case, there is still plenty to discover about the SCC*mec* system, since, nowadays there are many strains carrying non-typeable SCC*mec*, elements with two types of *ccr* and/or *mec* complexes or even SCC*mec*-llike elements that do not carry the *mec*A gene (IWG-SCC, 2009).

3. ANTIMICROBIAL RESISTANCE IN STAPHYLOCOCCI

3.1. THE GLOBAL PROBLEM OF ANTIMICROBIAL RESISTANCE

The discovery of antibiotics was one of the most important milestones in the history not only of medicine but also of humanity, given that they drastically reduced mortality rates during the first years of its introduction in the clinic, in the early 1940s. However, soon after its introduction, bacteria resistant to those antimicrobials began to evolve and spread. These microorganisms began to disseminate in hospitals; however, the hospital environment is not an isolated ecological niche but an atmosphere exposed to constant flow of genetic exchange with the environment. Thus, resistant bacteria were gradually

appearing and spreading also in the community, in animals and even in food (Finch et al., 2006).

The discovery and development of new families of antibiotics was very quick in the first decades after the discovery of penicillin, but this rate has stopped, and very few molecules with new activities or new families of antibiotics have been incorporated to the arsenal in recent decades (**Fig. 9**). Worryingly, the deployment of any novel antibiotic has been followed by the evolution of clinically significant resistance to that antibiotic in as little as a few years (**Fig. 9**).

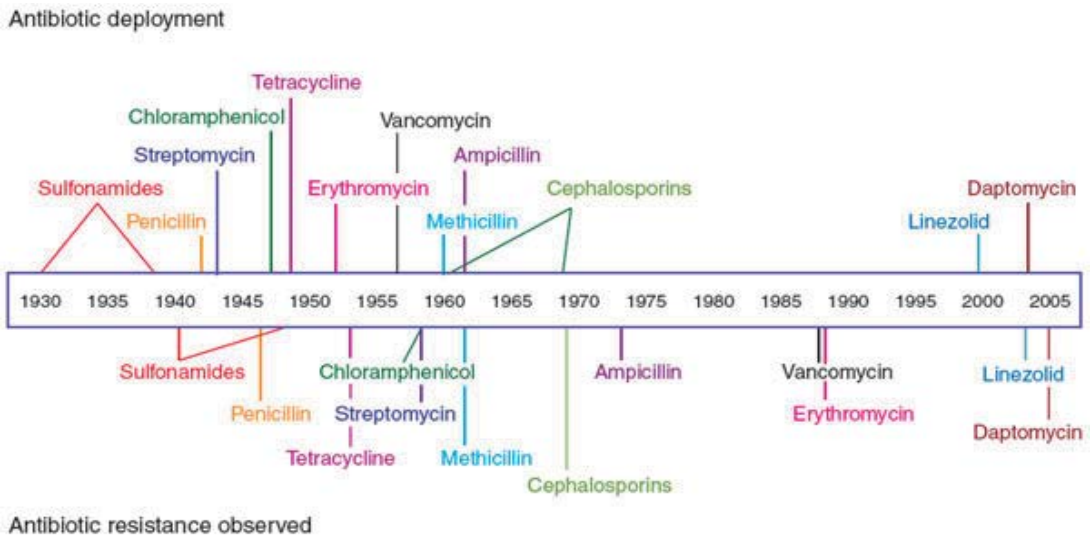


Figure 9. Timeline of antibiotic deployment and the evolution of antibiotic resistance. The year each antibiotic was deployed is depicted above the timeline, and the year resistance to each antibiotic was observed is depicted below the timeline (with the caveat that the appearance of antibiotic resistance does not necessarily imply that a given antibiotic has lost all clinical utility) (taken from Clatworthy et al., 2007).

This represents an important problem that greatly complicates the treatment of bacterial infections. Antimicrobial use in human and animal medicine and, to a lesser extent, in agriculture constitutes an important factor that exerts selective pressure on the bacterial populations, not only on those pathogenic, but also on the commensal endogenous microbiota of humans and animals. This selective pressure favours the selection and dissemination of commensal resistant bacteria that can later transfer the acquired resistant determinants to pathogenic bacteria (Fluit et al., 2006). Nowadays, antimicrobials are used in many fields, for prophylaxis and treatment of infections in humans and animals, or even in agriculture to prevent bacterial infections in crops. All these uses also generate selective

pressure of the environment in which they are used, and produce environmental contamination due to the persistence of certain amounts of antibiotic in sewage treatment plants, effluents, etc, which normally end up in rivers.

In the last decade, we witness a dramatic increment in the number of multiresistant bacterial pathogens that exhibit resistance to numerous families of antimicrobials. As an example, MRSP isolates not susceptible to any antimicrobials authorised for use in veterinary medicine have been published, what represents (i) a new challenge in veterinary medicine, and (ii) an additional pressure for veterinarians to use antimicrobials authorised for human medicine (EMAa, 2010). This rapid evolution of antimicrobial resistance represents a serious problem for public health, for the limited therapeutic options available. Consequently, the development of novel antimicrobial agent is imperative (Vila et al., 2010).

The disseminaton of antimicrobial resistance is an essential factor that helps bacteria evolve and adapt to the external conditions. It is due to the evolution of different MGEs and recombination processes between bacteria in contact, but also to additional anthropogenic factors such as the transfer of patients between hospitals, international travel and global food marketing (Vila et al., 2010).

Antimicrobial resistance appears to evolve towards (i) the global increment of multiresistant bacteria in different environments (hospital, community, environment), (ii) to the selection and dissemination of successful highly adapted clones, due to the acquisition and maintenance of MGEs of interest, and (iii) the appearance and spread of novel antimicrobial resistance genes and novel MGEs (Wright *et al*., 2010).

3.2. MECHANISMS OF ANTIMICROBIAL RESISTANCE

Antimicrobials damage bacteria by interfering in different essential cell processes (**Fig. 10**), which can lead to death of the organism (if is a bactericidal antimicrobial) or cessation of growth (bacteriostatic antimicrobial). Antimicrobial resistance may be **intrinsic** (innate resistance) or **acquired** (by mutation or horizontal gene transfer). This latter plays a more important role due to horizontal gene transfer between bacteria, which, although it depends on the elements that control the physical transfer of DNA (transformation, transduction or conjugation, see below) and the ability of cells to replicate and express the exogenous DNA, is highly prevalent in the bacterial populations (Barlow, 2009).

Staphylococci can then adapt to the selective pressure imposed by the use of antimicrobials by the development of multiple mechanisms of resistance that can affect different cellular structures or metabolic pathways. For this, this bacterium can suffer punctual mutations in their core genes or acquire genes from other bacteria resulting in the different antimicrobial resistance mechanims (**Fig. 10**):

- -Alteration of the permeability of the membrane or the wall-cell.
- -Active efflux of the antimicrobial from the cell.
- -Modification or protection of antimicrobial targets.
- -Enzymatic inhibition or inactivation of the antimicrobial.

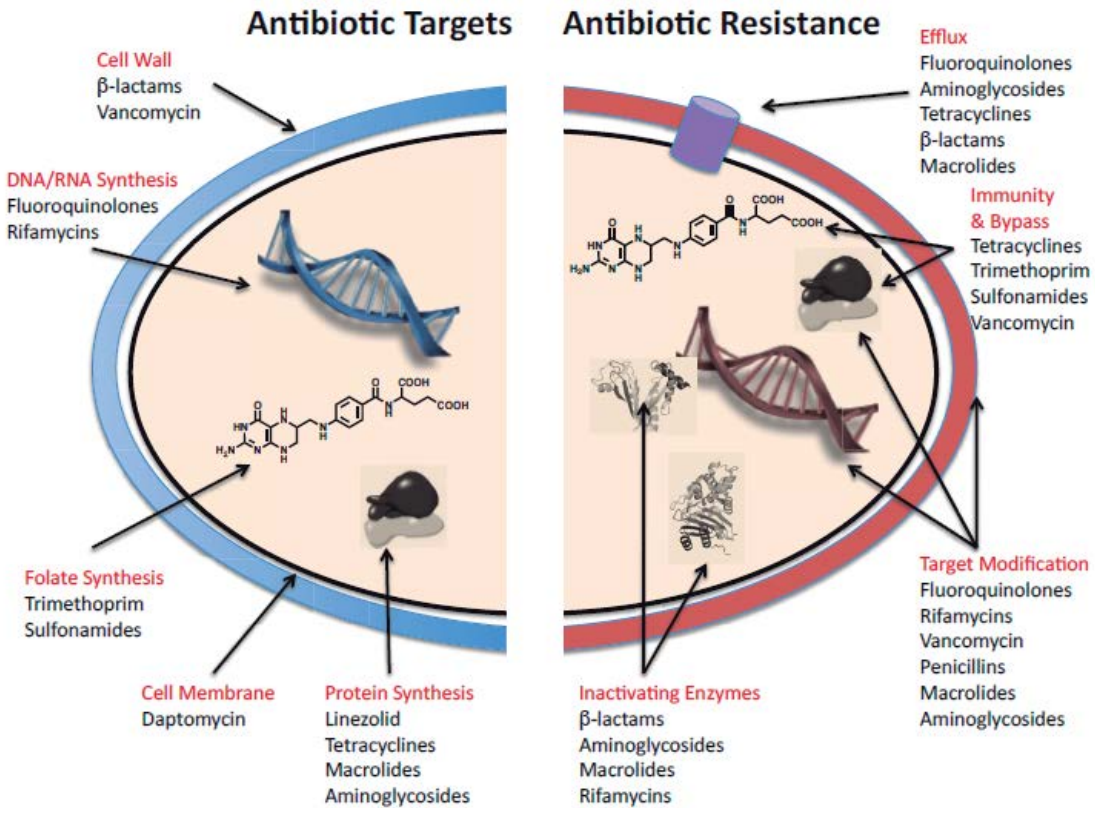


Figure 10. Antibiotic targets and mechanisms of resistance (Taken from Wright, 2010).

3.2.1. Methicillin resistance in staphylococci

β-lactams are bactericidal agents that inhibit the synthesis of the bacterial cell wall by blocking the final step of peptidoglycan synthesis (transpeptidation). These antimicrobials covalently associate to PBPs (penicillin-binding proteins), which are the enzymes (transpeptidases, transglucosilases and carboxypeptidases) involved in binding the different peptidoglycan components.

In the 1940s, penicillin was introduced for the treatment of staphylococcal infections and only a year later *S. aureus* strains resistant to penicillin began to appear. Penicillin resistance is mediated by the *blaZ* gene, with β-lactamase activity. This β-lactamase inactivates the β-lactam ring of penicillins by hydrolytic cleavage. Currently, over 90% of strains of *S. aureus* show penicillin resistance.

Due to the emergence of these enzymes, new antimicrobials were sought to treat infections caused by *S. aureus*, including methicillin, a semi-synthetic penicillin that is able to escape the action of these β-lactamases. In 1961, two years after the introduction of this drug, MRSA emerged. Since then, a widespread distribution of these strains occurred in many hospitals, and MRSA is nowadays one of the main pathogens that cause nosocomial infections worldwide (Weigelt, 2008).

In staphylococci, the main mechanism of resistance to methicillin is the synthesis of a PBP with low affinity to β-lactams, named PBP2a or PBP2'. This protein is encoded for the *mec*A gene, which is located within the chromosomal SCC*mec* aforementioned. Staphylococci produce at least 4 PBPs (PBP1, PBP2, PBP3 y PBP4) that are susceptible to the action of β-lactams by competitive inhibition for the natural substrate of these proteins (residues D-Ala-D-Ala of the peptidoglycane) (**Fig. 11**). The presence of the PBP2a allows the cell continue forming the peptidoglycane synthesis in spite of the presence of the antimicrobial (**Fig. 11**).

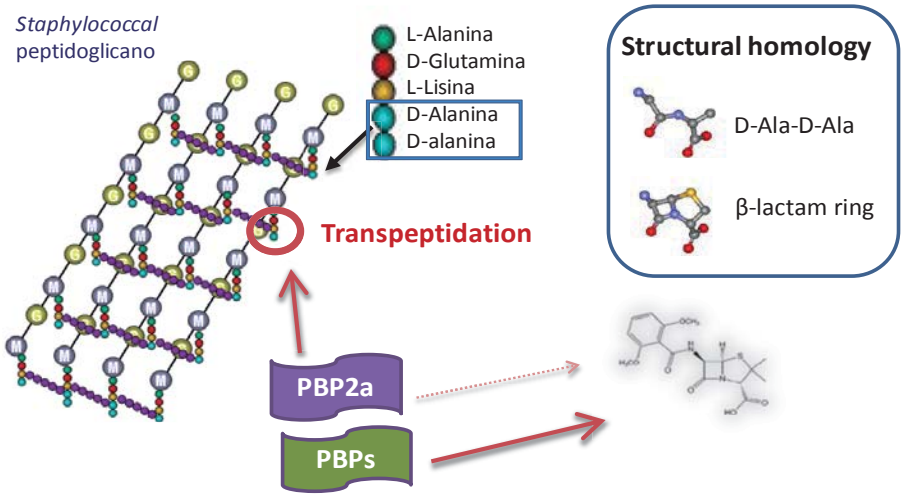


Figure 11. Mechanism of action of the normal PBPs and the PBP2a in the presence of β-lactams. In the presence of β-lactams, the PBP2a continue with its normal transpeptidase function.

MRSA strains are those that exhibit this PBP2a, which confers resistance to virtually all β-lactams, except for the novel cephalosporines ceftaroline and ceftobiprole (Cheng et al., 2008; Jacqueline et al, 2009). MRSA represents a major therapeutic problem, also due to the extraordinary ability of staphylococci to acquire resistance to other antimicrobials.

3.2.2. Resistance to other antimicrobials

Resistance to antimicrobials that inhibit the synthesis of bacterial cell wall

Glycopeptides. The mechanism of action of glycopeptide antibiotics is based on the inhibition of cell wall synthesis. Unlike β-lactams, glycopeptides act on the substrates of the PBPs. Within this group of antibiotics, vancomycin has been considered the primary treatment for MRSA infections. However, the first resistant strains have already appeared (Appelbaum, 2006). Two resistance mechanisms have been observed, (i) the thickening of the cell wall and an increase in the expression of PBP2a, what confers low level resistance, and (ii) due to the acquisition of *van*A gene, what confer high level resistance (CDC, 2002). Resistance to glycopeptides has not been detected in *S. pseudintermedius* to date.

Resistance to antimicrobials that inhibit protein synthesis

Tetracyclines. Tetracyclines act by inhibiting protein synthesis by binding to the 30S subunit of the ribosome. Tetracycline resistance in staphylococci is frequent and may be due to an increased active efflux or ribosomal protection. The genetic determinants

responsible for the first mechanism are the genes *tet*(K) and *tet*(L) and those for the second mechansims are *tet*(M) and *tet*(O). The gene most commonly found in *S. aureus* is *tet*(K) (which produces only tetracycline resistance) and the one most commonly detected in *S. pseudintermedius* is *tet*(M) (which involves cross-resistance to tetracycline, doxycycline and minocycline) (Guardabassi et al., 2004; Kadlec et al., 2012b; Schmitz et al., 2001).

Macrolides-Lincosamides-Streptogramins type B (MLS_B). These antimicrobials structurally differ but are part of the same group because they share mechanisms of action and resistance. All of them act on the 50S subunit of the bacterial ribosome, inhibiting the translocation of the peptide chain in protein synthesis, although each has a distinct binding site. Various resistance mechanisms have been described to these antimicrobials in Gram-positive bacteria, which are based on:

- Modification of the target (23S rRNA) by the action of methylases encoded primarily by *erm* genes, and rarely by the *cfr* gene or by mutations in the 23S rRNA and / or ribosomal proteins L3, L4 and L22.

- Active efflux of the antimicrobial from the cell, mediated by the genes *msr*(A)/*msr*(B), *mef*(A), *mef*(E), *erp*(B), *vga*(A), *vga*(B), *vga*(C), *vga*(D), *vga*(E), *lsa*(A), *lsa*(B), *lsa*(C) and *lsa*(E).

- Antimicrobial inactivation by the action of enzymes encoded by the genes *lnu*(A), *lnu*(B), *lnu*(C), *vat*, *vgb* and *mph*(C).

Different antimicrobial resistance phenotypes are exhibited depending on the antimicrobial affected. The most common is the so-called **MLS_B**, which consists of resistance to macrolides, lincosamides and streptogramin B, and it is due to the presence of *erm* genes. The genes *erm*(A) and *erm*(C) are the most frequently found in *S. aureus*. It is believed that the former is more common in MRSA and the in among MSSA (Schmitz et al., 2000), but many exceptions exit (Spiliopoulou et al., 2004). Other *erm* genes that have been also detected in staphylococci are *erm*(B), *erm*(T), *erm*(Y) and *erm*(TR) (Werckenthin et al., 2001). MLS_B phenotype can be constitutively or inducibly expressed. Macrolides are the inductor of the latter mechanism (Leclercq, 2002). Another phenotype of resistance is **MS**, which consists of resistance to 14- and 15-membered ring macrolides and to streptogramins B, but neither to 16-membered ring macrolides nor to lincosamides. In staphylococci, this phenotype is usually due to the presence of the genes *msr*(A) and *msr*(B), and is more

common among CoNS than in CoPS. Other phenotypes of resistance Other unusual resistance phenotypes are **L** (lincosamides resistance), **LS_A** (lincosamides and estretograminas A), **S_A** (streptogramin A) and **S_B** (streptogramin B). These phenotypes are due to the presence of genes such as *lnu*(A), *vga*(A), *vga*(C), *vga*(E), *lsa*(B) and *cfr*. These genes have been very occasionally detected in staphylococci and, in recent years, they have been associated with animal MRSA strains (Crombé et al., 2013; Kadlec et al., 2012a, Lozano et al., 2012a, Lozano et al., 2012b). In *S. pseudintermedius*, MLS_B resistance is predominant, which normally carry the *erm*(B) gene, while the other phenotypes are highly uncommon (Guardabassi et al., 2004; Kadlec et al., 2012b).

Aminoglycosides. These antimicrobials act by inhibiting protein synthesis after binding irreversibly to the 30s subunit of the bacterial ribosome. They interfere with the elongation of the peptide chain and induce mRNA mistranslation, causing erroneous amino acid incorporations in the forming protein. The mechanisms of resistance to these agents include (i) defects in the entrance of aminoglycoside into the bacterial cell, (ii) changes in the ribosome and (iii) enzymatic modification of the antimicrobial with loss of affinity for the ribosome. In staphylococci, resistance to these agents is mainly due to the action of modifying enzymes encoded by genes *aacA-aphD* (confers resistance to gentamicin, tobramycin and kanamycin), *aadD* (resistance to kanamycin, neomycin and tobramycin), *aphA3* (resistance to kanamycin) and *str*, *aadE* and *aadA* (resistance to streptomycin) (Van de Klundert et al., 1993). In *S. pseudintermedius*, *aadE* is predominant (Kadlec et al., 2012b) while the *str*, although not very prevalent, is more commonly detected in *S. aureus*.

Phenicols. These agents bind to the 50S subunit of the ribosome blocking protein synthesis by inhibiting the activity of the enzyme peptidyl transferase. Resistance to these antimicrobials is due by several mechanisms; (i) production of inactivating enzymes, (ii) modification of the target (which may imply other resistances) and (iii) reduced permeability of the bacterial cell wall. In staphylococci, phenicol resistance is mediated either by the chloramphenicol acetyltransferase encoding *cat* genes (cat_{pC194}, cat_{pC221} and cat_{pC223}) (Schnellmann et al., 2006), which confer resistance to non-fluorinated phenicols (e.g. chloramphenicol) or any of the two genes *fexA* (coding for a phenicol specific efflux pump) or *cfr* (coding for a rRNA methyltransferase), both of which mediate combined resistance to fluorinated (e.g. florfenicol) and non-fluorinated phenicols (Kehrenberg et al., 2006; Schwarz et al., 2004). In *S. aureus* and *S. pseudintermedius* the most common chloramphenicol

resistance gene is cat_{pC221}, while the phenicol resistance genes *fexA* or *cfr* had not been detected in *S. pseudintermedius* prior to the development of this thesis.

Mupirocin. This agent inhibits the bacterial protein synthesis by binding to isoleucyl-tRNA synthetase preventing the incorporation of isoleucine to the protein (Morton et al., 1995). There are two mechanisms of resistance: (i) mutation in the native gene encoding the enzyme isoleucyl-tRNA synthetase (*ileS*) and (ii) acquisition of the gene *mupA* (also known as *ileS2*) that encodes an enzyme isoleucyl-tRNA synthetase without affinity for mupirocin additional. The former mechanism produces low-level resistance and the latter high-level resistance (Cookson, 1998; Udo et al., 2001). In *S. pseudintermedius*, resistance to mupirocin is highly rare (Kadlec et al., 2012b).

Fusidanes. Fusidic acid is an inhibitor of bacterial protein synthesis that binds to the elongation factor G (EF-G) preventing it from being released from the ribosome. In staphylococci, this resistance may be due to (i) mutations in the gene encoding the EF-G (*fusA*) or (ii) acquisition of the gene *fusB* which encodes a protein that binds to EF-G and protects it from the action of the antimicrobial. The genes denominated *fusC* and *fusD* are very similar to the latter and have been described in different staphylococcal species (McLaws et al., 2008, O'Neill et al., 2007). A single study is available in which fusidic acid resistance, based on the gene *fusC,* was detected in two *S. pseudintermedius* isolates (O'Neill et al., 2007).

Resistance to antimicrobials that alter nucleic acid synthesis

Quinolones. The quinolones act by interfering with the action of the chromosomal topoisomerase II (or DNA-gyrase, encoded by genes *gyrA* and *gyrB*) and IV (*grlA* and *grlB*) of the bacteria, which are involved in the synthesis of DNA. The major mechanism of resistance to these agents in staphylococci is produced by modification of the target due to mutations within the quinolone-resistance-determining-region (QRDR) of GrlA and/or GrlB subunits (DNA topoisomerase IV) and/or GyrA and/or GyrB (DNA gyrase). In addition, in *S. aureus*, alteration of the entry of the agent by mutations of the *norA* gene have been detected (Nakanishi et al., 1991). Generally, the mutations responsible for the resistance in *S. aureus* occur first in GrlA protein (the most common substitution being Ser80Phe) and subsequently in GyrA (normally modification of Ser84Leu). Although quinolone resistance is common among MRSP, very scarce data is to date available at molecular level (Descloux et

al., 2008; Kadlec et al., 2012b). Amino acid substitutions at positions previously identified to play a role in quinolone resistance, such as Ser80Ile and Asp84Asn in topoisomerase IV (gene *grlA*) and Ser84Leu and Glu88Gly in topoisomerase II (gene *gyrA*) have been detected (Kadlec et al., 2012b). A couple of additional mutations at positions Thr678Ala and Glu714Lys outside the QRDR of the *gyrA* gene have been observed to result in higher MICs (>16 mg/L) to enrofloxacin among *S. pseudintermedius* strains (Descloux et al., 2008), but those strains also exhibited mutations within the QRDR region of those genes.

Resistance to antimicrobials that alter metabolic pathways

Diaminopyrimidines. Trimethoprim is a bacteriostatic diaminopyrimidine and inhibits the activity of the enzyme dihydrofolate reductase (DHFR), leaving the bacteria deficient in folate, which is essential for purine biosynthesis and thus of DNA. The most common resistance mechanisms against this agent are (i) antimicrobial efflux, (ii) reduced permeability, (ii) mutation of the natural *dfr* genes encoding the DHFR, (iv) overproduction of the enzymes or (v) production of additional DHFR enzymes that are resistant to the action of trimethoprim (Huovinen, 2001; Schwarz et al., 2001). The mechanism most frequently found in staphylococci is the production of additional DHFR enzymes encoded by the genes *dfrA* (also known as *dfrS1*), *dfrD*, *dfrG* and the recently described *dfrK* (Kadlec et al. 2009c; Schwarz et al., 2001). In *S. pseudintermedius*, the gene *dfrG*, which was described first in 2005 in a nosocomial *S. aureus* isolate (Sekiguchi et al., 2005), is predominant.

4. ELEMENTS OF DISEMINATION OF RESISTANCE AND VIRULENCE DETERMINANTS

The basic genetic mechanisms of resistance gene transfer between bacteria that live in the same environment - named horizontal gene transfer - are transformation, transduction and conjugation. **Figure 12** shows a schematic representation of the three processes.

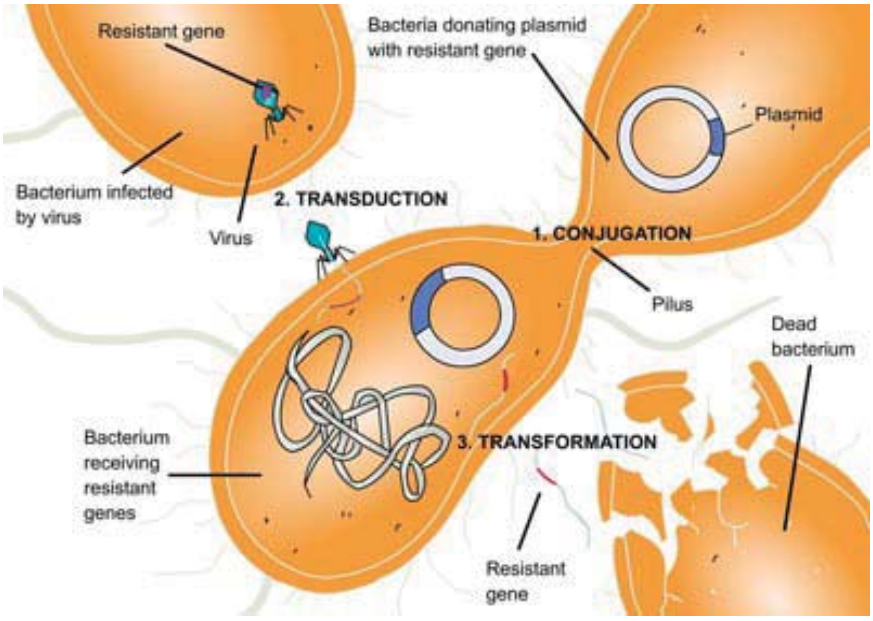


Figure 12. General mechanisms of acquisition of resistance genes.

Bacterial **conjugation** is the process by which plasmids and conjugative transposons can be transferred from a donor cell into a recipient cell by direct contact through the sex pili. This mechanism exists in a wide variety of bacterial genera and species. **Transduction** is the process by which a bacteriophage (bacterial virus) transfers exogenous DNA to a recipient bacterium. This phenomenon is usually restricted to bacterial clones and is confined to a range of hosts. Finally, **transformation** is a process that involves the uptake of naked DNA, usually from the environment, due to bacterial autolysis, to a competent bacterium (Davison, 1999).

Among these three possible ways bacteria can acquire genes, transduction by bacteriophages plays and important role in this genus. Conjugation does not occur frequently since conjugative plasmids and transposons are not spread among staphylococcal populations and transformation is not efficient (McCarthy et al., 2012).

Horizontal gene transfer is then normally mediated by mobile genetic elements (MGEs). Thanks to these elements, bacteria have the ability to evolve and adapt to the environment. Staphylococci, in special *S. aureus,* contain many types of MGEs, including plasmids, insertion sequences (IS), transposons (Tn), bacteriophages, pathogenicity islands, and staphylococcal cassette chromosomes. It is remarkable that most genes encoded by MGEs remain under the control of global regulators located within the core genome (Malachowa et al., 2010). Furthermore, although horizontal gene transfer is common among

organisms belonging to the same species or the same genus, this process has also been detected among bacteria of different genera, and even among Gram-positive and Gram-negative bacteria.

4.1. PLASMIDS

Plasmids are extrachromosomal doble strand auto-replicating DNA molecules. Plasmids do not confer essential functions for the survival of the cell, but they normally carry genes that confer an adaptive advantage to the bacteria, such as antimicrobial resistance genes, metal resistance genes and/or virulence genes. Plasmids are present in almost all bacterial species with highly variable sizes, from 2 kb to up to 400 kb (Schwarz et al., 2011). All plasmids present a core region or "backbone" and a variable region that serves the bacteria to acquire adaptive advantages. This backbone region is characteristic of each plasmid type and consists of a group of genes that serve for maintenance, replication and propagation of the bacteria (Garcillán-Barcia et al., 2011).

Staphylococci typically carry one or more plasmids per cell and these plasmids have varied gene content. Staphylococcal plasmids can be classified into one of the three following groups: (i) small multicopy plasmids that are cryptic or carry a single resistance determinant; (ii) larger (15–30 kb) low copy (4–6/cell) plasmids, which usually carry several resistance determinants; and (iii) conjugative multiresistance plasmids (Malachowa et al., 2010).

Larger plasmids undergo **theta** or **strand displacement replication**, while small plasmids replicate by a **rolling-circle replication** (RCR) mechanism (Malachowa et al., 2010). Staphylococci usually present small plasmids that present a RCR mechanism. RCR plasmids comprise a group of small high copy number plasmids that are common in Gram-positive bacteria (Khan, 2005). They are divided in families based on the Rep protein (with endonuclease activity) and the *dso* homologies and many of them carry antimicrobial and heavy metal resistance genes and/or insertion sequences and transposons, The *dso* region is essential for the replication of the leading strand of RCR plasmids (Khan, 2005). RCR plasmids contain an additional signal involved in initiation of the synthesis of the lagging-strand (**Fig. 13**), the *sso*, among them, the *ssoA* is only efficiently recognized by the machinery of its natural host (Del Solar et al., 1998; Khan, 2005; Kramer et al., 1998). Two segments, the recombination site B (RS_B) and a 6-bp sequence (5'-TAGCGT-3', termed CS-6),

are conserved in the *ssoA*s (Kramer et al., 1998). The RS_B, located in the basal part of the *ssoA* secondary structure, is important for binding of the RNA polymerase to generate an RNA primer for initiation of replication, while the CS-6 region, located in the terminal loop of the palindromic structure, functions as the termination site of such RNA primer synthesis (Kramer et al., 1998).

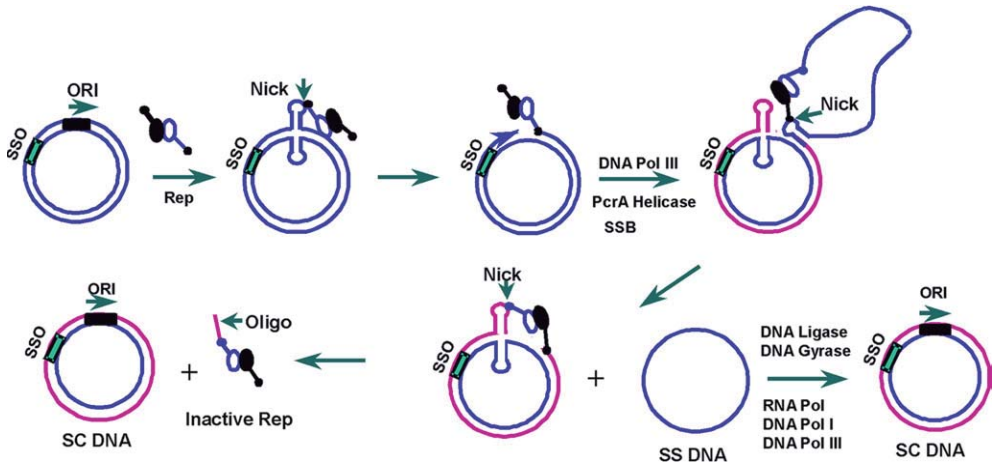


Figure 13. A model for RCR plasmid (Taken from Khan, 2005).

Plasmids can be also classified based on their transferability to other cells in **conjugative** and **mobilizable**. Conjugative plasmids are large plasmids that contain genes encoding proteins that allow its authonomous transfer to another cell. However, these plasmids are uncommon in staphylococci, and when present, they normally originate from other genera, such as *Enterococcus* spp. (Lindsay, 2010). Mobilizable plasmids do not possess the complete machinery for transfer, so they need part of the machinery of conjugative plasmids. This latter group can also be divided in **mobilizable**, for plasmids that present some of the genes for transfer (such as *mob* genes) and **non-mobile**, for very small plasmids that only present a basic replicon in addition to a single resistance element, and need to integrate in mobilizable or conjugative plasmids or in other MGEs for transference (normally RCR plasmids).

4.2. TRANSPOSONS

Transposable elements are MGEs that possess the required machinery to transposase from a donor to an aceptor site within the genome of the bacterium. Transposition can take place between plasmids or between a plasmid and the chromosomal DNA of the bacterium. Generally, these mechanisms do not require homology between both

sites (excision and integration sites) and have the ability to transpose randomly in the genome. However, some transposable elements exhibit a strong tendency to be inserted in specific nucleotide sequences or recognition sites (Roberts et al., 2008; Wagner, 2006). Enzymes that catalyze the transposition are called transposases (*tnp* genes). In general, the transposase cuts the donor DNA at the extremes of the transposable integrated element for excision and subsequent integration in the novel target DNA.

These elements can be classified in two major groups based on their genetic content:

Simple transposon or insertion sequence (IS): They are the simplest transposable elements, which range between 700 and 1000 bp. ISs consists of a gene encoding a transposase enzyme responsible for transposition, flanked by two inverted repeats of size between 10 and 40 bp, one at each end (Wagner, 2006). The ISs are distributed throughout the genome in variable locations and number according to species and strains. They are involved in deletions, insertions or bacterial genome rearrangements that have a large effect on its assembly and the clustering of genes with adaptive functions, such as antibiotic resistance, virulence or catabolic functions (Bennet, 2004; Haren et al., 1999) (**Fig. 14**). Some ISs may form an hybrid promoter with the native promoter of a gene, as is the case of IS*256* and IS*257*, and the resistance genes *aacA-aphD* and *dfrA* (Malachowa et al., 2010). In addition, IS inactivates genes by direct insertion or by having a polar effect on the transcription of nearby genes. It has been observed that some ISs constituting as part of transposons, may lead to free circular intermediates (Prudhomme et al., 2002). Although ISs can exist independently in the *S. aureus* genome, they are often present as pairs constituting a composite transposon (Byrne et al., 1989).

Composite transposon (Tn): Their size varies from 2 to 50 kb. They are formed by an IS at each end and a central DNA sequence that can locate a multitude of genes capable of modifying the bacterial phenotype, such as resistance genes. Transposons are flanked by two identical ISs in direct or inverse orientation, allowing transposition into different sites (Wagner, 2006) (**Fig. 14**). The smaller transposons are usually presented in multiple copies in the staphylococcal genome, either inserted into the chromosome or into MGEs, such as SCC or plasmids. In *S. aureus*, composite transposons carrying genes for resistance to trimethoprim, aminoglycosides or vancomycin have been described (Malachowa et al., 2010).

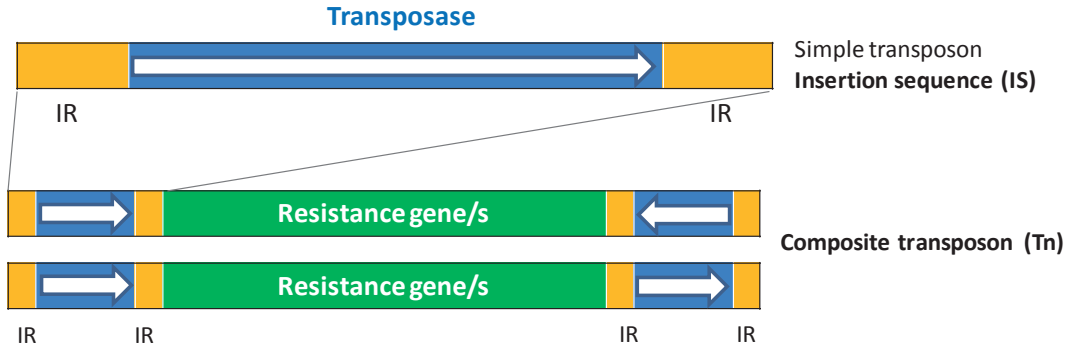


Figure 14. Basic structure of tranposable elements. IR, inverted repeat.

Both ISs and composite transposons are widely distributed among the genome of staphylococci. They may be present in a single copy or multiple copies on the chromosome or in association with other MGEs.

Conjugative Transposons: they are elements that are able of excise and integrate into a new genomal DNA by conjugation. They may contain a wide range of accessory genes including resistance genes. An example of such a transposon in *S. aureus* is the Tn*6014*, which contains the tetracycline resistance gene *tet*(M) (De Vries et al., 2009).

Mobilizable transposons: these transposons can be mobilized with the help of other elements that encode proteins required for the pore formation needed for conjugation, similar to what happens with plasmids.

Tn*3* family transposons: they are elements encoding an enzyme involved in the excision and integration (transposase), a site specific recombinase (resolvase) and a variable region that normally carry resistance genes (**Fig. 15**). This family of transposons are also flanked by two inverted repeats at the integration site. They are small transposons that normally have multiple copies in the genome. This type of transposons do not present conjugative properties but they show a widespread distribution given that many of them are part of conjugative plasmids. This group includes the staphylococcal Tn*554*, which harbour genes that confer resistance to macrolides and spectinomycin [*erm*(A) and *spc*]; the Tn*559*, responsible for thrimethoprim resistance due to the presence of the recently described *dfrK* gene (Kadlec et al., 2010b) and the Tn*558*, implicated in phenicol resistance (*fexA* gene) (Kehrenberg et al., 2005; Murphy, 1983).

Figure 15. Basic structure of the transposon family Tn3.

Transposition can occur by two mechanisms. A mechanism called **conservative transposition** where the transposon excise from the genome and integrate in another region, therefore without increasing the number of copies in the cell. The mechanism of **replicative transposition** implies that the transposon remains at its original location but, by a replication-recombination mechanism, a new copy of the same element appears and integrates elsewhere in the bacterial genome; thus, increasing the number of copies of the transposon within the bacteria (Bennett, 2004).

4.3. BACTERIOPHAGES

Bacteriophages or phages are viruses that exclusively infect bacteria and seem to have a great impact on staphylococcal diversity and evolution (Malachowa et al., 2010). All phages are classified into one of three distinctive groups: lytic, temperate, and chronic. **Lytic** phages have been used in phage therapy, because bacteria lyse completely during release of progeny phages. Bacteria infected with **chronic** phages release progeny into the extracellular environment without killing the host, which allows bacteria to grow and divide. **Temperate** phages, form the most numerous group among all phages. Temperate phages have the ability to lyse bacteria after infection, but they typically form a long-term relationship with the host cell, whereby the phage DNA integrates into the staphylococcal genome as a prophage (Goerke et al., 2009; Malachowa et al., 2010; Mann, 2008). Bacteriophages can encode different toxins such as enterotoxin A (SEA), the exfoliatin Eta, or leukotoxins such as PVL and LukM/LukF-PV and are widely distributed in strains of *S. aureus* (Kaneko et al., 2004; Lindsay, 2010). It is believed that all *S. aureus* strains carry a bacteriophage and some may contain up to four.

Prophages and prophage-encoded molecules also work in concert with other MGEs within staphylococci. For example, prophages create mobility for some staphylococcal pathogenicity islands (see below). Some phages also have the ability to transfer antibiotic resistance by transduction of plasmids or plasmid elements previously incorporated into chromosomal DNA. Plasmid pS194 with a chloramphenicol resistance determinant (cat_{pS194})

and pI258 containing erythromycin resistance [*erm*(C)] are likewise transduced (Jensen et al., 2009). A classification system based on the gene that determines the site of integration in the genome has been established (Lindsay et al., 2004).

Figure 16 shows the three MGEs described above that provide bacteria resistance and virulence properties by horizontal gene transfer, as well as their interactions.

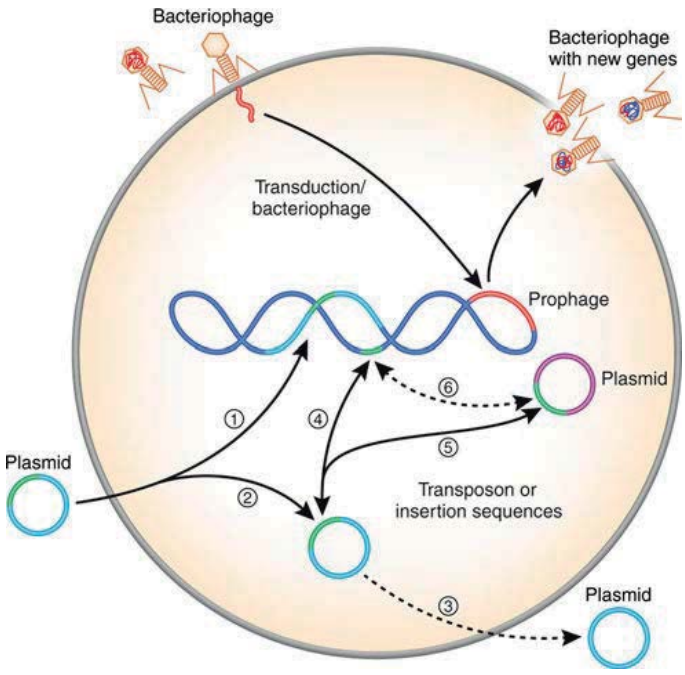


Figure 16. Acquisition of MGEs by Staphylococci. 1 Incorporation of plasmids or plasmid elements into genomic DNA. 2 Plasmids can be maintained as free circular DNA. 3 Suicide plasmid. 4 Transfer of a transposon or an insertion sequence between plasmid and genomic DNA. 5 Transfer of a transposon or an insertion sequence between plasmids within the cell. 6 Transfer of a transposon or an insertion sequence from genomic DNA to another plasmid. (Taken from Malachowa et al., 2010).

4.4. PATHOGENECITY ISLANDS

Staphylococcal pathogenicity islands (SaPIs) are MGEs of 14–17 kb in size. At least 16 SaPIs have been sequenced to date (Malachowa et al., 2010; Novick et al., 2007). In addition to core genes (common for all SaPIs), almost all SaPIs encode enterotoxins, the exfoliatin Etd or the TSST (Novick et al., 2007; Yarwood et al., 2002). Staphylococcal pathogenicity islands are integrated in one of six different specific sites on the chromosome (atts) and each is always in the same orientation (Novick et al., 2007). They differ from phages in the absence of genes necessary for the construction of the capsid, or the apparatus required for the horizontal transfer. Therefore, SaPIs are mobilized following

infection by certain staphylococcal bacteriophages or by induction of endogenous prophages (Lindsay, 2010).

4.5. GENOMIC ISLANDS

Three families of genomic islands exist among the *S. aureus* strains whose genomes have been sequenced (Baba et al., 2002; Gill et al., 2005; Ito et al., 2003; Lindsay et al., 2004). These genomic islands, named νSAα, νSAβ y νSAγ, are flanked by a broken transposase gene upstream and partial restriction-modification system type I downstream. Given the composition of genomic islands (remnant transposase genes and a G + C content that differs from the core genome), a current notion is that genomic islands were once mobile elements acquired by horizontal gene transfer (Dobrindt et al., 2004). νSAα carry superantigen-like genes (*ssl*) and νSAβ the leukocidin LukE/LukD, enterotoxins (SEG, SEI, SEM, SEN, SEU) and bacteriocins, among others (Argudín et al., 2011; Malachowa et al., 2010).

4.6. STAPHYLOCOCCAL CASSETTE CHROMOSOME

Staphylococcal cassette chromosomes (SCCs) are relatively large fragments of DNA that always insert into the *orfX* gene on the *S. aureus* chromosome. SCCs can encode antibiotic resistance and/or virulence determinants. SCCs are classified into staphylococcal cassette chromosome *mec* (SCC*mec*) or non-SCC*mec* groups. The later group contain virulence or fitness/survival determinants (Malachowa et al., 2010).

5. EPIDEMIOLOGY OF *S. aureus*

5.1. *S. AUREUS* IN HUMANS

S. aureus is a major cause of infections in humans for as long as we have historical records. With the evolution of man and medicine, *S. aureus* too has evolved and adapted to a wide variety of human conditions and medical innovations. Historically, *S. aureus* was certainly a significant human pathogen prior to the development of antibiotics. For example, in the last century, *S. aureus* was the major bacterial cause of death in the influenza pandemic of 1918, among those who developed secondary bacterial pneumonia. Following the introduction of antibiotics, *S. aureus* developed resistance to penicillin in the 1940s, and

then emerged as an important cause of serious nosocomial infections in the 1950s (Weigelt, 2008).

As mentioned above, the first report on clinical cases of MRSA dates back to 1961, in the United Kingdom (Jevons, 1961), while the first MRSA hospital outbreak was reported in a children's hospital in 1963 (Stewart et al., 1963). From then on, nosocomial MRSA infections emerged, though infrequently, worldwide. In the late 1980s and the early 1990s, MRSA gradually increased in frequency and became a serious pathogen in hospitals throughout the world, the so-called health-care or hospital-associated MRSA (HA-MRSA) (Enright et al., 2002; Grundmann et al., 2006). Nowadays, MRSA is one of the main causes of nosocomial infection worldwide (Grundmann *et al.,* 2006). In some hospitals, over half of the *S. aureus* infections are MRSA. The prevalence varies greatly depending on the country and even from one hospital to another. Globally, despite the lack of information from some countries, there is a gradient of prevalence, giving low values (5-10%) in Canada and Saudi Arabia and higher values (10-25%) in Mexico, Venezuela, Tunisia, Morocco and India. The prevalence increases significantly in the United States, Brazil, Chile, South Africa and Australia, among others, which reaches up to 50%. Colombia and Japan are among the countries that exceed the value of 50% (Grundmann et al., 2006). In Europe, according to data published by the European Surveillance System Resistance (EARSS) (www.earss.rivm.nl), there is a prevalence gradient from north to south (**Fig. 17**). In northern countries such as Finland, Sweden, Norway and Denmark, and the Netherlands, the incidence is lower (<1-5%), whereas in most Central European countries are average values (10-25%) and southern countries (Italy, Portugal, Croatia, Turkey) and the UK is estimated a percentage of MRSA between 25 and 50%. These differences may be due to several factors, (i) the different antimicrobial use policies, (ii) the subsequent antimicrobial selective pressure and (iii) different infection control and prevention programs, such as the strict Search and Destroy policies in some north European countries. Still, it is interesting to remark that Spain has reduced the number of MRSA cases of bacteraemia from 2010 (25-50%) to 2011 (10-25%) (**Fig. 17**).

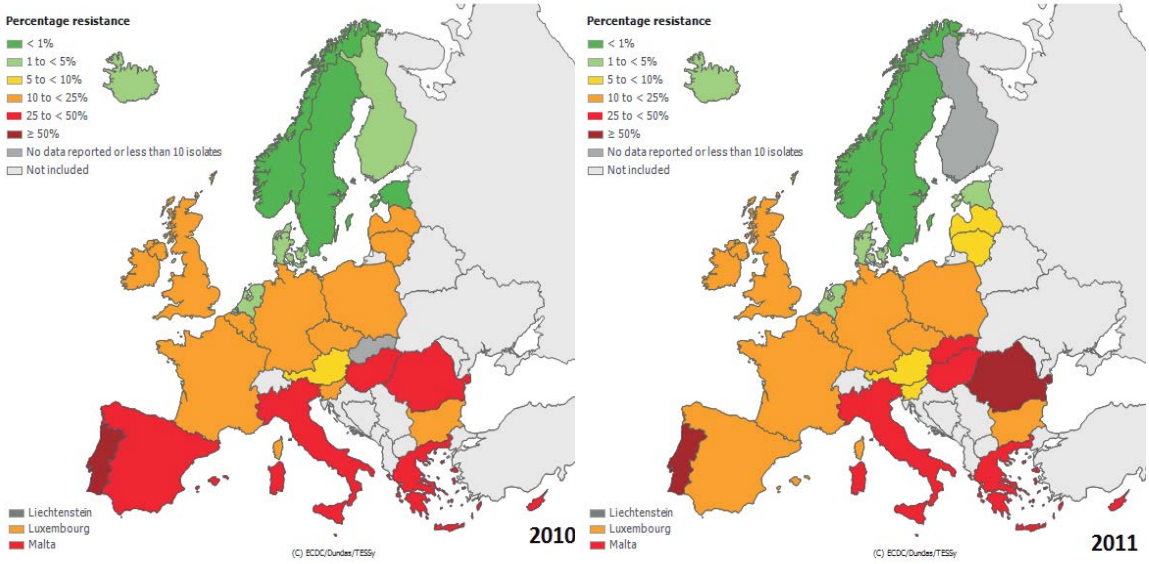


Figure 17. Geographic distribution of MRSA bacteremia rate during 2010 and 2011 at Europe. Adapted from European Antimicrobial Resistance Surveillance System (EARSS), 2011 (EARSS, 2011).

On the other hand, few data are concerning Africa and Asia but it is estimated that the prevalence may be very high, given the lack of restrictive measures on the use of antibiotics.

In many cases, these infections are associated with hospital outbreaks caused by certain epidemic clones (Deurenberg et al., 2008). There are several established risk factors for MRSA patients: immunosuppression, surgery, indwelling catheters or previous hospitalization, among others (**Table 4**). Control measures are needed because colonized patients and health professionals (as well as instrument material) are large reservoirs of MRSA in the hospital setting.

Since the 90s, however, the epidemiology of this microorganism is undergoing significant changes. The emergence of these bacteria is no longer a phenomenon exclusive to the hospital setting. In 1993, an infection caused by a community-acquired MRSA (CA-MRSA) was isolated from an Aboriginal in Australia (Udo et al., 1993). Today, there are numerous studies that report MRSA infections in community patients with neither previous contact with the health system, nor typical risk factors associated with infection by this organism (Lo et al. 2009; Makgotlho et al., 2009; Zaoutis et al., 2006). Clinically, CA-MRSA causes mostly skin and soft tissue infections (SSTIs), but it can also cause necrotizing pneumonia, or bacteraemia. Transmission is primarily spread by direct contact, and small

outbreaks have been described in certain population groups (contact sports players, soldiers, prisoners, tattooed, nurseries) (Deresinski et al., 2005).

Several clones that are associated with community-acquired infections are spread worldwide. In the USA, the clonal lineage ST8 (known as USA300) is endemic, which is leading cause of SSTIs, although cases have also been found in European countries (Faria et al., 2005), in Australia (Nimmo et al., 2008) and in Asia (Shibuya et al., 2008).

CA-MRSA strains differ from HA-MRSA strains not only in the clinical symptoms and its epidemiology, but they also typically present distinct genotypic characteristics. They have a different accessory genome, carry different staphylococcal cassette chromosome *mec* (SCC*mec*) elements, affect different populations, and cause other clinical symptoms (Enright et al., 2002; Grundmann et al., 2002; Ito et al., 2004; Naimi et al., 2003; Okuma et al., 2002; Tacconelli et al., 2008; Tenover et al., 2006; Vandenesch et al., 2003; Wijaya et al., 2006; Witte, 2009; Yamamoto et al., 2010) (**Table x**). Also, the PVL toxin is closely associated with CA-MRSA infections, which normally is absent in HA-MRSA (Vandenesch et al., 2003) (**Table x**).

Table 4. Major differences between typical HA-MRSA and CA-MRSA.

	HA-MRSA	**CA-MRSA**
Clonal lineage	CC5, CC8, CC22, CC30, CC45	CC1, CC8, CC30, CC59, CC80, CC93
SCC*mec*	I, II, III	IV, V, VII
agr	III	I, II
Virulence	Not PVL	PVL
Antimicrobial profile	Normally MDR	Narrow resistance profile. Usually only β-lactam resistant
Type of patient	Elderly, debilitated and / or chronic infections	Children and young people, athletes and sportsmen, military personnel, prisoners, drug users
Risk factors	Immunocompromised, surgery, catheters, previous hospitalization	Athletes and other sports, prisoners, child care, drug users, soldiers, tattooed persons
Clinical	Severe. Bacteraemia with no obvious source of infection, open sores, surgical wounds, urinary catheters. It may cause ventilator associated pneumonia	Skin and soft tissue infections (SSTIs) (cellulitis, abscesses) usually mild. Necrotizing pneumonia, bacteraemia and septic shock, bone infections, etc..

Presently, however, it becomes more difficult to distinguish HA-MRSA from CA-MRSA, since clones with a typical hospital-acquired genetic background enter the community and typical clones with community-acquired genetic background enter the hospital (Campanile et al., 2011; González-Domínguez et al., 2012; Song et al., 2011; Song et al., 2011; Stefani et al., 2012). This is also reflected in the fact that typical CA-MRSA clones can show MDR profiles, SCC*mec* types commonly found among HA-MRSA and lack the virulence genes commonly detected in CA-MRSA (Deresinski, 2005; Jeong et al., 2005).

While most countries have only very limited information on MRSA carriage rates in healthy people, estimates tend to be below 2%, even where MRSA is endemic in hospitals such as in the UK, the USA, Italy, Spain or Portugal (Abudu et al., 2001; Lozano et al., 2011a; Maudsley et al., 2004; Sa-Leao et al., 2001; Shopsin et al., 2000; Zanelli et al., 2002;).

5.2. *S. AUREUS* IN ANIMALS

AS mentioned before, *S. aureus* can also be isolated from animals either as part of the normal microbiota, mainly in the nasal mucosa, or as causative agent of infection. However, in contrast to humans, *S. aureus* isolates in animals, as well as its antimicrobial susceptibility, has been initially less continuously documented. Although there is an increasing interest to know the circulating lineages and molecular characteristics of *S. aureus* in animals, both MSSA and MRSA, there is still very scarce data on MSSA, which makes difficult addressing its epidemiology.

The first isolation of MRSA was reported in 1972 from milk samples from cases of bovine mastitis, with isolates that were believed to belong to human associated lineages (Devriese et al., 1972; Devriese et al., 1975). Since then MRSA has been found in a variety of other domestic species including dogs (Loeffler et al., 2005; Pak et al., 1999), cats (Bender et al., 2005), horses (Anzai et al., 1996; Hartmann et al., 1997), sheep (Gharsa et al., 2012; Goni et al., 2004) pigs (Voss et al., 2005) and chickens (Lee, 2003) leading to an upsurge of reports and interest in MRSA colonization and infection in animals. The presence of *S. aureus* in animals plays an important role in its global epidemiology since they may represent secondary reservoirs or vectors, and contact with positive animals may pose a risk to human health.

In relation to the presence of MRSA in animals, it is important to distinguish between companion animals and livestock (or food-producing animal). They constitute two different situation and strains normally exhibit different molecular characteristics.

5.2.1. MRSA in companion animals

Although the major microorganism isolated from dogs and cats is *S. pseudintermedius*, *S. aureus* can be also present at relatively high frequencies (<20%) in the skin and mucous of these animals (Weese et al., 2010a). *S. aureus* causes a wide range of opportunistic infections in companion animals, such as superficial skin infections, urinary, respiratory and postoperative infections and sometimes it is also the cause of major diseases.

MRSA was first detected in pets in the nose and skin of healthy dogs in Nigeria in 1972 and phage-typing suggested a human origin of the strains (Ojo, 1972). During the 1980s and early 1990s, sporadic case reports of MRSA isolated from animals were published, mainly in human medical journals. These referred to MRSA-contaminated or carrier pets implicated as vectors for human infection where infection control during human outbreaks proved difficult (Cefai et al., 1994; Scott et al., 1988). However, over the past 10 years, in line with the appearance and dramatic increase in CA-MRSA infections, MRSA has emerged as an important pathogen in veterinary medicine, especially in countries with a high MRSA burden in human hospitals (Leonard et al., 2008; Loeffler et al., 2010a). Since then, MRSA has been isolated from many other companion animal species including cats, rabbits, a guinea pig, a turtle and a chinchilla and also from birds, both as colonizer or as causative agent of infections (Leonard et al., 2008; Loeffler et al., 2005; Loeffler et al., 2010a).

Even though the incidence of infection remains largely unknown, MRSA can still be considered an uncommon pathogen in dogs and cats based on the frequency of MRSA isolation from clinical submissions to veterinary laboratories (<2%) (Haenni et al., 2012; Loeffler et al., 2010a) and slightly higher values in horses (<5%) (Cuny et al., 2006). As for MRSA carriage, prevalence rates vary between countries, regions and groups of animals sampled, with normally low ranges of <1% (Loeffler et al., 2010a). In contrasts, in animals admitted to referral hospitals, carriage rates are significantly higher (9-20%) (Loeffler et al., 2010a; Weese et al., 2010b).

Molecular typing studies reveal that the majority of MRSA strains isolated in companion animals show similar characteristics to those isolates from humans, especially HA-MRSA lineages (Loeffler et al., 2010a; Strommenger et al.; 2006; Weese et al., 2010). In fact, it has been observed that dogs normally carry the MRSA strains spread in the human population of the same geographic region (Weese et al. ,2010a; 2010b). In addition, although scarce data is available on methicillin-susceptible strains, it is expected to present a similar epidemiology. Therefore, it seems that humans are the source for *S. aureus* acquisition in pets (Van Duijkeren et al. 2004; Walther et al., 2009; Weese et al., 2005) and some authors have termed this transmission as "humanosis" (anthropozoonosis) (Morgan et al., 2008). Simultaneously, positive animals have been implicated as sources and vectors for recurrent re-colonization and re-infection human infection (Cefai et al., 1994; Manian et al., 2003; Scott et al., 1988; Sing et al., 2008; Walther et al., 2009). A recent study reported that 6.1% of dogs from MRSA-infected outpatients carried identical strains to those from their infected owners, representing a potential source of infection or re-infection for humans (Pinto-Ferreira et al. 2011). However, available data on the general *S. aureus* transmission between humans and companion animals are limited and the public healthy impact of such transmission needs to be the subject of more detailed epidemiological studies. MRSA contamination of veterinary clinic, hospital environments or household settings has also been recognized, what suggest that contaminated environments may also play an underestimated role in the transmission routes of *S. aureus* between in-contact persons (owners, veterinary staff) and pets. (Loeffler et al., 2010a).

While MRSA strains isolated from pets tend to be of human origin, those from horses are of a more diverse genetic background and their origin remains largely unknown. Genetic analysis have identified a major predominant clone among horses, CC8, including ST8, ST254 and ST247, mostly lineages that had been associated with human HA-MRSA in the past but which have since been superseded (Loeffler et al., 2010a). However, equine-to-human transmission has been suggested by several groups (Seguin et al., 1999).

How far companion animals provide a true reservoir for *S. aureus*, both MRSA and MSSA, or whether they should only be considered as contaminated living vectors remains unclear. While the definition of a reservoir implies that the host animal can maintain the pathogen indefinitely (Ashford, 2003), this has not been investigated for any of the companion animal species to date (Loeffler et al. 2010a). On the contrary, there are suggestions that MRSA carriage is not sustained for long periods by companion animal hosts

in a clean environment, as it has been observed in several studies after cleaning, disinfection and/or prevention control measures. To this respect, MRSA carriage resolved in 16 healthy rescue dogs identified during cross-sectional screening with daily cleaning and disinfection of the kennel environment alone (Loeffler et al., 2010b).

Since these species, especially dogs and cats, are typically kept for companionship, and the social behaviours with pets has substantially evolved in the last decades, transmission of *S. aureus* between humans and their pets may increase either by direct contact or by sharing the same environment in the household (sofas, beds, etc). These factors, together with the fact that *S. aureus* is not the predominant microorganims in these animals, place dogs and cats in a special position with respect to other animal species in the epidemiology of human *S. aureus*. Colonized companion animals play an important role, and sometimes underestimated, in the epidemiology of human *S. aureus*. Still very limited information is available on the occurrence of methicillin-susceptible strains among these animals and their possible implications in the global epidemiology of *S. aureus* (Boost et al., 2008; Hanselman et al., 2009; Walther et al., 2012).

5.2.2. Livestock associated MRSA (LA-MRSA) ST398

In recent years, a new MRSA clone associated with production animals has spread in Europe and is emerging worldwide (Crombé et al., 2013). This clone differs from the typical HA-MRSA and CA-MRSA lineages; is PFGE not typeable by standard methodology, presents a number of specific related *spa* types (t011, t034, t108, t1197, t2346, t899, t567, etc) and belongs to the MLST type ST398 (CC398). The pig is the major reservoir of this clone, which has been designated livestock-associated (LA) MRSA ST398. The first description of this novel wave in the global epidemiology of MRSA (LA-MRSA ST398) was reported among a family of pig farmers and their pigs in The Netherlands in 2005 (Voss et al., 2005). In parallel, in France, pig farming was observed to be a risk factor for *S. aureus* colonization in humans, where ST398 was included among several lineages identified in pigs and pig farmers (Armand-Lefevre et al., 2005). Since their discovery, LA-MRSA ST398 has been steadily reported among livestock, especially pigs, in numerous European countries (De Neeling et al., 2007; Lozano et al., 2011b; Lozano et al., 2011c; Pomba et al. 2009; Witte et al., 2007), Canada (Khanna et al., 2007), the USA (Smith et al., 2009), Peru (Arriola et al., 2011), Singapur (Sergio et al., 2007), China (Cui et al., 2009) and recently in Korea (Lim et al., 2012).

Although in Asia, ST9 constitutes the predominant LA-MRSA clone among swine (Crombé et al., 2013).

In 2008, the European Food Safety Authority (EFSA) underwent a European baseline study to determine the MRSA occurrence among breeding and production pig holdings among 24 European Union Member States (EUMS) and 2 non-EUMS states (Switzerland, Norway) (EFSA, 2009b). This long-range study used dust samples to estimate the MRSA and MRSA ST398 prevalence and revealed highly variable rates depending on the country (from 0-46% in breeding holdings; from 0-50.2% in production holdings), with Spain as the country with highest MRSA ST398 occurrence [breeding 46%; production 50.2% (**Fig. 18**)]. In spite of the high values recovered in some countries, it is estimated that due to the nature of the methodology (pooling of environmental wipes) the true LA-MRSA ST398 values were underestimated.

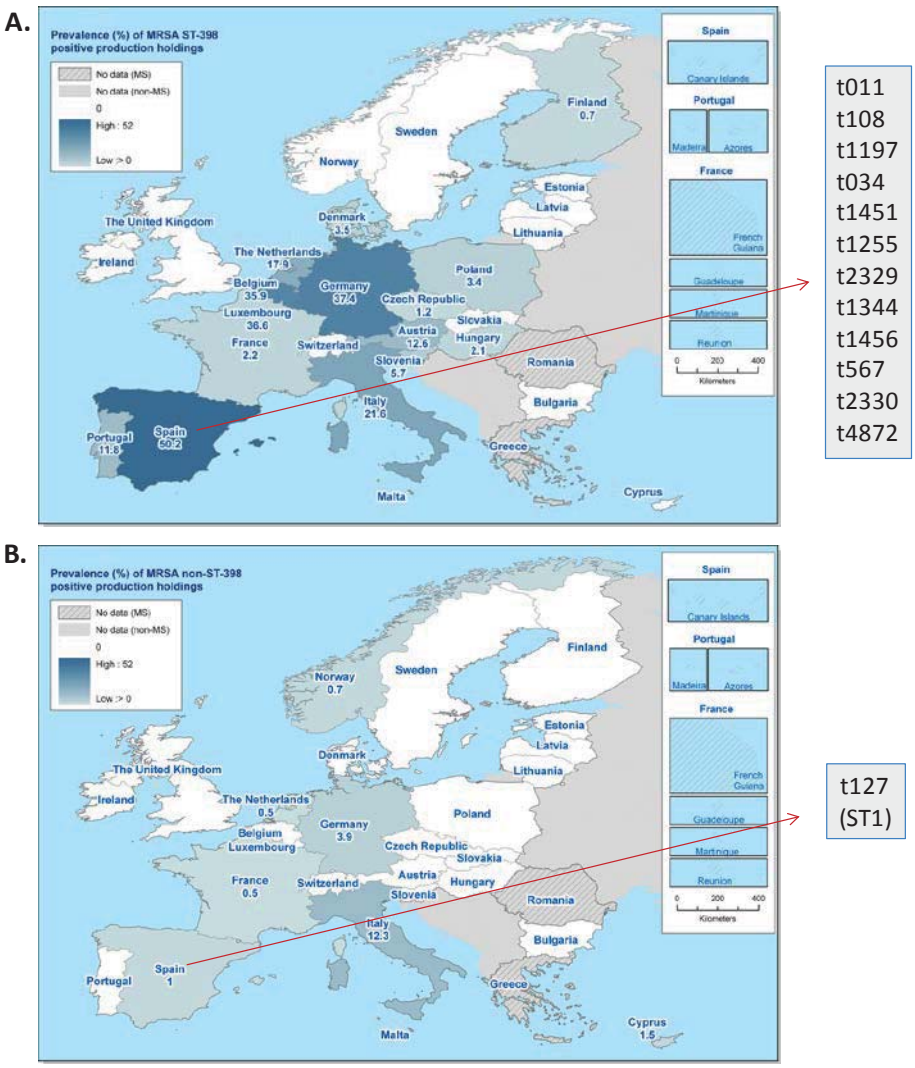


Figure 18. Prevalence (%) of **(A)** MRSA ST398 and **(B)** MRSA non-ST398 positive production holdings according to the EFSA report in 2008 (EFSA, 2009b). The different *spa* types detected in Spain are indicated. Source EFSA, 2009.

Colonization rates at animal level vary widely across studies, ranging from 6-80% (Weese et al., 2010; Crombe et al., 2013). However, caution must be taken when comparing results due to differences in sampling and isolation procedures, number of pigs sampled, sample size and sample populations (finishing vs. breeding pigs, piglets vs. older pigs, open vs. closed farms, pigs at the abattoir vs. pigs at the farm, etc.) (Broens et al., 2011a).

Although LA-MRSA ST398 is more associated with colonization in farm animals, especially in pigs, it has also been isolated as etiologic agent of infection in these animals (Armand-Lefevre et al, 2005; Kadlec et al., 2009; Meemken et al., 2010; Sergio et al., 2007; Schwarz et al., 2008; van der Wolf et al., 2012).

LA-MRSA ST398 in other animals

Healthy carriage of LA-MRSA ST398 has also occasionally been reported in bovines. Carriage rates among veal calves and dairy cows have been reported ranging from 1% in Switzerland (Huber et al., 2010), 6.5% in France (Haenni et al., 2011) to 28–50% in the Netherlands (Graveland et al., 2009; Graveland et al., 2010). LA-MRSA ST398 has been also reported in cows with mastitis or milk samples in Switzerland (1.4%) (Huber et al., 2010; Sakwinska et al., 2011), Germany (1.4-16.7%) (Fessler et al., 2010; Monecke et al., 2007; Spohr et al., 2011) and Belgium (10% of tested farms) (Vanderhaeghen et al., 2010).

Poultry appear to carry MRSA, though with a lower prevalence compared to pigs residing on the farm (Monecke et al., 2013; Mulders et al., 2010; Nemati et al., 2008; Persoons et al., 2009). Very limited studies investigated the LA-MRSA ST398 carriage rates in poultry, mainly in Belgium and The Netherlands, with rates of 6.9-10.7% positive animals and values of 12.8-35% among the flocks sampled (Mulders et al., 2010; Persoons et al., 2009). In spite of these values, the majority of isolates reported, in both diseased and healthy chickens, belonged to the CC5 (Monecke et al., 2013), which is also one of the most successful human-associated lineages (Lowder et al., 2009).

Concerning horses, LA-MRSA ST398 has mainly been reported in equine clinics (Cuny et al., 2008; Hermans et al., 2008; Sieber et al., 2011; Van den Eede et al., 2009) but limited data is available at farm level (Van den Eede et al., 2012; 2013). In West-European horses admitted to a Belgian veterinary clinic, a LA-MRSA ST398 carriage rate of 10.9% was found (Van den Eede et al., 2009). However, (Van den Eede et al. 2012) recently reported a low prevalence (<1%) at farm level in Belgium. Similarly, very low and even absent farm level carriage rates have been reported in the Netherlands (Busscher et al., 2006), Slovenia (Vengust et al., 2006), and Canada (Burton et al., 2008).

LA-MRSA ST398 can be also present, although at lower rates, in other animal species. As mentioned above, pets (cats and dogs) are recognised as vectors or sources of MRSA from human origin. However, LA-MRSA ST398 has occasionally been detected in cats and dogs, normally causing infections in these animals (Floras et al., 2010; Haenni et al., 2012; Nienhoff et al., 2009; Witte et al., 2007). Until now, the prevalence of LA-MRSA ST398 in companion animals residing on farms is unknown. Yet, (Denis et al., 2009) reported positive MRSA carriage in dogs living in pigs farms, and (Fessler et al., 2012) reported two

positive dogs that carry identical MRSA ST398 clones to the pigs in the farm in Germany. Accordingly, companion animals residing on the farm could act as vectors, transporting MRSA from one area of the farm to another.

Rodents, due to its abundance in farm environments, are recognized for their role in transmission and persistence of zoonotic bacteria on livestock farms (Meerburg et al., 2006). Rodents may easily be contaminated by direct contact with contaminated faeces, dust or by inhalation when roaming around in MRSA-positive stables. From then on, they can transport MRSA to other pig units or even beyond farms. It was not surprising then that LA-MRSA ST398 appeared in rats living on pig farms (van de Giessen et al., 2009).

Very recently, LA-MRSA ST398 has been described in a pet rabbit and in a goat in the USA (Loncaric et al., 2013a; Loncaric et al., 2013b).

LA-MRSA ST398 in humans

People exposed to livestock have a greater risk of colonization, and subsequently of infection, specially in farms with a high prevalence of LA-MRSA ST398 (Armand-Lefevre et al., 2005; Khanna et al., 2008; Lozano et al., 2011b; Lozano et al., 2011c; Lozano et al., 2011d). Indeed, a study revealed a 760-fold higher MSA carriage rate among a group of regional pig farmers compared to the general Dutch population (Voss et al., 2005). Pig-farmer family members also have been observed to present higher LA-MRSA ST398 nasal occurrences (Aspiroz et al., 2009; Khanna et al., 2008; Lewis et al., 2008; Voss et al., 2005), what suggest the human-to-human transmission within the household setting. Although few data is available on the transmissibility of LA-MRSA ST398 among humans, despite some cases of human to human transmission (Huijsdens et al., 2006), as well as a hospital outbreak (Wulf et al., 2008), data indicate that the ability of LA-MRSA ST398 to spread in the community is low, similarly to its ability to spread in hospital settings (5.9 times less transmissible than non-ST398) (Boostma et al., 2011). However, the occupational risk for people exposed to livestock and those in direct contact with them has been repeatedly shown and the mentioned risk of infections has become a reality. An interesting fact is that the prevalence of LA-MRSA ST398 in the farmers and their families appears to decrease in periods of contact with animals, as is the case for extended vacations or between production cycles (Graveland et al., 2011).

Several cases of human infections caused by this clonal lineage have been described in some European countries (Krziwanek et al., 2009; Voss et al., 2005; Witte et al., 2007; Wulf et al., 2008), including Spain (Lozano et al., 2011b; Lozano et al., 2011c). These are normally SSTIs but sometimes they have been associated with severe infections (Hartmeyer et al., 2010; Krziwanek et al., 2009; Lozano et al., 2011d; Mammina et al., 2010; Pan et al., 2009, Wulf et al., 2008). It is important to highlight several recent cases of infections caused by MSSA ST398 in people without direct contact with farm animals (Davies et al., 2011; Jimenez et al., 2011; Price et al., 2012; Uhlemann et al., 2012; Rasigade et al., 2010).

LA-MRSA ST398 in food

Not many studies have focused on the detection of MRSA in food samples but the recent emergence of LA-MRSA ST398 has favoured the increase of these works. To this respect, EFSA conducted a surveillance study in 2009 in order to know the current situation and potential control and prevention measures that should be implemented (EFSA, 2009a). Focussing on meat products, contamination rates varied from absence of MRSA (Huber et al., 2010; Kaszanyitzky et al., 2003) or very low detection rates (<2%) (Hanson et al., 2011; Kerouanton et al., 2007; Kitai et al., 2005; Lozano et al., 2009), to higher values (2-12%) (De Boer et al. 2009; Molla et al., 2012; O'Brien et al., 2012; Pu et al., 2009; van Loo et al., 2007; Weese et al., 2010). Furthermore, recent studies generally show higher values, being remarkable a study conducted in meat products in Germany, which revealed a MRSA prevalence of 37% (Fessler et al., 2011).

Strains recovered have been associated with not only typical HA-MRSA (Pu et al., 2009) and CA-MRSA (Lozano et al., 2009; Molla et al., 2012; O'Brien et al., 2012; Pu et al., 2009; Waters et al. 2011) but also with LA-MRSA ST398 (Boost et al., 2012; de Boer et al., 2009; Fessler et al., 2011; Lozano et al., 2009; Molla et al., 2012; O'Brien et al., 2012; van Loo et al., 2007; Weese et al., 2010).

It can be assumed that the food chain can be a vehicle for the LA-MRSA ST398 transmission, with the subsequent risk implied for people exposed to these products, both by occupational exposure and subsequently consumers. On the other hand, LA-MRSA ST398 risk for the consumer derives from their ability to produce enterotoxins and is not higher than MSSA strains or other MRSA lineages.

Figure 19 shows a graphical summary of the different human and animal LA-MRSA ST398 transmission routes that are involved in the global epidemiology of LA-MRSA ST398.

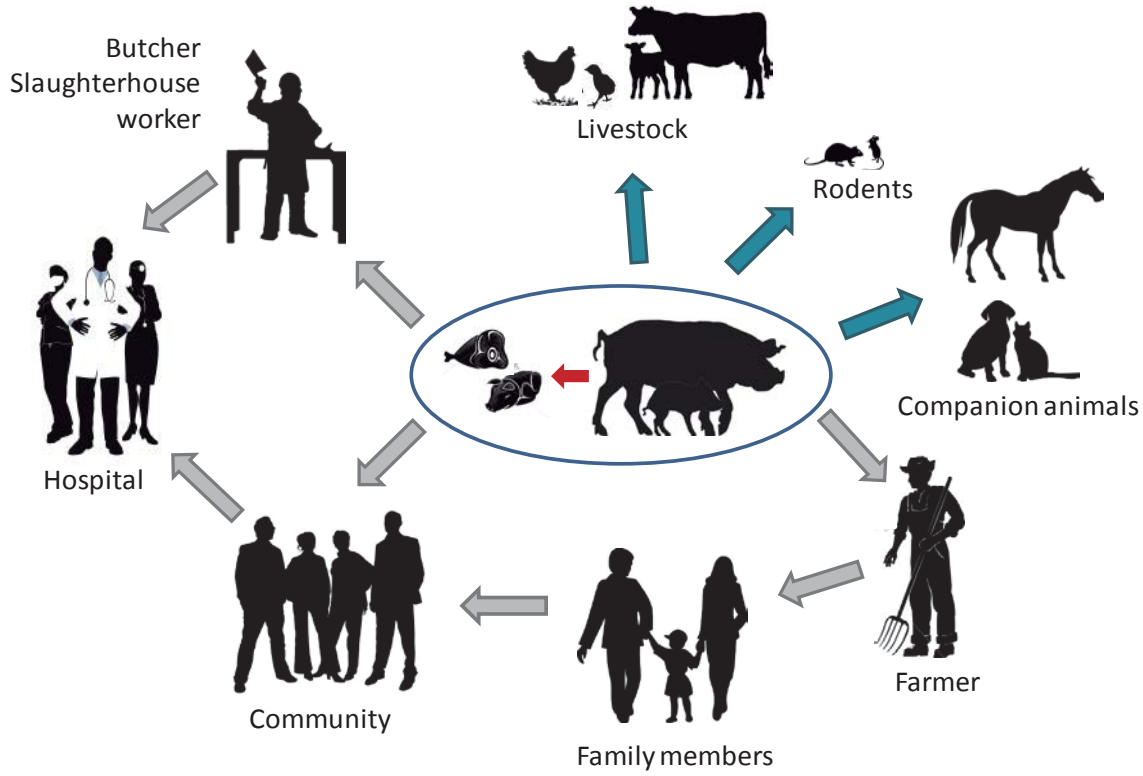


Figure 19. LA-MRSA ST398 transmission routes between livestock, companion animals, rodents, people within the farming environment or food sector and community and healthcare environment.

In addition to LA-MRSA ST398, there are few other LA-MRSA lineages spread among farm animals. In recent years, in line with the spread of LA-MRSA ST398 and the increasing number of reports that focus on this topic, several studies revealed that LA-MRSA ST9 is the major lineage detected in swine in Asia (China, Malaysia, Thailand and Taiwan) (Cui et al., 2009; Lo et al., 2012; Neela et al., 2009; Tsai et al., 2012; Wagenaar et al., 2009), with a similar epidemiology as LA-MRSA ST398. This lineage has already been detected among farmers and among butchers of wet markets (Boost et al., 2012; Guardabassi et al., 2009), showing also zoonotic potential. MRSA lineages CC97 and CC151 are also closely associated with cattle, showing a remarkable pathogenic potential, since they are normally recovered from cases of bovine mastitis (Sung et al., 2008).In addition, CC97 has been detected among swine in Europe (Battisti et al., 2010; EFSA, 2009b). Lineage ST133 has been reported as lineage adapted to small ruminants (Guinane et al., 2010) and is known to cause mastitis in goats, sheep and cows (Monecke et al., 2007; Smyth et al., 2009).

Role of antimicrobials and metals in the dissemination of LA-MRSA ST398

Numerous factors are involved in the appearance, maintenance and dissemination of LA-MRSA ST398 (Crombé et al., 2013), but two anthropogenic factors are of special interest for the development of this thesis (i) the use of antimicrobials and (ii) the feed supplementation of metals (such as copper and zinc).

Antimicrobial use has been associated with the emergence and spread of LA-MRSA ST398 (Kadlec et al., 2012a; Tacconelli et al., 2008; van Duijkeren et al., 2008b). In a recent longitudinal field study, higher transmission rates were observed when tetracycline and β-lactams were used (Broens et al., 2012b). In addition, feed supplemented with tetracycline appeared to increase the nasal MRSA CC398 load of piglets in an experimental study (Moodley et al., 2011). On the other hand, it has been shown that MRSA can be present in pigs with no antimicrobial use at all (Weese et al., 2011), suggesting that antimicrobial use is not an essential factor for MRSA acquisition but it is likely to have some influence on the MRSA load and/or to predispose animals to MRSA colonization.

The broad use of tetracycline in pig farming seem to have played an important role in the selection and increase of transmission rates of ST398 isolates, since tetracycline resistance genes are present in virtually all LA-MRSA ST398 (kadlec et al., 2012a). With this respect, the last European Medicines Agency (EMAb, 2010) report on sales of antimicrobial veterinary medical products in 19 European Countries 2010 shows that tetracyclines, followed by penicillins and lincosamides, are the antimicrobials most commonly employed in food-producing animals (**Fig. 20**).

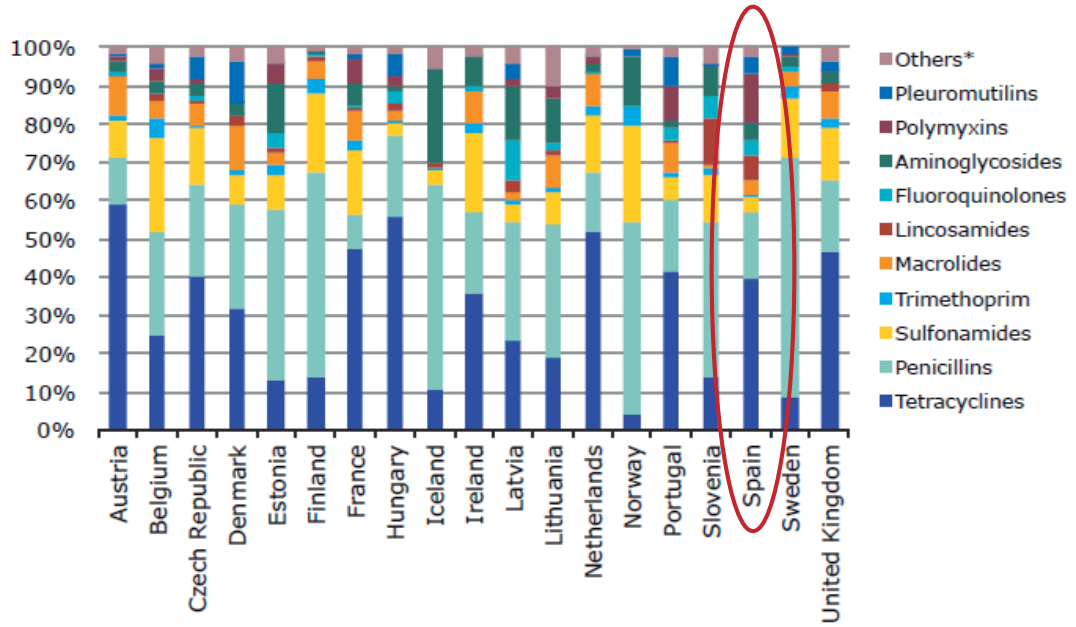


Figure 20. Proportion of the total sales of the different veterinary antimicrobials classes, in mg/PCU, by country, for 2010. "Others" refers to amphenicols, cephalosporins, other quinolones and other antibacterials (adapted from EMAb, 2010). PCU, population correction unit. (proxy of the size of the animal population at the time of treatment).

To this respect, LA-MRSA ST398 with decreased susceptibility to lincosamides, pleuromutilins and streptogramins A has recently been detected among LA-MRSA ST398 (Kadlec et al., 2009d; Kadlec et al., 2010; Kehrenberg et al., 2009; Lozano et al., 2012a; Mendes et al., 2011; Schwendener et al., 2011), and novel resistance genes to these combined agents have been found in LA-MRSA ST398 isolates, such as the *vga*(C), *vga*(E) and *lsa*(E) (Kadlec et al., 2009d; Schwendener et al., 2011; Wendlandt et al., 2013b). In addition, a novel trimethoprim resistance gene, named *dfrK*, and a novel spectinomycin resistance gene, designated *spw*, have been also described in this clonal lineage (Kadlec et al., 2009c; Wendlandt et al., 2013a). Also, antimicrobial resistance genes from other Gram-positive bacteria (such as *Streptococcus*, *Bacillus* or *Enterococcus*) have been recently reported for the first time in staphylococci in LA-MRSA ST398, such as the MLS_B resistance gene *erm*(T) (Kadlec et al., 2010a) and the lincosamides resistance gene *lnu*(B) (Lozano et al., 2012b). These data point to the suggestion that LA-MRSA ST398, in spite of its to date low virulence properties, possesses an outstanding ability to acquire antimicrobial resistances, with the subsequent risk implied for human health.

In addition to this, the use of metals in pig farming has been addressed as potential promoters for the co-selection and spread of antimicrobial resistance (Hasman et al.,

2006b). A large number of metals play a vital role as trace elements in the physiology of animals and they must be present in adequate quantities in feed in order to maintain a normal healthy state. However, copper and zinc are added to the feed in larger quantities than can be explained by the physiological needs of the animals. In addition to their role in the normal growth of the animal, they also seem to have additional effects when used in high doses. Further, livestock producers in the US, but not in Europe, also use the nonessential metal arsenic as a feed supplement to chicken, turkeys and pigs (Hasman et al., 2006a). The overuse of these metals perform a dual purpose, as they seem to increase the daily growth rate in newly weaned pigs, reduces the incidence of diarrhea as well as control the cases of postweaning scouring (Hasman et al., 2006a).

Hence, it has been hypothesized that the emergence of LA-MRSA ST398 in pigs is also driven by the use of these compounds (Aarestrup et al., 2010; Cavaco et al., 2011; de Neeling et al., 2007; van Duijkeren et al., 2008b). To this respect, zinc-oxide appeared to increase the nasal LA-MRSA ST398 load of piglets (Moodley et al., 2011). In addition, zinc-oxide has the potential to co-select specifically LA-MRSA ST398 since the zinc and cadmium resistance gene *czrC*, is present within the SCC*mec* V (5C2&5) element in ST398 (Cavaco et al., 2010; Cavaco et al., 2011). On the other hand, similar to tetracycline treatment, it has been reported that transmission of MRSA between positive and negative animals was not influenced by the short-term exposure to zinc-oxide, showing to play a non essential role in propagation (Moodley et al., 2011). Interestingly, as indicated above, the novel SCC*mec* elements IX and X, detected among LA-MRSA ST398 human isolates, harbour resistance determinants to other metals, such as copper (*copB*, *mco*), cadmium (*cadDX* operon) and arsenate (*arsRBC* or *arsDARBC* operons). Copper sulphate resistance has been described in other gram-positive livestock-associated bacteria (i.e. enterococci) (Aarestrup et al., 2004). However, very limited data do currently exists on the possible influence of copper, zinc or other metal exposures in the selection and spread of LA-MRSA ST398, what warrant further investigations.

5.3. HOST JUMPS AND HOST SPECIFICITY

Association between exposure to pigs and human carriage of *S. aureus* ST398 has remained a constant since its emergence in 2005, leading to hypothesize a pig origin of this lineage (Armand-Lefevre et al., 2005; Voss et al., 2005). However, until recently there were no phylogenetic studies of ST398 that supported this hypothesis. (Price et al., 2012) recently

suggested that ST398 is formed by two distinct clusters. One cluster is associated to livestock where isolates carry genes for methicillin and tetracycline resistance and the second cluster is associated to humans where isolates carry human host-adaptation genes (IEC) and lack tetracycline resistance (McCarthy et al., 2012; Price et al., 2012; Uhlemann et al., 2012). Phylogenetic analyses showed that the presumed LA-MRSA ST398 most likely emerged from the human-associated MSSA ST398 strains, leading to hypothesize a human origin of this lineage (Price et al., 2012). The studies that proposed a human origin of ST398 suggested that it acquired methicillin and tetracycline resistance after the host jump to pigs, probably driven by the selection pressure that antimicrobials pose in pig farming. To this respect, several studies have detected the so called "animal-independent" MSSA ST398 strains among, both healthy and clinically diseased, humans with not contact to livestock (Bhat et al., 2009; David et al., 2013; Jimenez et al., 2011; Lozano et al., 2011a; Mediavilla et al., 2012; Valentin-Domelier et al., 2011; van Belkum et al., 2008; Vandendriessche et al., 2011; van der Mee-Marquet et al., 2011; Zhao et al., 2012). Worryingly, this sublineage normally present higher virulence potential, since several cases of PVL positive isolates have been recently described (Rasigade et al., 2011; Zhao et al., 2012), while its presence remains exceptionally unusual in its LA-MRSA counterpart (Welinder-Olsson et al., 2008).

The adaptation ability of *S. aureus* ST398 provides an excellent example of how bacterial populations can explore a wide range of potential niches and acquire optimal adaptations for life in alternative hosts. This ability was termed the "shifting balance theory" by Sewall Wright 80 years ago, for which Baquero (2012) has resurged for the life example of ST398. The author raises concerns on the possibility that ST398 might readapt to human hosts without losing fitness in livestock; since a double-host adapted organism could certainly be in an optimal position for increasing its virulence and antibiotic resistance (Baquero, 2012).

Also, this would not be the first time that animals carry strains originated in humans. The poultry ST5 was also reported to have undergone genetic diversification from its human progenitor strain reflecting avian host adaptive evolution (Lowder et al., 2009). The small ruminant associated ST133 has been shown to be evolved as the result of a human to ruminant host jump followed by adaptive genome diversification (Guinane et al., 2010), and the newly described MRSA ST130 carrying the novel *mec*C has been suggested to have a human origin based in the finding of a human blood stream infection with this strain in 1975

(García-Álvarez et al., 2011), although it is unknown if this *S. aureus* lineage was present in animals at that time.

Some other *S. aureus* lineages seem to have an extended host spectrum genotype (EHSG), since they have been shown, both MSSA and MRSA, in different mammalian species, including humans. Lineages more widely distributed in different hosts are CC1, CC5, CC8, CC22, CC30, CC45, CC15 and CC398, among others (Vinzce et al., 2012; Walther et al., 2009a). MRSA CC22 seems to be also able to cause a wide range of infectious diseases in several mammalian species; in humans (Witte et al., 2008), in dogs and cats, but also in exotic animals like turtles and bats as well as in pet birds (Loeffler et al., 2005; Strommenger et al., 2006; Walther et al., 2008, Walther et al., 2009a). Occasionally, this genotype was also found to be associated with isolates derived from pigs (Sergio et al., 2007) and horses (Sung et al., 2008; Walther et al., 2009b).

The recent emergence of MRSA in food and companion animals has greatly accentuated the interest of the research community in the population genetic structure of *S. aureus* in different animal reservoirs. However, the current knowledge on the occurrence and diversity of *S. aureus* in animals is largely biased in favour of MRSA. Yet, several typical human or animal associated lineages have been recently detected in different animal species as MSSA. CC133 has been described as the major lineage in cats in Japan (Sasaki et al. 2012) and the predominant circulating lineage detected in Tunisian donkey (Gharsa et al., 2012). In addition, this lineage has been detected among zoo animals in several different species, such as lions, capybara, maras or pygmy goats (Espinosa-Góngora et al., 2011). Also, the human associated CC8 (ST72) has been detected as lethal causative agent of infection in a gorilla in Gabon and as colonizers in related chimpanzees (Nagel et al., 2013). Other human associated lineages, CC6, CC30, CC152 have been recovered recently from camels in United Arab Emirates (Monecke et al., 2011b). These data reflects the overall EHSGs of *S. aureus* and the importance to tackle its spread in different animal ecosystems.

The epidemiology of *S. aureus* is broader than believed and it cannot be limited to MRSA strains, since some methicillin-susceptible isolates in both animals and humans seem to be reservoirs of subsequent MRSA emerging lineages.

6. EPIDEMIOLOGY OF *S. pseudintermedius*

6.1. *S. PSEUDINTERMEDIUS* IN ANIMALS

As indicated previously, the family of *Canidae*, such as dogs and foxes, are the natural host for *S. pseudintermedius*, being firstly described as *S. intermedius* in dogs in 1976 (Hajek, 1976). In recent years, since its reclassification as *S. pseudintermedius* (Devriese et al., 2005) and in parallel with the alarming increment of methicillin-resistant strains (MRSP), a number of studies have focused on the occurrence and epidemiological aspects of this opportunistic pathogen. *S. pseudintermedius* is both a skin and a mucous membrane commensal in the dog and the most frequent bacterial pathogen isolated from clinical canine specimens (Bannoehr et al., 2012).

S. pseudintermedius seems to be more common in dogs than *S. aureus* in humans, with average carriage rates of 40% among healthy dogs (Bannoehr et al., 2012). However, highly variable values have been observed depending on the body site tested and the methodology employed. The carriage rates reported by four cross-sectional studies conducted on large populations of healthy dogs including multiple body sites are quite variable and range between 46 and 92% (Devriese et al., 1987; Griffeth et al., 2008; Hanselman et al., 2009; Rubin et al., 2011). It has been also observed higher carriage rates in dogs with atopic dermatitis (87%) than in healthy individuals (37%) (Fazakerley et al., 2009). Although *S pseudintermedius* presents more restricted host specificity than *S. aureus*, it has been recovered from a number of animal species, including cats, horses and cows, on which this bacterium is also able to cause infections (Bannoehr et al., 2012).

Remarkable high genetic diversity is observed among *S. pseudintermedius* isolates, with a highly heterogeneous population structure (Bannoehr et al., 2012; Black et al., 2009). While a single *S. aureus* clone is usually present in a colonized human, from two to four *S. pseudintermedius* clones have shown to be normally present in a positive dog (Bannoehr et al., 2012). In addition, similarly to *S. aureus*, *S. pseudintermedius* can survive for extended periods in the environment (Laarhoven et al., 2011), what is relevant when addressing indirect zoonotic transmissions.

6.1.1. MRSP and its clonal spread

Since 2006, methicillin-resistant *S. pseudintemedius* (MRSP) has emerged as a significant animal health problem in veterinary medicine (Van Duijkeren et al., 2011; Weese et al., 2010). The emergence and spread of MRSP is internationally recognised (Van Duijkeren et al., 2011a). One veterinary laboratory noted a 272% increase in MRSP cases from 2007-2008 through 2010-2011 (Steen, 2011). One characteristic of MRSP is that they are normally MDR, showing resistance to many classes of antimicrobials, limiting the therapeutic option available and becoming a challenge for veterinary medicine. Moreover, MRSP isolates resistant to all antimicrobial classes available for veterinary use have been described (EMAa, 2010), what has resulted in a potential pressure for veterinarians to use antimicrobials authorized for human medicine (EMAa, 2010; van Duijkeren et al., 2011a).

MRSP has been detected to be present in 0-4.5% of healthy dogs in the community and upon admission to veterinary hospitals (Griffeth et al., 2008; Hanselman et al., 2009; Hanselman et al., 2008; Murphy et al., 2009; Vengust et al., 2006) and in 0-7% in dogs with skin disease (Griffeth et al., 2008, Kania et al., 2004, Medleau et al., 1986). Although an unexpectedly high prevalence of 30% was detected in dogs at a veterinary clinical in Japan (Sasaki et al., 2007). Lower values have been detected in cats and horses (<4%) (Abraham et al., 2007; Couto et al., 2011; Ruscher et al., 2009). MRSP colonization and infection has been described in dogs, cats, horses, birds, and humans (Kadlec et al., 2010c; Ruscher et al., 2010; Weese et al., 2010). Also, very recently, the first description of MRSP among urban rats has been reported in Canada (Weese et al., 2012).

In contrast to MSSP, MRSP is highly clonally spread, with MRSP ST71 disseminated in Europe and ST68 in the US (Black et al., 2009; Kaldec et al., 2010c; Perreten et al., 2010; Ruscher et al., 2010; van Duijkeren et al., 2011a). In addition, the novel SCC*mec* II-III, which was described for the first time in the MRSP clone ST71 (Descloux et al., 2008), is virtually present in all MRSP ST71 isolates of different geographic regions, evidencing its highly clonal distribution (Bannoehr et al., 2012; van Duijkeren et al., 2011a). It is noteworthy that none of these two lineages has been detected so far among meticillin susceptible isolates. This is a remarkable difference from *S. aureus*, because the first MRSA lineages that emerged in humans (the archaic clone ST250) and in animals (LA-MRSA ST398) originated from common meticillin-susceptible lineages in their respective host populations. On the other hand, the new MLST scheme proposed by (Solyman et al., 2013), have detected diversity within the

American MRSP clone ST68 clone, which has been divided in three related STs (ST29, ST30 and ST68), revealing that this lineage is not as clonal as revealed by the previous approach.

6.2. *S. PSEUDINTERMEDIUS* IN HUMANS

S. pseudintemedius colonization in humans seems to be uncommon and transient, since these bacteria are not part of our normal nasopharyngeal microbiota (<1%) (Talan et al., 1989a, Talan et al., 1989b). However, transmission between dogs and in-contact humans is possible and has recently been reported more frequently. MSSP rates of up to 4.5% have been reported among persons with direct contact to dogs (Boost et al., 2008, Hanselman et al., 2009; Mahoudeau et al., 1997; Talan et al., 1989b; Walther et al., 2012). However, very limited data are available on the possible persistence of *S. pseudintermedius* in the human host, and reports are largely biased in favour of MRSP (Bannoehr et al., 2012; Harvey et al., 1994). On the other hand, it is a common and potential invasive pathogen of dog-bite wounds in humans (Lee, 1994). It has additionally been reported in a number of clinical human infections, and has been associated with bacteraemia (Chuang et al., 2010; Vandenesch et al., 1995), a brain abscess (Atalay et al., 2005), pneumonia (Gerstadt et al., 1999), ear infections (Kikuchi et al., 2004, Tanner et al., 2000), varicose leg ulcers (Lee, 1994), an infected suture line (Lee, 1994), and an infected implantable defibrillator (Riegel et al., 2010; van Hoovels et al., 2006). However, in most of these cases the origin of the organism remained unknown and zoonotic transmission was not proven.

MRSP is also uncommon in humans, but it has been reported in recent years in some European countries (De Martino et al., 2010; Frank et al., 2009; Guardabassi et al., 2004; Hanselman et al., 2009; Morris et al., 2010; van Duijkeren et al., 2008a; van Duijkeren et al., 2011a), in China (Boost et al., 2011) and Japan (Ishihara et al., 2010; Sasaki et al., 2007) with rates of 0.4-5.3%, always among humans in contact (either occupation or ownership) with healthy or diseased animals. Reports on infections of humans with MRSP haven described in few occasions as causative agent of a gastric adenocarcinoma (Campanile et al., 2007), pneumonia (Gerstadt et al., 1999) and post-operative sinus infections (Kempker et al., 2009; Stegmann et al., 2010) (the last two cases reported direct contact to dogs or cats). Intra-household transmission from MRSP-infected or colonized animals to healthy contact animals has also been described (van Duijkeren et al., 2011b).

The transfer of SCC*mec* elements between different staphylococcal species is a concern. Although colonization or infection with MRSP is not common in humans, the potential transfer of SCC*mec* elements from MRSP to *S. aureus* and the subsequent clonal spread of such a new MRSA clone might be a threat for human health in the future.

7. JUSTIFICATION FOR THE THEMATIC UNIT OF THE THESIS

As previously indicated, *S. aureus* and *S. pseudintermedius* are common colonizers of the skin and mucosal surfaces of humans and dogs, respectively, but they are also important opportunistic pathogens that cause numerous infectious diseases. While *S. aureus* is a well-known human and animal pathogen, *S. pseudintermedius* has been only recognised as a zoonotic agent in the last few years.

For several decades, we witness a worldwide problem, both in the clinical and the veterinary sector. In the last years, there has been a dramatic increment in the antimicrobial resistance occurrence in both *S. aureus* and *S. pseudintermedius*, especially to β-lactams. The selective pressure from external factors, such as the misuse of antimicrobials in the human, animal and - to a lesser extend - the agricultural sector (**Fig. 21**), together with the broad machinery of bacteria to mobilize distinct genetic elements that carry antimicrobial and virulence resistance determinants, contribute significantly to the current situation.

Antimicrobial resistance in the animal sector has gained attention in the last years due to the alarming increase in these and other bacteria, for the regulatory limitations available on antimicrobial use and for the close contact to animals, both occupational exposure and co-inhabitation with companion animals. Further, the spread of LA-MRSA ST398, especially in pigs, and the emergence of MDR MRSP in dogs, have increased the alarm when addressing their zoonotic capacities. Besides antimicrobials, heavy metals such as copper and zinc, which are added as feed supplements to livestock, have been shown to promote co-selection of antimicrobial resistance and probably contribute also to the spread of antimicrobial resistant bacteria.

On the other hand, the molecular ecology of MSSA and MSSP in different hosts has traditionally been biased in favour of methicillin-resistant isolates, and scarce data is available on the circulating MS lineages and their antimicrobial resistance and virulence properties. However, due to the recent studies that suggest a possible human origin of LA-

MRSA ST398 as MSSA ST398 (Price et al., 2012; Uhlemann et al., 2012), the interest in methicillin-susceptible strains of different ecosystems has increased as an essential tool to help understand the appearance and evolution of successful methicillin-resistant lineages.

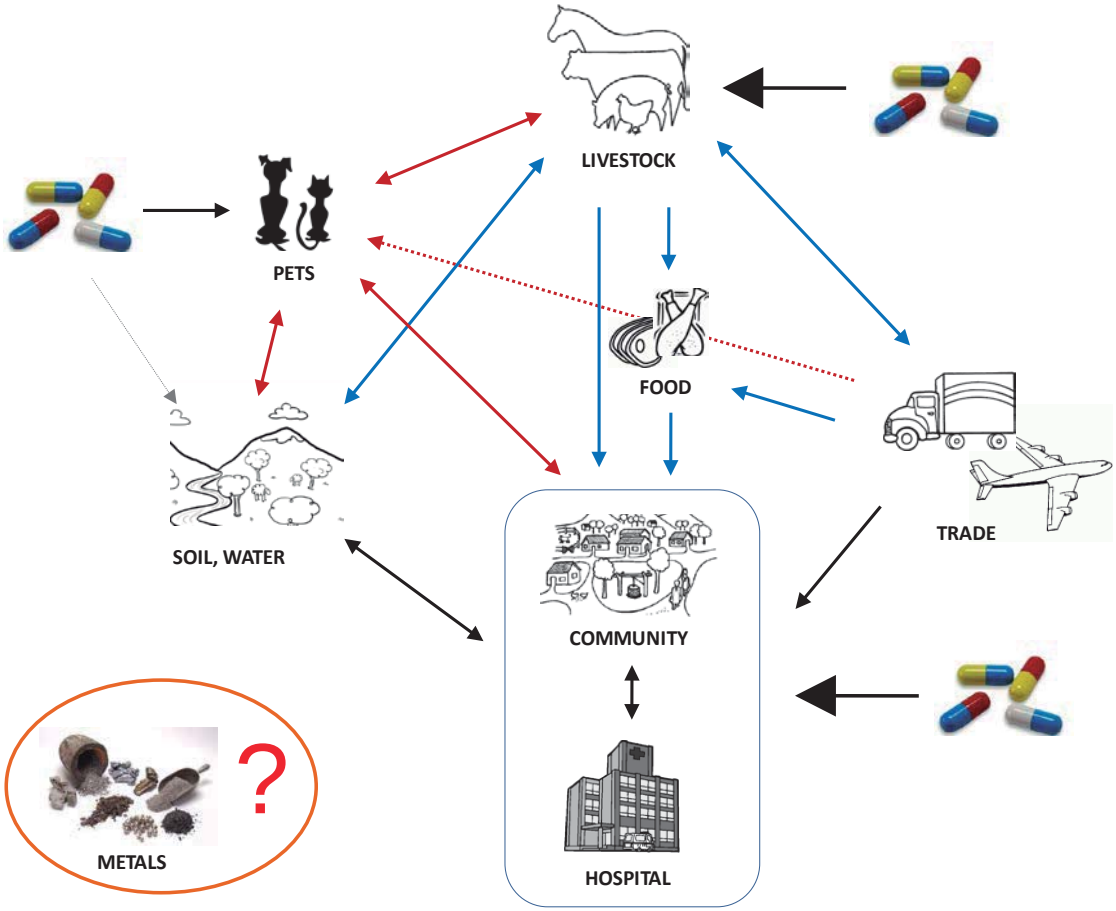


Figure 21. Routes of dissemination of of antimicrobial resistant bacteria and antimicrobial resistance genes in the different ecosystems as well as factors that influence its emergence.

The development of this thesis will contribute to the global results on the molecular ecology of *S. aureus* and *S. pseudintermedius*, both methicillin-resistant and methicillin-susceptible, in different animal species such as companion (dogs, cats, horses) and food producing animals (pigs), as well as on their capacity to be present in not natural hosts. Part of this work represents a first approach to understand the role that pet animals, especially dogs, may play in the propagation of these bacterial species among the different household inhabitants. This thesis will also deepen on the current knowledge of elements of dissemination of antimicrobial resistance, what contributes to the global spread of these bacteria.

Objectives

To determine the nasal carriage rate of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in healthy dogs and their owners in the household (**Chapter 1**).

1.1. To perform the molecular characterization of isolates.

1.2. To detect possible cases of transmission between humans and animals.

1.3. To study the carriage dynamics over time of *S. aureus* and *S. pseudintermedius* from cases of interspecies transmission.

1. To study the nasal carriage rate and molecular characterization of *S. aureus* and/or *S. pseudintermedius* in different animal species:

   5.1. Pound dogs (**Chapter 2**)

   5.2. Slaughter pigs of different age groups (**Chapter 3**).

   5.3. Clinical samples of horses (**Chapter 3**).

2. To characterize the genetic environment and location of antimicrobial resistance genes of interest in *S. aureus* and *S. pseudintermedius* as well as its possible association with metals resistance genes (**Chapter 4**).

Results

Graphical summary

Chapter 1

43 households:

Paper 1
CoPS

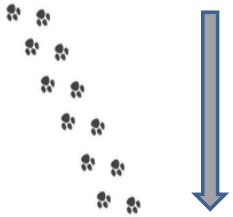
66 pets
(54 dogs, 12 cats)

+

67 owners

5 cases direct interspecies transmission

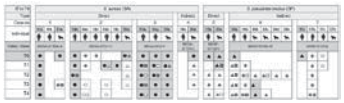
5 cases possible indirect interspecies transmission



Paper 2
CoPS
+
co-MRCoNS

Dynamics of colonization of 7 cases (4 direct, 3 indirect)

16 owners
10 dogs

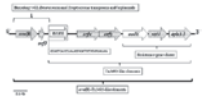


★ Selected strains that were investigated in **Chapter 4**.

Chapter 2

98 kennel dogs

Paper 3
MRCoPS



Paper 4
MSCoPS

★

Chapter 3

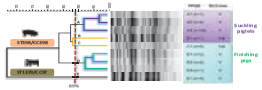
Paper 5
MRSA

★

53 finishing pigs

+

53 sucking pigs

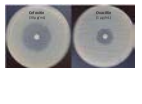


8 CoPS:
7 horses

+

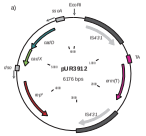
1 donkey

Paper 6
Clinical
CoPS

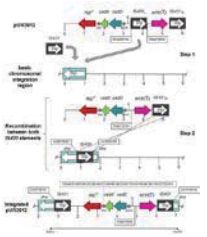


Chapter 4

pUR3912



Paper 7
MSSA ST398
pUR3912

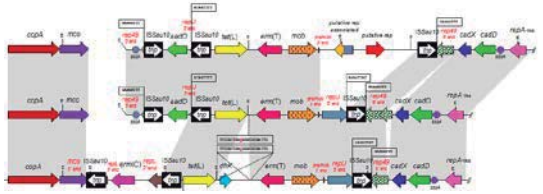


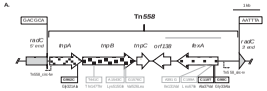
Paper 8
cointegrate

pUR1902

pUR2940
pUR2941

Paper 9
MRSA ST398
pUR1902
pUR2940
pUR2941





C2719 (Tn*558*)

Paper 10
MSSP
fexAv

Chapter 1

S. aureus and *S. pseudintermedius* in dogs and their owners at the household and colonization dynamics of cases of interspecies transmission

CHAPTER 1. *S. aureus* AND *S. pseudintermedius* IN DOGS AND THEIR OWNERS AT THE HOUSEHOLD AND COLONIZATION DYNAMICS OF CASES OF INTERESPECIES TRANSMISION.

Very limited data are available on the occurrence and molecular characteristics of *S. aureus* and *S. pseudintermedius* in healthy pets and their owners, and none on the dynamics of colonization of both bacterial species among individuals at the household. However, analysis of the colonization dynamics over time is essential to address the real carriage status of a positive individual and for a better understanding of the interaction among staphylococci in the distinct hosts. The aim of this chapter was to gain knowledge on the role that pet-owning may play in the epidemiology of *S. aureus* and *S. pseudintermedius* in healthy owners and their pets at the household.

Individuals from 43 pet-owning households were sampled for the presence of nasal *S. aureus* and *S. pseudintermedius* isolates. Sixty-seven healthy owners and 66 healthy pets (52 dogs and 12 cats) were investigated. Molecular characterization of isolates was performed by different molecular typing techniques, antimicrobial resistance profile and virulence traits. Clonal relatedness of isolates was investigated by SmaI/PFGE.

S. aureus was detected in 41.8% of owners (1 MRSA/ST8/PVL-positive; 27 MSSA) and 12.1% of dogs (8, all MSSA), who came from 51.2% of investigated households. *S. pseudintermedius* was recovered from 22.7% of dogs (2 MRSP/ST71 or ST92, 13 MSSP) and 4.5% of owners (3, all MSSP), who came from 30.2% of households. Both bacterial species were simultaneously detected in 16.7% of residences. Nineteen different *spa*-types were detected among *S. aureus* from humans and seven *spa*-types were observed in pets, five of them being common in both owners and pets. Sixteen distinct STs were recognized among *S. aureus* from humans and pets, with all but one ST present in pet isolates also revealed in those from humans. In total, 31.3% of STs showed novel allele or allele combinations. Twelve different STs were detected among the 18 *S. pseudintermedius* of animal origin. As for those detected in humans, one *S. pseudintermedius* isolate showed identical ST (ST142) to one related animal. In total, 33.3% of *S. pseudintermedius* STs were novel. High clonal diversity by PFGE was observed among *S. pseudintermedius*. Only the MRSA strain was MDR, with the majority of MSSA only resistant to penicillin. Interestingly, both MRSP and 39% of MSSP were MDR. All *S. aureus* harboured some of the virulence genes tested, with unexpected elevated rate of enterotoxin genes among both, human (93%) and animal (75%) isolates. All *S. pseudintermedius* harboured the *lukS/F-I*, *seit* and

se-int virulence genes, while 22.2% presented the *expA* or *expB* genes, half of them from human origin.

Ten cases of possible interspecies transmission (owner⇔pet) were observed (23.3% of households). Five cases of direct interspecies transmission - four caused by *S. aureus* and one by a MDR *S. pseudintermedius* - were detected in both owner and pet. In addition, five additional cases were suspected for indirect *S. aureus* or *S. pseudintermedius* interspecies transmission, in which 3 pets were positive for *S. aureus* and two owners for *S. pseudintermedius*.

Seven of these ten possible cases (4 direct, 3 indirect) could be followed up in a one-year longitudinal study. The investigated cases were of suspected *S. aureus* anthropozoonotic (human→dog, n = 4) or *S. pseudintermedius* zoonotic (dog → human, n = 3) origin. Concurrent carriage of *S. aureus* or *S. pseudintermedius* with MRCoNS was also evaluated. Sixteen owners and 10 dogs from the 7 residences were sampled every three months for one year. All households presented individuals (human and/or dog) positive for *S. aureus* or *S. pseudintermedius* in every sampling. All owners involved in cases of direct transmission were persistently colonized by the initial *S. aureus* clone, whereas only one dog remained positive throughout the sample year (anthropozoonotic origin). Both owner and his dog were persistent *S. pseudintermedius* carriers in the unique direct zoonotic transmission case. Additionally, in one of both indirect zoonotic transmission cases studied, the owner resulted persistent *S. pseudintermedius* carrier, while their dogs were intermittent carriers. Between 2 and 5 clones were present per household, with *S. pseudintermedius* being more clonally diverse in time than *S. aureus*. All clones investigated but one MRSP were methicillin-susceptible and harboured several virulence genes. Pandemic clones of *S. aureus* were detected: CC45, CC121, CC30, CC5 and CC398. An elevated rate of human persistent nasal carriers (43.8% of *S. aureus*; 12.5% of *S. pseudintermedius*) and a moderate rate for dogs (10% *S. aureus*, 30% *S. pseudintermedius*) were observed. Throughout the sampling year, 71.2% of owners and 11.1% of dogs were only *S. aureus* carriers sometime, while only *S. pseudintermedius* was recovered in 23.1% of owners and 44.4% of dogs. Both bacterial species were present in 7.7% of owners and 44.4% of dogs. Concurrent carriage of *S. aureus* or *S. pseudintermedius* with MRCoNS was common (30.7%).

The detection of two *S. aureus* ST398 clones with the typical human associated characteristics (MSSA, presence of IEC, *spa*-type t1451 or t571) in 3 owners (two related) and one unrelated dog is notable. Interestingly, all of them carried the MLS_B resistance gene *erm*(T) gene alone.

Our results give evidence that pet-owning may play a relevant role in the staphylococcal species distribution and maintenance of in-contact individuals.

These studies have led to one published paper and another at present, in advanced stage of revision, in international peer-reviewed journals.

1.1. PAPER 1.

Gómez-Sanz E, Torres C, Lozano C, Zarazaga M. High diversity of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* lineages and toxigenic traits in healthy pet-owning household members. Underestimating normal household contact? Comp Immunol Microbiol Infect Dis. 2013;36(1):83-94.

1.2. PAPER 2.

Gómez-Sanz E, Torres C, Ceballos S, Lozano C, Zarazaga M. Clonal dynamics of nasal *Staphylococcus aureus* and *S. pseudintermedius* in pet-owning household members. PloS One (advanced stage of revision).

Paper 1

Comparative Immunology, Microbiology and Infectious Diseases 36 (2013) 83–94

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High diversity of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* lineages and toxigenic traits in healthy pet-owning household members. Underestimating normal household contact?☆

Elena Gómez-Sanz, Carmen Torres, Carmen Lozano, Myriam Zarazaga *

Área Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain

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ABSTRACT

Forty-three unrelated pet-owning households were screened in Spain to study the *Staphylococcus aureus* and *Staphylococcus pseudintermedius* nasal carriage, their genetic lineages and virulence traits. Sixty-seven healthy owners and 66 healthy pets were investigated. Isolates characterization was performed and potential interspecies transmission was assessed. *S. aureus* was present in 51.2% of households studied while *S. pseudintermedius* in 30.2%. Twenty-eight owners (41.8%) carried *S. aureus*: one methicillin-resistant *S. aureus* (MRSA) [t5173-ST8-SCC*mec*IVa] and 27 methicillin-susceptible *S. aureus* (MSSA). Three owners (4.5%) were colonized by methicillin-susceptible *S. pseudintermedius* (MSSP). Fifteen pets (22.7%) carried *S. pseudintermedius*: two methicillin-resistant *S. pseudintermedius* (MRSP) [ST71-SCC*mec*II/III; ST92-SCC*mec*V] and 13 MSSP; in addition, 8 pets (12.1%) presented MSSA. High diversity of *spa* and sequence types (STs) was detected. Typical livestock-associated *S. aureus* lineages (CC398, CC9) were observed in humans and/or companion animals and hospital and/or community-acquired *S. aureus* lineages (CC45, CC121, CC5, CC8) were detected among pets. Almost 40% of *S. pseudintermedius* were multidrug-resistant. *S. aureus* isolates harboured a remarkable high number of virulence genes. The *expA* gene was detected in 3 *S. pseudintermedius* isolates. Identical strains from both owners and their pets were identified in 5 households (11.6%): (a) four MSSA (t073-ST45/CC45, t159-ST121/CC121, t209-ST109/CC9, t021-ST1654$_{[new]}$/singleton) and (b) one multidrug-resistant MSSP (ST142$_{[new]}$). Highly clonally diverse and toxigenic *S. aureus* and *S. pseudintermedius* are common colonizers of healthy humans and pets. The presence of these bacterial species, virulence genes, and interspecies transmission detected, points out to consider pet ownership as a risk factor to acquire, maintain and spread, potential pathogenic bacteria.

1. Introduction

Staphylococcus aureus (SA) and *Staphylococcus pseudintermedius* (SP) are common colonizers and opportunistic pathogens of the skin and mucouses of humans and animals, especially mammals. SA is the most common coagulase positive staphylococci (CoPS) isolated from humans with approximately 25% of healthy persons persistently colonized [1–4]. Instead, SP is the major CoPS that colonizes healthy dogs and cats [5,6], although SA can be also

☆ These data were presented as oral exposition at the 2nd ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals, Washington, September 2011.

* Corresponding author at: Área Bioquímica y Biología Molecular, Universidad de La Rioja, Madre de Dios 51, 26006 Logroño, Spain.
Tel.: +34 941 299751; fax: +34 941 299721.
E-mail address: myriam.zarazaga@unirioja.es (M. Zarazaga).

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isolated from these animals, particularly those coexisting with their owners (<20%) [5,7]. In addition, the presence of SP in humans in contact with these animals should not be underestimated, moreover taking into account that potential misidentifcation of SP with SA or *S. intermedius* (SI) occurs [5,8,9].

Methicillin-resistant SA (MRSA) is also found in a small fraction of healthy dogs (0–4%) [5,7,10–12]. In this regard, based on typing data and clonal relatedness studies, it is considered that MRSA in household pet animals has emerged as a consequence of MRSA in humans [10–12], pointing out an anthropozoonotic origin. In addition, recent reports on MRSA and methicillin-resistant SP (MRSP) in dogs and cats show that these resistant microorganisms are emerging as pathogens of these animals [2,6,13–15]. Alternatively, a clear zoonotic origin is predictable for MRSP, given that its prevalence in humans seems to be especially rare [5,8,9,16]. Further, MRSP human infection has been already recognized among individuals in contact with pet animals [8]. On top of this, both species can be especially pathogenic due to the potential presence and production of numerous virulence factors such as leukocidins, haemolysins, exfoliatins, and/or pyrogenic toxins superantigens (PTSAgs), among others [3,6,17,18].

All these data emphasize the importance of addressing the presence of SA and SP at the household setting, putting special attention on the possibility of human to animal bacterial transmission and vice verse.

Based on these data, and due to lack of studies focused on this topic in Spain, the aims of this study were to investigate the nasal occurrence of SA and SP in healthy humans and their healthy companion animals residing within common households. We also focus on the molecular characterization of all isolates obtained to analyze the circulating genetic lineages at the household setting and to enable us evaluate potential cases of interspecies transmission between humans and their pets.

2. Materials and methods

2.1. Study population and sampling criteria

Forty-three unrelated pet-owning households were sampled in La Rioja region (Northern Spain) for the nasal carriage of SA and SP. Individual swabs were taken from March 2009 to February 2011. Inclusion criteria for households tested included healthy humans whose profession did not involve any direct animal contact. None of individuals tested (neither humans nor animals) had received antimicrobial treatment within the 4 months prior sampling. Household recruitment was on voluntary basis. Sixty-seven humans and 66 animals (54 dogs, 12 cats) were included. All owners gave their consent to participate in this study, which is included in a project approved by the Medical Ethical Committee of La Rioja (Permit Number: METC 09-399/C). One to 5 owners and 1–5 pets were tested from each household, showing 10 different combinations. In most cases (20, 46.5%), only one person and one animal were sampled per household. Tested samples were transported to the laboratory within 5 h after sampling and were either immediately analyzed or stored at −20 °C until further study.

2.2. Isolation and identification of SA and SP isolates

Sampled nasal swabs were inoculated into Brain Heart Infusion broth (BHI; Difco) supplemented with 6.5% NaCl and incubated at 37 °C for 24 h. One-hundred microliters were inoculated on Oxacillin Resistant Staphylococcal Agar Base (ORSAB; OXOID) plates supplemented with 2 mg/L of oxacillin for the isolation of MRSA and MRSP. Seventy microliters were seeded on Manitol Salt Agar (MSA; BD) plates for the isolation of SA and SP. Plates were incubated at 35 °C for 24–48 h. Representative colonies were subcultured and further studied. Identification of isolates was based on colony morphology, Gram staining, and catalase and DNase activities. A multiplex PCR that amplifies the species specific *nuc* gene of SA or SI/SP was conducted to identify such species [18]. Discrimination between SI and SP was conducted by digestion of *pta* gene amplicon with *Mbo*I endonuclease [19]. The presence of *mec*A gene was investigated by PCR [20].

2.3. Molecular typing of SA and SP isolates

Spa-typing on SA isolates was performed following standard methodology [21] and sequences were analyzed using Ridom Staph-Type software version 1.5.21 (Ridom GmbH). Determination of the *agr* allotype of SA and SP was achieved by specific PCRs as previously described [22,23]. Multi Locus Sequence Typing (MLST) and subsequent assignment of clonal complexes (CC) was undergone as recommended (www.mlst.net; http://eburst.mlst.net) on all SA isolates. As for SP, 5 housekeeping genes were amplified and sequenced (*pta*, *cpn60*, *tuf*, 16S rRNA and *agrD*) followed by assignment of alleles by comparison to allele sequences present in the NCBI nucleotide database and using a key table for MLST typing of *Staphylococcus intermedius* group (SIG) isolates [22]. MRSA and MRSP were additionally typed by determination of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) described for MRSA and MRSP by multiplex PCRs [20].

2.4. Antimicrobial resistance pheno- and genotype of isolates

Susceptibility to 17 antimicrobial agents was performed by agar disk-diffusion method [24]. Antimicrobials tested were as follows (μg/disk): penicillin (10U), oxacillin (1), cefoxitin (30), erythromycin (15), clindamycin (2), gentamicin (10), kanamycin (30), streptomycin (10U), tobramycin (10), tetracycline (30), trimethoprim–sulfamethoxazole (1.25 + 23.75), chloramphenicol (30), ciprofloxacin (5), mupirocin (200), fusidic acid (10), vancomycin (30), and linezolid (30). For streptomycin and fusidic acid, methods and breakpoints employed were those recommended by the Société Française de Microbiologie (http://www.sfm.asso.fr). The double-disk diffusion test (D-test) was performed on all isolates to detect inducible clindamycin resistance.

The presence of 31 antimicrobial resistance genes was investigated by PCR: *mec*A, *blaZ*, *tet*(K), *tet*(M), *tet*(L), *erm*(A), *erm*(B), *erm*(C), *erm*(T), *mph*(C), *msr*(A), *msr*(B), *lnu*(A), *vga*(A), *vga*(C), *aacA-aphD*, *aphA3*, *aadE*, *aadD*, *aadA*, *str*, *sat4* (even though streptothricin susceptibility was not tested), *dfr*(A), *dfr*(D), *dfr*(G), *dfr*(K), *fexA*, *cfr*, cat_{pC194}, cat_{pC221}, and cat_{pC223} [20,21,25]. Positive controls from the collection of the University of La Rioja were included in each reaction.

The presence of the multidrug resistance-gene-cluster *aadE-sat4-aphA3*, in addition to its potential physical linkage to *erm*(B) gene [known as *erm*(B)-Tn*5405*-like element], was investigated in all isolates by 2 specific PCRs; one for the detection of *aadE-sat4-aphA3* cluster, and a second PCR for the identification of the physical linkage between *erm*(B) gene and such cluster [20,26].

Mutations in the Quinolone Resistance Determining Region (QRDR) of *gyrA* and *grlA* genes of SP and SA isolates, as well as a relevant outside-QRDR of *gyrA* in SP, were determined by PCR and sequencing following previous recommendations [3,20].

2.5. Presence of virulence genes

PCR-based determination of the leukocidin genes *lukS/F-PV*, *lukM*, and *lukE/D* was implemented on all isolates. They were also screened for the presence of *hla*, *hlb*, *hld*, *hlg*, and *hlgv* haemolysin genes; and *eta*, *etb*, and *etd* exfoliative genes by specific PCRs. The PTSAgs: *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu* enterotoxin genes as well as the toxic shock syndrome toxin gene *tst* were likewise investigated [20,21]. The presence of the *sec* canine variant (sec_{canine}) was investigated by sequencing and comparison of *sec* amplicon (GenBank accession number U91526). Overlapping PCRs were conducted to detect the presence of the enterotoxin gene cluster operon *egc*, which consists of the enterotoxin genes *seg*, *sei*, *sem*, *sen* and *seo* or the egc_{like} (*egc* + *seu*) operon. In addition, the presence of the leukocidin genes *lukS/F-I*, the exfoliative genes *siet*, *expA* (formerly named *exi* gene), and *expB* [27,28], and the enterotoxin gene *se-int* were tested by PCR on all SP isolates [20].

2.6. Pulsed field gel electrophoresis (PFGE)

Clonal relatedness of all methicillin-susceptible SP (MSSP), in addition to all isolates involved in cases of potential interspecies transmission, was analyzed by PFGE of total DNA restricted with *Sma*I enzyme as previously described [20,21]. PFGE was run for 24 h at 5.6 V/cm and using pulsed time ramping from 2 to 5 s [15]. Additionally, GelCompar software package (Applied Maths, Kortrijk, Belgium) was used to analyze all MSSP PFGE profiles. The Unweighted Pair Group Method using Arithmetic averages (UPGMA) based on Dice coefficient was implemented, with a maximum position tolerance of 2.5%. A similarity coefficient of 77% was selected to define pulsed-field groups.

3. Results

3.1. Occurrence of SA and SP in individuals and households

Twenty-eight owners (41.8%) were positive for SA, one of them (1.5%) being MRSA. In addition, 3 owners (4.5%) were positive for MSSP, but no MRSP was obtained from humans (Table 1). Methicillin-susceptible SA (MSSA) were isolated in 5 dogs and 3 cats (12.1% of tested animals; 9.3% of dogs and 25% of cats), but MRSA was not detected in pets. Moreover, SP was recovered from 14 dogs and one cat (22.7% of sampled animals; 26.9% of dogs and 8.3% of cats), MRSP being identified in 2 dogs (3% of investigated animals; 3.7% of dogs tested) (Table 1). In total, 36 SA (35 MSSA, 1 MRSA) obtained from 28 owners and 8 pets, and 18 SP (16 MSSP, 2 MRSP) coming from 15 pets and 3 owners were obtained and completely characterized. Only one individual (dog from household number 4) was colonized by two different bacterial species, presenting a MSSA and a MRSP isolate (Table 2).

SA was present in at least one individual (either owner or pet) in 22 of the 43 (51.2%) households studied while SP was isolated in 13 (30.2%) of these residences. Alternatively, both bacterial species were detected in 7 (16.3%) households. At least one owner and one pet were colonized by SA in 5 (11.6%) households, while SP was concurrently present in owner and pet in another residence (2.3%) (no. 1–6) (Table 2). Instead, in 12 (27.9%) of sampled households (no. 11–22) only owners were colonized by SA or SP whereas in 6 (14%) residences (no. 23–28) only pets carried SA or SP (Table 2). Neither SA nor SP was obtained in 15 households (35%).

3.2. Molecular typing of MRSA and MRSP isolates

The MRSA isolate obtained from a human (7-H) was typed as t5173(*spa*)-I(*agr*)-ST8/CC8(MLST)-SCC*mec*IVa. Both MRSP obtained from dogs ($4\text{-}D_{1b}$ and 7-D) were typed as III-ST71-SCC*mec*II/III or II-ST92-SCC*mec*V (5C2&5) (Table 2).

3.3. Molecular typing of MSSA and MSSP isolates

Eighteen distinct *spa* types were identified among the 27 MSSA obtained from humans. Major *spa* types identified among humans were t002 (4 isolates), t073 (3), and t159 (3). As for pets, 7 different *spa* types were obtained from the 8 MSSA positive animals. Fifteen different STs were recognized among the human MSSA isolates with the presence of 4 novel STs registered on the SA MLST database as ST1654, ST1733, ST2175, or ST2177 (Table S1). STs obtained from SA of human origin belonged to 9 distinct clonal complexes (CCs) with CC121 (5 isolates), CC30 (5), CC45 (4), and CC5 (4) as predominant. In relation to companion animals, 6 distinct STs were observed, with the presence of 2 novel STs, named ST1654 and ST2176 (Table S1). STs from animal MSSA belonged to 5 CCs: CC45 (2 isolates), CC121 (2), CC5 (1), CC8 (1), CC9 (1). In total, five of the 16 STs (31.3%) exhibited by our MSSA strains presented novel characteristics.

Table 1
Colonization rates by *S. aureus* and *S. pseudintermedius* and number of isolates obtained.

	No. tested	Colonization % (number of isolates)				
		CoPS	SA		SP	
			MSSA	MRSA	MSSP	MRSP
Owner	67	46.3 (31)	40.3 (27)	1.5 (1)	4.5 (3)	–
Dog/cat	66	33.3 (23[a])	12.1 (8[b])	–	19.7 (13[c])	3.0 (2)
Household	43	65.1	48.8	2.3	25.6	4.7

[a] One dog carried 1 MSSA and 1 MRSP.
[b] 5 MSSA from dogs and 3 MSSA from cats.
[c] 12 MSSP from dogs and 1 MSSP from cat.

Ten distinct STs were detected among the MSSP of animal origin, with 4 novel STs registered on the MLST database for SP as ST142, ST146, ST154, and ST155 (Table S2). Concerning the 3 human MSSP isolates, 2 were ascribed to ST21 or ST100, and one to the novel ST142 (Table S2). In total, four of the 12 STs (33.3%) revealed in our MSSP strains were new.

3.4. Clonal relatedness of MSSP and correlation with other typing techniques

High variety of MSSP profiles was revealed (Fig. 1). Cluster analysis generated 5 major groups (A–E) with 15 individual patterns. Group A was prominent with 6 individual profiles. Group D clustered strains of human, dog and cat origin, all ascribed to lineage ST142. Strains with novel STs were present in 3 different groups. The 3 MSSP of human origin were grouped in 3 different clusters revealing lack of clonal relatedness. Interestingly, both MSSP ST7 strains were clustered in distant groups. Different *agr* allotypes were present in strains of the same clusters.

3.5. Antimicrobial resistance pattern

Antimicrobial resistance phenotype of all SA and SP isolates investigated is shown in Fig. 2. The unique MRSA strain (*mec*A-positive) obtained was multidrug-resistant (MDR) (resistance to at least 3 families of antimicrobials), showing additional resistance to erythromycin, kanamycin, and ciprofloxacin (strain 7-H in Table 2). MSSA isolates showed resistance to the following antimicrobials (% of resistant isolates): penicillin (88.6), tetracycline (5.7), erythromycin–clindamycin (14.3), and streptomycin (5.7). None of the 35 MSSA obtained were MDR.

Both MRSP (*mec*A-positive) were MDR showing resistance to at least 6 distinct families of antimicrobial agents (Table 2). MSSP isolates showed the following resistances (% of resistant isolates): penicillin (93.8), tetracycline (37.5), erythromycin–clindamycin (31.3), gentamicin (6.3), kanamycin–tobramycin (31.3), streptomycin (31.3), cotrimoxazol (12.5), and chloramphenicol (25). The complete multidrug resistance-gene-cluster *erm*(B)-Tn*5405*-like element was present in MRSP ST71 and in only one MSSP (3-D_4). Instead, the *aadE-sat4-aphA3* cluster was detected in 4 additional MSSP and in the MRSP ST92. In total, 39% of all SP isolates were MDR.

Inducible clindamycin resistance was observed in all erythromycin resistant MS isolates while both MRSP showed a constitutive resistance pattern.

3.6. Toxin gene profile

In relation to SA, the unique MRSA isolated harboured the Panton–Valentine leukocidin (PVL) toxin gene (*lukS/F-PV*) being the only positive strain (Table 3). High rate of isolates (46.4% of human and 50% of animal SA) harboured the *lukE/D* gene. The exfoliative toxin genes *eta* or *etb* were observed in 8.3% of SA (detected in both animal and human isolates). Twenty-five percent of SA harboured the *tst* gene, all of them recovered from humans. High diversity of enterotoxin genes and gene combinations were observed (Table 3). In total, 93% of human and 75% of animal SA presented at least one enterotoxin gene with 30.6% of SA carrying the *egc* operon and 11.1% the egc_{like} operon. Additionally, 41.7% of SA showed variants of such clusters (*seg-sei-sem-sen-seu* in 25% and *seg-sei-sem-sen* in 16.7%). Altogether, 83.3% of SA carried *egc*, egc_{like} or incomplete clusters.

All SP isolates, independently of their origin and resistance pattern, harboured the leukocidin *lukS/F-I*, the exfoliatin *siet* and the *se-int* enterotoxin gene. One isolate harboured the sec_{canine} variant. Interestingly, 4 isolates also carried the recently described exfoliatin genes *expA* or *expB*, two of which were of human origin.

3.7. Concomitant colonization by identical isolates

Based on all molecular techniques performed, identical human and animal strains were detected in 5 of the 28 positive households (11.6% of tested households) (Table 2). Clonal relatedness of such strains is shown in Fig. 3. Individuals from household no. 1 harboured an identical MSSA of a novel lineage (ST1645), whereas individuals from 3 households (nos. 2, 3 and 4) presented MSSA of lineages CC45, CC9 or CC121 (Table 2). All MSSA strains presented exfoliatin genes and/or clustered enterotoxin genes, among others. In fact, all strains involved carried *egc*, egc_{like} or *egc* variants. Further, in one household (no. 5), a toxigenic MDR MSSP strain of a novel lineage (ST142) was isolated in both owner and dog (Table 2).

Table 2
Molecular characterization of the *S. aureus* and *S. pseudintermedius* strains isolated from the 28 positive households.

House	Strain name[a]	Bacterial species	*spa-agr*-MLST	CC for SA	Antimicrobial resistance phenotype[b]	Resistance genes detected	Toxin genes investigated:			
							Leukocidins	Exfoliatins	Haemolysins	PTSAgs
1	1-H	MSSA	t021-III-ST1654$_{[new]}$	Singleton	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seu*]
	1-D	MSSA	t021-III-ST1654$_{[new]}$	Singleton	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seu*]
2	2-H$_1$	MSSA	t073-I-ST45	CC45	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seo*]
	2-H$_2$	MSSA	t073-I-ST45	CC45	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seo*]
	2-D	MSSA	t073-I-ST45	CC45	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seo*]
	2-C	MSSA	t073-I-ST45	CC45	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seo*]
3	3-H	MSSA	t209-II-ST109	CC9	P-E-C$_{ind}$	*blaZ-erm*(A)	-	***eta***	*hla, hld, hlgv*	[*seg, sei, sem, sen, seo, seu*]
	3-D$_1$	MSSA	t209-II-ST109	CC9	P-E-C$_{ind}$	*blaZ-erm*(A)	-	***eta***	*hla, hld, hlgv*	[*seg, sei, sem, sen, seo, seu*]
	3-D$_2$	MSSP	IV-ST42		P	*blaZ*	*lukS/F-I*	*siet*	-	*se-int*
	3-D$_3$	MSSP	II-ST141		P-T	*blaZ-tet*(M)	*lukS/F-I*	*siet*	-	*se-int*, (*sec$_{canine}$*, *sel*)
	3-D$_4$	MSSP	III-ST154$_{[new]}$		P-E-C$_{ind}$-To-S	*blaZ-*[*erm*(B)*-aphA3-aadE-sat4*]	*lukS/F-I*	*siet*	-	*se-int*
	3-D$_5$	MSSP	IV-ST33		P-T	*blaZ-tet*(M)	*lukS/F-I*	*siet*	-	*se-int*
4	4-H$_1$	MSSA	t159-IV-ST121	CC121	P-T	*blaZ-tet*(K)	*lukE/D*	***etb***	*hla, hld, hlgv*	[*seg, sei, sem, sen*]
	4-D$_{1a}$	MSSA	t159-IV-ST121	CC121	P-T	*blaZ-tet*(K)	*lukE/D*	***etb***	*hla, hld, hlgv*	[*seg, sei, sem, sen*]
	4-H$_2$	MSSA	t002-II-ST5	CC5	P	*blaZ*	*lukE/D*	-	*hla, hld, hlgv*	[*seg, sei, sem, sen, seo, seu*]
	4-H$_3$	MSSA	t002-II-ST5	CC5	P	*blaZ*	*lukE/D*	-	*hla, hld, hlgv*	[*seg, sei, sem, sen, seo*]
	4-D$_{1b}$	**MRSP**	III-ST71		P-O-F-T-E-C-G-To-K-S-X-Cp[c]	*blaZ-**mecA**-tet*(K)-[*erm*(B)*-aphA3-aadE-sat4*]*-aacA-aphD-dfr*(G)	*lukS/F-I*	*siet*	-	*se-int*
5	5-H	MSSP	III-ST142$_{[new]}$		P-T-E-C$_{ind}$-K-S-X-Ch	*blaZ-tet*(K)*-tet*(M)*-erm*(B)-[*aphA3-aadE-sat4*]*-dfr*(G)*-cat$_{pC221}$*	*lukS/F-I*	*siet*	-	*se-int*
	5-D	MSSP	III-ST142$_{[new]}$		P-T-E-C$_{ind}$-K-S-X-Ch	*blaZ-tet*(K)*-tet*(M)*-erm*(B)-[*aphA3-aadE-sat4*]*-dfr*(G)*-cat$_{pC221}$*	*lukS/F-I*	*siet*	-	*se-int*
6	6-H	MSSA	t440-III-ST30	CC30	P-S	*blaZ-str*	-	-	*hla, hld, hlg*	***tst***, *sea, see, sep*, [*seg, sei, sem, sen, seu*]
	6-C	MSSA	t002-II-ST5	CC5	-	-	*lukE/D*	-	*hla, hlb, hld*	*sep*, [*seg, sei, sem, sen, seo*]

Table 2 (*Continued*)

House	Strain name[a]	Bacterial species	*spa-agr*-MLST	CC for SA	Antimicrobial resistance phenotype[b]	Resistance genes detected	Toxin genes investigated:			
							Leukocidins	Exfoliatins	Haemolysins	PTSAgs
7	7-H	**MRSA**	t5173-I-ST8	CC8	P-O-F-E-K-Cp[c]	*blaZ*-***mecA***-*msr*(A)-*msr*(B)-*mph*(C)-*aphA3*	***lukS/F-PV***, *lukE/D*	-	*hla, hld, hlgv*	*sec, sek, seq*
	7-D	**MRSP**	II-ST92		P-O-F-E-C-T-G-To-K-S-X-Ch-Cp[c]	*blaZ*-***mecA***-*tet*(M)-[*erm*(B)-*aphA3-aadE-sat4*]-*aacA-aphD-dfr*(G)-cat_{pC221}	*lukS/F-I*	*siet*	-	*se-int*
8	8-H	MSSA	**t571**-I-ST398	**CC398**	E-C_{ind}	***erm*(T)**	-	-	*hla, hld, hlg*	-
	8-D	MSSP	II-ST155$_{[new]}$		P	*blaZ*	*lukS/F-I*	*siet*	-	*se-int, seh*
9	9-H	MSSA	t1054-III-ST2175$_{[new]}$	Singleton	P	*blaZ*	-	-	*hlb, hld*	***tst***, *seh*, [*seg, sei, sem, sen, seu*]
	9-D	MSSP	IV-ST42		P	*blaZ*	*lukS/F-I*	*siet*	-	*se-int*
10	10-H	MSSA	t073-I-ST45	CC45	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seo*], (*sec, sel*)
	10-C	MSSP	III-ST142$_{[new]}$		P-T-E-C_{ind}-K-S-Ch	*blaZ-tet*(M)-*erm*(B)-[*aphA3-aadE-sat4*]-cat_{pC221}	*lukS/F-I*	*siet*	*hla, hld*	*se-int*
11	11-H_1	MSSP	IV-ST21		P	*blaZ*	*lukS/F-I*	*siet*, ***expA***	-	*se-int*
	11-H_2	MSSA	t012-III-ST30	CC30	P	*blaZ*	-	-	*hla, hld, hlg*	*tst, sea*, [*seg, sei, sem, sen, seu*]
	11-H_3	MSSA	t002-II-ST5	CC5	P-E-C_{ind}	*blaZ-erm*(C)	*lukE/D*	-	*hla, hld, hlgv*	-
12	12-H	MSSP	IV-ST100		P	*blaZ*	*lukS/F-I*	*siet*, ***expA***	-	*se-int*
13	13-H_1	MSSA	t1077-IV-ST2177$_{[new]}$	Singleton	P	*blaZ*	*lukE/D*	-	*hla, hld, hlgv*	***tst***, [*seg, sei, sem, sen*]
	13-H_2	MSSA	t1077-IV-ST95	CC121	P-E-C_{ind}	*blaZ-erm*(C)	*lukE/D*	-	*hla, hlb, hld, hlgv*	***tst***, [*seg, sei, sem, sen*]
14	14-H_1	MSSA	t3916-III-ST34	CC30	P	*blaZ*	-	-	*hla, hld, hlg*	***tst***, *seh*, [*seg, sei, sem, sen, seu*]
	14-H_2	MSSA	t037-III-ST30	CC30	P	*blaZ*	-	-	*hla, hlb, hld, hlg*	***tst***, [*seg, sei, sem, sen, seu*]
	14-H_3	MSSA	t3711-III-ST34	CC30	P	*blaZ*	-	-	*hla, hld*	***tst***, *seh*, [*seg, sei, sem, sen*]
15	15-H_1	MSSA	t7901-I-ST1733$_{[new]}$	Singleton	P	*blaZ*	-	-	*hla, hld, hlg*	***tst***, [*seg, sei, sem, sen, seo*]
	15-H_2	MSSA	t7901-I-ST1733$_{[new]}$	Singleton	P	*blaZ*	-	-	*hla, hld, hlg*	[*seg, sei, sem, sen, seo*]
16	16-H	MSSA	t148-I-ST72	CC8	P-S	*blaZ-str*	*lukE/D*	-	*hla, hlb, hld, hlgv*	[*seg, sei, sem, sen, seo*], (*sec, sel*)
17	17-H	MSSA	t159-IV-ST121	CC121	P	*blaZ*	*lukE/D*	***eta***	*hla, hlb, hld, hlgv*	[*seg, sei, sem, sen, seu*]
18	18-H	MSSA	t015-I-ST45	CC45	P	*blaZ*	-	-	*hld, hlg*	[*seg, sei, sem, sen, seo*], (*sec, sel*)

Table 2 (*Continued*)

House	Strain name[a]	Bacterial species	*spa-agr*-MLST	CC for SA	Antimicrobial resistance phenotype[b]	Resistance genes detected	Toxin genes investigated:			
							Leukocidins	Exfoliatins	Haemolysins	PTSAgs
19	19-H	MSSA	t091-I-ST7	CC7	P	*blaZ*	*lukE/D*	-	*hla, hlb, hld, hlgv*	*sep*
20	20-H	MSSA	t002-II-ST146	CC5	-	-	*lukE/D*	-	*hla, hld, hlgv*	[*seg, sei, sem, sen, seo, seu*]
21	21-H	MSSA	t2155-IV-ST121	CC121	P	*blaZ*	*lukE/D*	-	*hla, hld, hlgv*	*seb*, [*seg, sei, sem, sen*]
22	22-H	MSSA	t159-IV-ST121	CC121	P	*blaZ*	*lukE/D*	***etb***	*hla, hld, hlgv*	[*seg, sei, sem, sen, seu*]
23	23-D	MSSA	t151-IV-ST121	CC121	-	-	*lukE/D*	-	*hla, hlb, hld*	-
24	24-C	MSSA	t711-I-ST2176$_{[new]}$	CC8	P	*blaZ*	*lukE/D*	-	*hla, hlb, hld, hlgv*	-
25	25-D$_1$	MSSP	III-ST29		P	*blaZ*	*lukS/F-I*	*siet*	-	*se-int*
	25-D$_2$	MSSP	IV-ST7		-	-	*lukS/F-I*	*siet*, ***expB***	-	*se-int*
26	26-D	MSSP	III-ST77		P-T-E-C$_{ind}$-G-K-S-Ch	*blaZ-tet*(M)-*erm*(A)-*erm*(B)-*aacA-aphD*-[*aphA3-aadE-sat4*]-*cat*$_{pC221}$	*lukS/F-I*	*siet*	-	*se-int*
27	27-D	MSSP	II-ST146$_{[new]}$		P	*blaZ*	*lukS/F-I*	*siet*	-	*se-int*
28	28-D	MSSP	IV-ST7		P	*blaZ*	*lukS/F-I*	*siet*, ***expA***	-	*se-int*

Lines in dark and faint grey shaded correspond to those individuals suspected for cases of direct or indirect interspecies transmission, respectively. Resistance and virulence genes delimited by square brackets are those proved to be physically linked, and those virulence genes within brackets are those supposed to have a physical linkage. Especially remarkable data is in bold type.

[a] Strain nomenclature: household number - origin of isolate [Human (H); dog (D); cat (C)]. If necessary for proper discrimination of strains an additional subscript number is included (1-5). For strains obtained from the same individual an extra letter is also subscripted.

[b] P, penicillin; O, oxacillin; F, cefoxitin; T, tetracycline; E, erythromycin; C, clindamycin; C$_{ind}$, inducible clindamycin resistance; G, gentamicin; To, tobramycin; K, kanamycin; S, streptomycin; X, cotrimoxazol; Ch, chloramphenicol; Cp, ciprofloxacin; -, susceptible to all antimicrobials tested.

[c] Strain 7-H: Ser80Tyr in GrlA; Ser84Leu in GyrA. Strain 7-D: Ser80Ile in GrlA; Ser84Leu and silent mutation C2196T (outside QRDR) in GyrA. Strain 4-D$_{1b}$: Ser80Ile in GrlA; Ser84Leu in GyrA QRDR; Glu678Ala, Glu714Lys, and C2196T (silent mutation) outside GyrA QRDR.

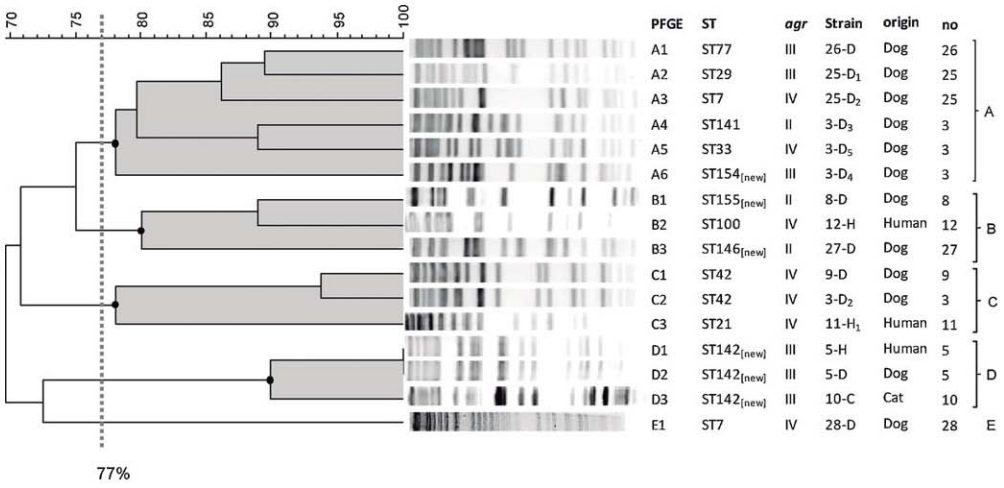

Fig. 1. Dendrogram showing the relatedness of the resultant *SmaI* macrorestriction patterns after PFGE of all MSSP obtained: 13 from animals (12 dogs and 1 cat) and 3 from humans. A 77% cut-off similarity was applied to differentiate the 5 major groups (A–E). The ST and *agr* allotype of all MSSP isolates is also indicated. No refers to the number of positive household from which MSSP isolate was recovered.

4. Discussion

An elevated CoPS prevalence (46.3%) is observed among tested owners, while almost 42% carried SA. Of these, one isolate (1.5%) corresponded to a MRSA strain. This prominent human SA occurrence might be attributed to the coexistence with pet animals, suggesting that, even though they are expected to be colonized not for extended periods, cohabitation with animals may represent an intermittent source of SA for human recurrence, although other causes such as the role of contaminated surfaces were not investigated in these households. Although previous studies on SA in healthy pet-owning household members have shown not elevated SA colonization rates in pet owners, there are very limited reports on this subject [5,7]. In contrast, MRSA occurrence observed here is consistent with previous reports on the general healthy population (1%) [3,4,7]. Nevertheless, one-point prevalence data cannot discern between real colonization, transient carriage or mere contamination and only longitudinal insights are capable of recognizing the real burden of pets as sources of SA. Surprisingly, 4.5% of tested humans carried SP. Hanselman et al. [5] detected an unpredicted 4.1% SP human prevalence; however, in that study, 42% of tested owners were veterinarians. The prevalence of SP nasal carriage in healthy humans is estimated to be very low (<1%) [16]. Moreover, studies addressing this topic assumed SP human carriage to be related to pet-ownership, and not to be prolonged in time [8,13]. Nevertheless, the high MSSP human rate detected here suggests that the presence of this species in humans, at least in those coexisting with pet animals, may be overlooked.

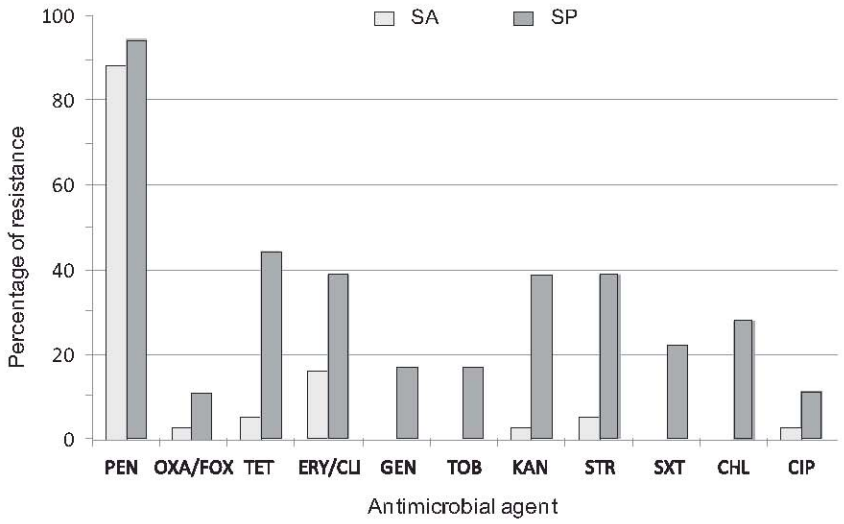

Fig. 2. Percentage of resistance to different antimicrobials of the 36 SA isolates versus that of the 18 SP obtained. PEN, penicillin; OXA/FOX, oxacillin/cefoxitin; TET, tetracycline; ERY/CLI, erythromycin and/or clindamycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; SXT, cotrimoxazol; CHL, chloramphenicol; CIP, ciprofloxacin. All isolates showed susceptibility to vancomycin, mupirocin, fusidic acid, and linezolid.

Table 3
Percentage of different virulence genes and/or gene combinations detected, isolate origin and genetic lineages of the 36 *S. aureus* and 18 *S. pseudintermedius* obtained.

Virulence gene	SA (no. H/A)[a]	SP (no. H/A)[a]	ST (no.[b])	CC[c] (no.[b])
Leukocidin				
lukS/F-PV	2.8 (1/0)	0	ST8	CC8
lukE/D	47.2 (13/4)	0	ST121 (6), ST5 (4), ST8, ST2177, ST95, ST72, ST7, ST146, ST2176	CC121 (7), CC5 (5), CC8 (3), CC7, Sn[d]
lukS/F-I	0	100 (0/18)	ST142 (3), ST42 (2), ST7 (2), ST141, ST154, ST33, ST71, ST155, ST21, ST100, ST29, ST92, ST77, ST146	
Exfoliatin				
eta	8.3 (2/1)	0	ST109 (2), ST121	CC9 (2), CC121
etb	8.3 (2/1)	0	ST121 (3)	CC121 (3)
siet	0	100 (0/18)	ST142 (3), ST42 (2), ST7 (2), ST141, ST154, ST33, ST71, ST155, ST21, ST100, ST29, ST92, ST77, ST146	
expA	0	17 (2/1)	ST21, ST100, ST7	
expB	0	6 (0/1)	ST7	
Toxic-shock syndrome toxin				
tst	25 (9/0)	0	ST30 (3), ST34 (2), ST95, ST1733, ST2175, ST2177	CC30 (5), Sn (3), CC121
Haemolysin combination				
hla, hld, hlg	36.1 (10/3)	0	ST45 (5), ST1654 (2), ST30 (2), ST1733 (2), ST398, ST34	CC45 (5), Sn (4), CC30 (3), CC398
hla, hld, hlgv	33.3 (10/2)	0	ST121 (4), ST5 (3), ST109 (2), ST8, ST2177, ST146	CC121 (4), CC5 (4), CC9 (2), CC8, Sn
hla, hlb, hld, hlgv	13.9 (4/1)	0	ST95, ST121, ST72, ST2176, ST7	CC121 (2), CC8 (2), CC7
hla, hlb, hld	5.6 (1/1)	0	ST5, ST121	CC5, CC121
hla, hlb, hld, hlg	2.8 (1/0)	0	ST30	CC30
hla, hld	2.8 (0/1)	0	ST34	CC30
hlb, hld	2.8 (1/0)	0	ST2175	Sn
hld, hlg	2.8 (1/0)	0	ST45	CC45
Enterotoxin combination [e] (f)				
[*seg, sei, sem, sen, seo*]	19.4 (5/2)	0	ST45 (4), ST1733 (2), ST5	CC45 (4), Sn (2), CC5
[*seg, sei, sem, sen, seu*]	13.9 (4/1)	0	ST1654 (2), ST121 (2), ST30	Sn (2), CC30, CC121 (2)
[*seg, sei, sem, sen, seo, seu*]	11.1 (3/1)	0	ST109 (2), ST5, ST146	CC9 (2), CC5 (2)
[*seg, sei, sem, sen*]	11.1 (3/1)	0	ST121 (2), ST95, ST2177	CC121 (3), Sn
[*seg, sei, sem, sen, seo*], (*sec, sel*)	8.3 (3/0)	0	ST45 (2), ST72	CC45 (2), CC8
seh, [*seg, sei, sem, sen, seu*]	5.6 (2/0)	0	ST2175, ST34	CC30, Sn
sea, see, sep, [*seg, sei, sem, sen, seu*]	2.8 (1/0)	0	ST30	CC30
sep, [*seg, sei, sem, sen, seo*]	2.8 (0/1)	0	ST5	CC5
sea, [*seg, sei, sem, sen, seu*]	2.8 (1/0)	0	ST30	CC30
seh, [*seg, sei, sem, sen*]	2.8 (1/0)	0	ST34	CC30
seb, [*seg, sei, sem, sen*]	2.8 (1/0)	0	ST121	CC121
sec, sek, seq	2.8 (1/0)	0	ST8	CC8
sep	2.8 (1/0)	0	ST7	CC7
se-int	0	100 (0/18)	ST142 (3), ST42 (2), ST7 (2), ST141, ST154, ST33, ST71, ST155, ST21, ST100, ST29, ST92, ST77, ST146	
se-int, (*sec_{canine}, sel*)	0	5.6 (0/1)	ST141	
se-int, seh	0	5.6 (0/1)	ST155	

Data of *S. pseudintermedius* isolates are indicated in grey shaded.
[a] H, human; A, animal.
[b] Number of strains when more than one.
[c] Clonal complex (CC) of *S. aureus* isolates.
[d] Sn, singleton.
[e] Overlapped PCR probed physical linkage of such genes.
[f] Physical linkage of such genes is suspected based on bibliography.

SP colonization is frequent in companion animals, especially dogs, and values vary greatly depending on the sampling site [5,8,29]. However, the SP nasal prevalence in our tested pet population was somewhat low (23%). This value could underestimate the real occurrence, maybe due to the single body-site tested and/or the followed methodology. Alternatively, the 3% of MRSP carriage detected here among tested pets is in accordance to

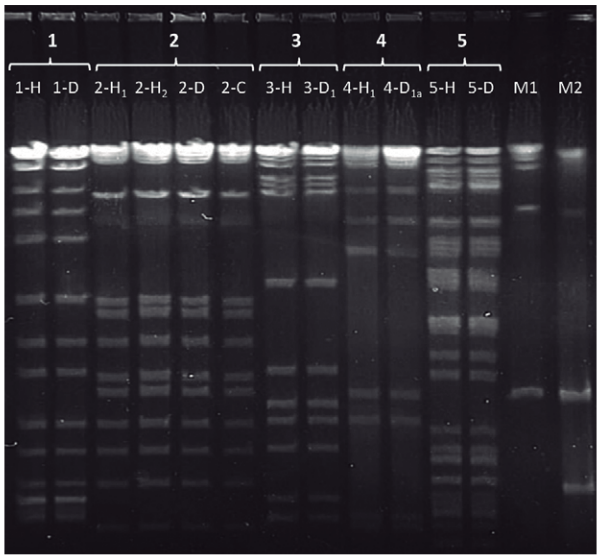

Fig. 3. PFGE profile of chromosomal DNA digested with *SmaI* of all SA and SP isolates obtained from individuals involved in possible cases of direct interspecies transmission. Numbers (1–5) above the brackets indicate the positive household number from which cases were detected. Isolate identification of involved strains is indicated on each band pattern. M1, lambda ladder marker; M2 low range ladder marker.

reported data on MRSP occurrence among healthy dogs (0–4.5%) [20,30]. The presence of SA in companion animals is relatively common, with rates of <20%, although it has traditionally been associated with the presence of SA in their owners, mostly documented when it comes to MRSA [5,7,11,30,31]. On the other hand, the prevalence of MRSA among pets without any risk factor (previous hospitalization, MRSA-positive owner, etc.) is relatively low (0–4%), what is in harmony with our results [7,11–13].

Concurrent SA or SP colonization among at least one owner and one pet was detected in 6 of investigated households (14%), with individuals carrying indistinguishable strains in five cases (11.7%). Hospital and/or community associated SA lineages (CC45, CC121) were implicated in two cases, suggesting a human origin. Conversely, both owner and dog shared an identical MSSA CC9 in another household. Even though CC9 is nowadays a well-known livestock associated MRSA lineage, mainly detected in China and in Southeast Asia [32,33], our positive individuals neither had contact with livestock nor with rural areas. Although acquisition of this lineage via contaminated meat cannot be discarded, the host tropism of MSSA of this lineage is yet not properly studied. The presence of an identical MSSP in both pet and owner in one household is of relevance. This MSSP strain showed resistance to a wide variety of antimicrobial agents and represents the first case in literature of a MSSP MDR involved in a suspected zoonotic case of interspecies transmission between owner and contact healthy domestic pet, and brings concern on the importance of MS isolates. Several suspected cases for indirect zoonotic (2 unrelated owners with SP) or anthropozoonotic transmission (2 unrelated cats and one dog with SA carrying lineages CC5, CC121 or CC8) were additionally observed. However, the origin of SA cannot be always attributed to humans, and dogs carrying MRSA of CC5 or CC8 are not exceptional [2,12,14,31]. These data could also reflect false negative results in the tested individuals, transient colonization or acquisition from different in-contact individuals. In any case, direction of transmission cannot be inferred based on the expected biology of the organism, and host tropism of the strains can be only unveiled on longitudinal studies.

The finding of 65% of tested households positive for SA and/or SP is remarkable, and suggests that there is a flow of these bacterial species at the household setting.

High diversity of genetic lineages is detected in both bacterial species investigated. Eight distinct clonal complexes (CC45, CC9, CC121, CC5, CC30, CC8, CC398, and CC7) were observed among our SA strains. The majority of these lineages are extended pandemic hospital and/or community acquired MRSA clones [32], but the presence of MSSA of these lineages in healthy individuals has been also documented [3]. Even though the incidence of MSSA t571-CC398 in humans is becoming not rare [34], it is important to underline its presence in one human isolate with no contact with livestock. The elevated rate of novel MSSA STs (31.3%) is noteworthy, and together with the presence of novel singletons highlights the capacity of this species to evolve and diverge. Alternatively, all SA animal isolates belonged to CCs also present within our human tested population, what supports the idea that SA lineages from companion animals usually reflects the epidemiology of human clones within the same geographic region [2,10]. In relation to SP, our 2 MRSP showed different characteristics, one belonged to the clonally spread European lineage ST71 [8,15,30], which has recently been detected in Spain [20]. Additionally, high number of STs (33.3%) presented novel characteristics stressing also the ability of this species to diverge. The elevated clonal variety (STs and PFGE patterns) observed among our MSSP population is in line with previous studies that indicate greater genetic diversity among MSSP than MRSP [8,22,29]. There was reasonably good correlation between the PFGE pattern and the ST detected and conjugation of results provided by these two techniques shows good discriminatory power.

The unique MDR SA isolate was our MRSA strain, while most MSSA showed only penicillin resistance. The presence of macrolide–lincosamide resistance in 14.8% of MSSA of human origin is not unexpected and it may be attributed to the extense use of these antimicrobials to treat human infections caused by Gram-positive bacteria. It is interesting to remark that our MSSA strain CC398 carried the *erm*(T) gene. This gene has been recently described among MRSA CC398 of pig origin, but its presence in CC398 human isolates, both MRSA and MSSA have been already detected [3,21,35,36].

Both MRSP of dog origin were MDR and showed resistance to a wide range of antimicrobials, implying limited therapeutic options for treatment. All MSSP but one were resistant to penicillin and were, in general, more resistant to the antimicrobials tested than MSSA isolates. Remarkably, MDR was always associated with the presence of the

aadE–sat4–aphA3 cluster, in addition to the gene *erm*(B). The presence of the complete *erm*(B)-Tn*5405*-like element has been recently documented at a high prevalence in MRSP ST71 of dog origin [15,20]. In addition, the fact that almost 40% of SP was MDR increases concerns when addressing the zoonotic potential of this bacterium, but also for the possible resistance gene transference between distinct microorganisms.

High number and variety of virulence factors has been detected among our staphylococcal isolates. As for SA, the presence of a community associated MRSA CC8 PVL-positive strain of human origin is notable due to its greatly leukotoxic properties. Human and canine MRSA PVL-positive infections, including CC8 strains, have been detected in different pet-owning households members (dogs and/or owners) [2,37], suggesting host transmissibility of these toxigenic MRSA clone. In addition, isolates of CC121 carried in most cases the leukocidin gene *lukE/D*, a toxin gene which virulence properties have been recently reviewed considering it important for lethal SA bloodstream infection [38]. The detection of the exfoliatin toxin genes *eta* and *etb*, some of which belonged to the CC121, is remarkable, given that SA of this lineage commonly causes skin and soft tissue infections worldwide [32]. PTSAgs were detected in the vast majority (92.6%) of isolates. Unpredictably, the gene *tst* was only detected among human isolates suggesting a possible preference of *tst*-positive SA for humans. However, the number of SA from pets was very limited and this hypothesis should be supported by extended epidemiological data. In addition, as previously reported [3], most of these isolates belonged to CC30. Enterotoxins were unexpectedly highly prevalent in both human and animal isolates. Additionally, the elevated rate of SA isolates that harboured the complete *egc* operon, egc_{like}, or *egc* variants, is exceptional. These rates are notoriously higher than earlier reports from both healthy humans and animals [3,14,39,40]. The elevated rate of *egc* variants detected is noteworthy and reveals that gene composition of this chromosomal enterotoxin gene operon frequently occurs [40]. The high occurrence of enterotoxigenic SA among our tested population underlines the possibility that companion animals play relevant role in the epidemiology of household food poisoning outbreaks.

AS for SP, most isolates harboured the same virulence factors, showing limited toxin diversity. However, it is important to underline the presence of the recently described exfoliatin genes *expA* or *expB* in 4 isolates, two of human origin. This represents the first report of *expA* gene among healthy animals. Since ExpA protein is involved in canine pyoderma and impetigo [17,27], further epidemiological and virulence analysis of ExpA is required to identify the pathogenic role of this protein in cutaneous infections in distinct mammal species.

In conclusion, an elevated nasal carriage rate of toxigenic SA and a moderate rate of SP have been detected in pet-owning household members. This is one of the first comprehensive studies on the SA and SP colonization at the household setting, addressing molecular characterization of all strains. Further, the transmission of SA and SP between humans and companion animals is strongly suspected. These data suggest companion animals to be vehicles for transmission of these microorganisms, and pet ownership as a possible risk factor for such acquisition. In addition, the evolution of not only novel but also already described lineages detected here should be tracked to evaluate their risks and incidences of colonization, transmissibility and pathogenicity. These findings strengthen the need for further research on staphylococcal ecology and its virulence traits for a better understanding of the emergence of toxigenic SA and SP in different hosts in order to determine appropriate household infection prevention and control practices.

Nucleotide sequence accession numbers

Novel *S. pseudintermedius* alleles sequences obtained from this study have been deposited in the GenBank database with accession numbers JN792402 (*cpn60*_36) and JN792403 (*pta*_25).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cimid.2012.10.001.

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Annex to paper 1

Table S1. Characteristics of the novel sequence types (ST) detected among *S. aureus* isolates obtained in **paper 1**.

ST	CC	*arcC*	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqil*	Novel characteristics	Strain
ST1654	singleton	2	239	2	5	8	5	2	Novel *aroE* allele (99% similarity to *aroE*_2)	1-H, 1-D
ST2176	CC8	3	3	1	1	4	243	3	Novel *tpi* allele (99% similarity *tpi*_4)	24-C
ST2175	singleton	8	2	2	166	6	8	2	Novel *gmk* allele (99% similarity to *gmk*_2)	9-H
ST2177	singleton	6	5	6	2	7	244	34	Novel *tpi* allele (99% similarity to *tpi*_14) SLV[a] of ST95	13-H_1
ST1733	singleton	7	6	1	4	8	5	6	Novel allele combination SLV of ST217	15-H_1, 15-H_2

New alleles are indicated in grey shaded

[a] SLV; Single Locus Variant

Table S2. Allele combination and sequence type (ST) of the 18 *S. pseudintermedius* (SP) isolates obtained in **paper 1**.

ST	*16S* rRNA	*tuf*	*cpn60*	*pta*	*agrD*		Novel characteristics	Strain
					allele	allotype		
ST42	1	1	2	2	3	IV		3-D_2, 9-D
ST141	1	1	9	1	2	II		3-D_3
ST154	1	2	39	1	1	III	New *cpn60* allele_39 Genbank accession no. JN792402	3-D_4
ST33	1	2	2	1	3	IV		3-D_5
ST71	1	1	9	2	1	III		4-D_{1b}[a]
ST142	1	1	2	25	1	III	New *pta* allele_25 Genbank accession no. JN792403	5-H, 5-D, 10-C
ST92	1	1	8	1	2	II		7-D[a]
ST155	1	1	6	2	2	II	New allele combination	8-D
ST29	1	2	2	1	1	III		25-D_1
ST7	1	1	13	1	3	IV		25-D_2, 28-D
ST77	1	1	13	1	1	III		26-D
ST146	1	1	9	2	2	II	New allele combination	27-D
ST21	1	2	7	2	3	IV		11-H_1
ST100	1	2	7	1	3	IV		12-H

New alleles and STs are indicated in grey shaded

[a] MRSP isolate.

Paper 2

Clonal dynamics of nasal *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in dog-owning household members.

Elena Gómez-Sanz, Carmen Torres, Sara Ceballos, Carmen Lozano, Myriam Zarazaga[#]

Área Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain

The objective of this study was to investigate the dynamics of nasal colonization by *Staphylococcus aureus* (SA) and *Staphylococcus pseudintermedius* (SP) among healthy dog-owning household members involved in 7 previous index cases of suspected anthropozoonotic (n=4) and zoonotic (n=3) interspecies transmission [4 direct cases, identical SA (n=3) or SP (n=1) in owner and dog; three indirect, SP in owner (n=2) or SA in dog (n=1)] and coexisting individuals. Concurrence with methicillin-resistant coagulase-negative staphylococci (MRCoNS) was also evaluated. Sixteen owners and 10 dogs were sampled once every three months for one year. In total, fifty SA and 31 SP were analysed by MLST, and SA also by *spa* typing. All isolates were subjected to *Apa*I/*Sma*I-PFGE, antimicrobial resistance and virulence profiles. All index owners were persistent SA carriers in all direct-anthropozoonotic transmission cases, while only one dog was persistent SA carrier. Owner and dog exhibited a persistent SP carriage status in the direct-zoonotic transmission case. SP was maintained in the index human over time in one indirect-zoonotic transmission case. Only one SP was methicillin-resistant. All representative SA and SP isolates exhibited toxigenic potential. SA pandemic clones were detected: CC45, CC121, CC30, CC5 and CC398). Three individuals carried a SA t1451-ST398 clone with the *erm*(T)-*cadD/cadX* resistance genes. SA or SP was persistently detected in the nasal cavity of 7 (43.8%) and 2 (12.5%) owners, and in one and 2 dogs, respectively. SA was recovered as the single species in 10 of 14 positive owners and one of 9 positive dogs; SP in 3 owners and 4 dogs; and both bacterial species in one owner and 4 dogs. Concurrent carriage of SA or SP with MRCoNS isolates was common (30.7%). This is the first study on the dynamics of colonization of SA and SP in healthy pet-owning household members. Dog-contact may play a relevant role in the staphylococcal species distribution of in-contact individuals. Further studies with a larger cohort should be performed based on this finding.

Key words: *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, nasal carriage, dogs, dog-

Introduction

Staphylococcus aureus (SA) and *Staphylococcus pseudintermedius* (SP) form part of the normal microbiota of the nares, skin and mucous of humans and dogs, respectively, but they are also opportunistic pathogens [1, 2]. SA can be also found in dogs at moderate rates (<20%) with a suggested anthropozoonotic origin, although the direction of transmission has not yet been elucidated [3-7]. Humans can carry SP at very low frequencies [8, 9], while higher rates are generally detected among individuals with regular dog contact (≤4.5%) [2, 3, 7, 10, 11].

Analysis of the colonization dynamics over time is essential to address the real colonization status of a positive individual and for a better understanding of the interaction among staphylococci in the distinct hosts. However, very limited data do exist on the longitudinal colonization of SP in humans in contact to dogs [12-14], and none among healthy individuals. A couple of recent studies on the colonization dynamics of methicillin-resistant SP (MRSP) in dogs and in-contact humans reported humans to be rarely colonized by MRSP for prolonged periods of time [13, 14]. Various reports have focused on the SA colonization dynamics in humans, including specific populations, such as homeless people, drug users, healthy infants, health care workers, subjects with staphylococcal (previous) infection [15-22] or, more recently, in individuals in contact to livestock [23, 24]. Nonetheless, to our knowledge, no longitudinal studies on SA from

pet-owning humans and their pets are reported to date.

On the other hand, humans are common carriers of methicillin-resistant coagulase negative staphylococci (MRCoNS), which normally harbored multiple antimicrobial resistance genes [25-27]. Although these bacteria are considered reservoirs for the exchange of genetic material between different staphylococcal species [28-31], concurrence of SA or SP and MRCoNS in humans and in-contact pets has not been previously analyzed.

In this study, we investigated the dynamics of SA and SP colonization of dog-owning household members with previous suspected cases of SA or SP interspecies transmission [7] to evaluate the individual colonization status and to estimate the direction of bacterial transmission. In addition, we sought to assess the co-carriage of these bacterial species and MRCoNS, to appraise the possible risk of gene transfer between those microorganisms. Our results give evidence that pet-owning may play a relevant role in the staphylococcal species distribution of in-contact individuals.

Materials and Methods

Description of investigated households and index cases

Healthy owners and dogs coming from 7 households with previous description of suspected SA (4 cases, 1-4) or SP (3 cases, 5-7) interspecies transmission, recently detected in a study performed at the University of La Rioja (Spain) [7], were further investigated in the present study. Index cases were defined as cases in which SA or SP interspecies transmission is presumed to have occurred in original sampling (named T0), between at least one owner and one pet. All individuals tested were healthy and none has suffered from previous SP or SA infection. Direct interspecies transmission was considered when both, owner and dog, exhibited an identical SA or SP clone. Indirect interspecies transmission was defined as the presence of SA in dog but not in human or SP in owner but not in dog. Figure 1 shows the composition of the residences, the bacterial species responsible and the different types of suspected interspecies transmission.

In the former study, household number assigned to the different index cases was as follows (case number in the present study): household no.1 (case 1), household 4 (case 2), household 2 (case 3), household 23 (case 4), household 5 (case 5), household 12 (case 6) and household 11 (case 7) [7]. Clone nomenclature used in this study is indicated as bacterial species and methicillin resistance pattern + Multilocus Sequence Type (MLST) + Pulsed-Field-Gel-Electrophoresis (PFGE) pattern. Index clones, described as clones detected in index cases are shown in Figure 1 [7]. Several further isolates, not involved in the interspecies transmission index cases, were detected in T0 in the coexisting individuals (Figure 1). In total, 17 isolates (12 SA and 5 SP) obtained in T0 from the 7 households were included in the current study.

None of individuals tested presented occupational exposure to pets or the Health Care system. Both owners from cases 1, 3 and 6 were couples (range of age 27-30) while those from cases 2 and 7 were mother and/or grandmother (50-70 years old) plus 2 or 3 adult children (22-28). Owners from cases 4 and 5 (aged 27-35) lived alone with their dog. Individuals from cases 6 and 7 lived in rural areas.

Study design

In addition to owners (n=7) and dogs (n=5) involved in T0 index cases (all index cases present the letter “a” as part of nomenclature and are gray shaded in Figure 1), all coexisting individuals were also investigated (n=9 and n=5) (Figure 1). Nasal samples from the anterior nares of 16 owners and 10 dogs were studied once every 3 months for one year (5 sampling times, T0 to T4) with a total of 130 samples analyzed. Sixty-four samples were recovered from owners and 40 samples from dogs during T1-T4 and completely characterized in this study. In addition, twenty-six samples from T0 from the former study were considered. Nasal concurrence of SA and/or SP with MRCoNS was also investigated. All persons completed a written informed consent and agreed that their dog/s were also included. Sampling was approved by the Medical Ethical Committee of

La Rioja (Permit Number: METC 09-399/C). Sterile swabs were provided to the owners in addition to detailed description of the sampling procedures. The owners swabbed themselves and the nares of their dogs. Nasal swabs were collected from the household owner within 24-48 h after sampling and they were immediately processed or eventually stored at -20ºC until further analysis.

Definition of the carriage status and interspecies transmission dynamics

Subjects positive for SA or SP in at least four of the five samplings (including T0) were considered persistent carriers; those positive in two or three samplings were defined intermittent carriers; individuals positive in a single sampling were reported sporadic carriers; and those negative throughout the study were defined as non carriers. It is important to remark the possibility that individuals who carry both bacterial species exhibited different carriage status per bacterial species. Dynamics of the interspecies transmission cases over time was defined likewise (persistent, intermittent, and sporadic).

Isolation and identification of SA, SP and MRCoNS isolates

Samples were inoculated into Brain-Heart-Infusion broth (BHI, Difco) supplemented with 6.5% NaCl and incubated at 37ºC for 24h. One-hundred microliters were seeded on Oxacillin-Resistant-Staphylococcal-Agar-Base (ORSAB; OXOID) plates supplemented with 2 mg/L of oxacillin for the isolation of MR staphylococcal isolates, either SA, SP or CoNS. Seventy microliters were inoculated in parallel on Manitol-Salt-Agar (MSA; BD) plates for the isolation of SA and SP. Plates were incubated at 35ºC for 24-48h. All colonies with different appearance were subcultured on BHI agar (Difco) and further studied. Preliminary identification of isolates was based on colony morphology, Gram staining, and catalase and DNase activities. A species-specific multiplex PCR was performed to identify SA and *S. intermedius*/SP isolates [32]. Discrimination between *S. intermedius* and SP was conducted by restriction fragment length polymorphism of the *pta* gene [33]. The presence of *mec*A gene was investigated by PCR in all isolates [34]. Identification of MRCoNS was performed by amplification and sequencing of the *sodA* gene in all *mec*A positive CoNS isolates [35]. Only one MRCoNS isolate per individual was further characterized when isolates belonged to the same bacterial species.

Analysis of the clonal relatedness of isolates

The genetic relatedness of all isolates obtained (SA, SP and MRCoNS) was investigated by PFGE of total DNA restricted with *Sma*I or *Apa*I macrorestriction enzymes as previously described [36]. For SA and MRCoNS isolates, PFGE running conditions were those recommended by the HARMONY protocol [36]. SA isolates non-typeable with *Sma*I were subjected to *Apa*I-PFGE and run for 20h at 6V/cm using pulsed time ramping from 2 to 5 s [37]. *Sma*I-digested Plugs obtained from SP isolates were run for 24h at 5.6V/cm using pulse times from 2 to 5 s [38]. Isolates were considered a unique clone when they showed indistinguishable PFGE band patterns and subclones of the major clone when closely related patterns (1-3 band differences) were observed [39]. The different SA patterns were distinguished by capital letters, major SP patterns by Roman numbers and MRCoNS by Arabic numbers. Subclones were indicated with the major lettering type followed by a lower case letter.

Molecular typing of SA and SP isolates

All SA isolates were subjected to *spa* typing as previously described [40] and sequences were analyzed using Ridom Staph-Type software version 2.0.21 (Ridom GmbH). MLST was performed as recommended (http://www.mlst.net) on one representative SA isolate per clone and per household. MLST was determined in all SP isolates; for this, five housekeeping genes were amplified and sequenced (*pta*, *cpn60*, *tuf*, 16S rRNA and *agrD*) followed by assignment of alleles by comparison to those present in the NCBI nucleotide database and using a key table for MLST typing of *S. intermedius* group (SIG) isolates [38].

Antimicrobial resistance profile of isolates

Susceptibility testing to 17 antimicrobial agents was performed in all

isolates obtained by agar disk-diffusion method [41]. Antimicrobials tested were as follows (µg/disk): penicillin (10U), oxacillin (1), cefoxitin (30) erythromycin (15), clindamycin (2), gentamicin (10), kanamycin (30), streptomycin (10U), tobramycin (10), tetracycline (30), trimethoprim-sulfamethoxazole (1.25+23.75), chloramphenicol (30), ciprofloxacin (5), mupirocin (200), fusidic acid (10), vancomycin (30), and linezolid (30). Methods and breakpoints followed for streptomycin and fusidic acid were those recommended by the Société Française de Microbiologie (http://www.sfm-microbiologie.org). Inducible or constitutive clindamycin resistance was determined by the double-disk diffusion test (D-test) [41].

The last recovered isolate of each novel clone per household was chosen as representative strain for in-depth molecular characterization. Presence of 32 antimicrobial resistance genes was investigated by PCR in all representative SA and SP (SA/SP) strains and in all MRCoNS isolates [34, 42]. Antimicrobial resistance genes tested were as follows: *mec*A, *blaZ*, *tet*(K), *tet*(M), *tet*(L), *tet*(O), *erm*(A), *erm*(B), *erm*(C), *erm*(T), *mph*(C), *msr*(A), *msr*(B), *aacA-aphD*, *aphA3*, *aadE*, *aadD*, *aadA*, *str*, *sat4*, *dfr*(A), *dfr*(D), *dfr*(G), *dfrK*, cat_{pC221}, cat_{pC194}, cat_{pC223}, *fexA*, *cfr*, *mupA*, *fusB* and *fusC*.

Detection of virulence genes

Presence of the Panton Valentine leukocidin (PVL) genes (*lukS/F-PV*) was determined in all isolates by PCR [7]. The leukocidins *lukE/D* and *lukM*, exfoliatins *eta* and *etb*, the toxic-shock syndrome toxin gene *tst* and 18 enterotoxin genes were likewise investigated in all representative SA strains [7]. These isolates were additionally tested by PCR for the presence of the human-specific immune evasion cluster (IEC) genes encoding the chemotaxis inhibitory protein (*chp*), staphylococcal complement inhibitor (*scn*) and staphylokinase (*sak*), enclosed within prophage φ3 [43].

PCR-based determination of the leukocidin *lukS/F-I*, exfoliatins *siet*, *expA* and *expB,* and 18 enterotoxin genes plus the species specific sec_{canine} and *se-int* genes were also investigated in all representative SP strains [7].

Results

SA isolates and clones recovered and SA population structure

All isolates and clones recovered from the 7 households are schematically represented in Figure 1.

Thirty-two SA isolates were obtained from 16 owners and 6 isolates were recovered from 10 dogs. The 12 SA isolates obtained in T0 were also considered. In total, 50 SA isolates were recovered from the 7 cases.

Based on the PFGE band profile of the 50 SA isolates, 10 SA clones were detected (Table 1). Ten different *spa* types (Table 2) and 7 STs (ST1654, ST121, ST5, ST398, ST45, ST30, and a new ST registered as ST2619) were observed. Clone MSSA-ST45-F in owner $H3a_1$, exhibited 3 different but related *spa* types along the sample year (T0→t073; T1→t116; T2→t026; T3-4→t073) (Table 2).

SP isolates and clones recovered and SP population structure

Eight SP isolates were recovered from 16 owners and 18 isolates were recovered from 10 dogs. Including the 5 SP isolates obtained in the former report (T0) [7], a total of 31 SP isolates were recovered from the 7 cases.

Based on their PFGE band profile, 9 SP clones and 9 distinct STs (ST71, ST77, ST6, ST42, ST142, ST185, ST100, ST70 and ST21) were identified (Table 1). Four subclones (VIIIa to VIIId) of MSSP-ST70-VIII were detected (Table 1).

Antimicrobial resistance profile of SA and SP isolates

All SA isolates were susceptible to oxacillin and cefoxitin and were *mec*A negative (Table 1). All SA isolates from the same clone presented identical antimicrobial resistance phenotypes. None of the SA clones were multidrug resistant (MDR) (resistance to at least three classes of antimicrobials). The majority of the SA clones (6/10) was susceptible to all antimicrobials or showed only

penicillin resistance harboring the *blaZ* gene. Two clones (MSSA-ST398-E, MSSA-ST5-J) presented additional MLS_B resistance [*erm*(C) or *erm*(T)], and a single clone (MSSA-ST121-C) tetracycline resistance [*tet*(K)].

All SP isolates from the same clone presented identical antimicrobial resistance phenotypes. A single sporadic MRSP clone (MRSP-ST71-I) and 2 MSSP (MSSP-ST77-II, MSSP-ST142-V) were MDR (Table 1). The remaining SP clones obtained were susceptible to all antimicrobials or showed only penicillin resistance (*blaZ*).

Presence of virulence genes in SA and SP

Virulence traits of SA and SP clones are shown in Table 1. All isolates lacked the PVL-toxin genes *lukS/F-PV*. All SA representative strains (one isolate per clone, per household) but one carried enterotoxin genes, with 63.6% of them harboring different enterotoxin-gene-cluster combinations. Further, 45.5% of them exhibited the leukocidin *lukE/D* genes and two clones the exfoliatin gene *etb*. All SP representative isolates tested harbored the *lukS/F-I*, *siet* and *se-int* and 3 (33.3%) the *expA* gene.

SA and SP species distribution along the sample year

Five of the 7 households presented both bacterial species in at least one sampling. Most positive owners (10/14) carried only SA throughout the sampling year. However, three owners only exhibited SP (Figure 3). Both SA and SP were obtained along the study in only one person. In contrast, both bacterial species were recovered from a high number of positive dogs (4/9) along the study, with a single dog carrying only SA. Four animals were only positive for SP.

SA and SP carriage status

The carriage status of the investigated subjects over time is shown in Figure 2. Among the 16 owners, seven were persistent carriers of SA and 2 of SP. None of these individuals presented SA or SP co-carriage along the sampling period (Figure 1). Two humans were intermittent SA carriers. Two owners were sporadic SA carriers and 2 sporadic SP carriers. A single owner (H2b) was carrier of both bacterial species along the year (intermittent SA carrier + concurrent sporadic SP carrier) (Figure 1). Only 2 owners were non-carrier throughout the sample year.

The majority of dogs (6/10) were sporadic nasal carriers of SP (n=3) or SA (n=3). Three dogs were intermittent SP carriers and one dog was a SA intermittent carrier, with 2 of these animals being also sporadic SA carriers. Two animals were SP persistent carriers and one was a persistent carrier of SA. Among these, two dogs were additional sporadic concurrent SA/SP carriers (Figure 1). Remarkably, 40% of dogs belonged to 2 distinct carriage types depending on bacterial species. A single dog was non-carrier.

Dynamics of the interspecies transmission cases over time

Cases of direct SA transmission

All three households with presumed SA direct transmission (cases 1, 2 and 3) maintained the original SA clone in the owners implicated in the index cases while only one index dog (D3a) was a persistent carrier (MSSA-ST45-F) (Table 1, Figure 1).

In case 2, index dog D2a revealed to be a persistent SP carrier due to the presence of a MDR SP clone (MSSP-ST77-II) in samplings T1, T2 and T3 (Table 1, Figure 1). A coexisting owner (H2b) harbored the aforementioned SP clone in sampling T1, what represents a novel sporadic case of direct SP interspecies transmission. It is interesting to underline the presence of a SA t1451 clone (MSSA-ST398-E) that belongs to the lineage ST398 in two owners (H2b and H2d) in two consecutive samplings (T3-T4).

Indirect SA anthropozoonotic transmission

The dog from case 4 (D4a), positive for a SA ST121 isolate only in the initial sampling, was positive for a SP clone (MSSP-ST42-IV) in the three following samplings (Table 1, Figure 1), while its owner was a non SA or SP carrier.

Direct SP transmission

In case 5, owner and dog (H5a and D5a) were persistent SP carriers. Remarkably, in the last 3 samplings (T2 to T4) a different SP

clone (MSSP-ST185-V) from the index case was observed. (Table 1, Figure 1).

Cases of indirect SP zoonotic transmission

As for case 6, the index human (H6a) was permanently colonized by SP, with MSSP-ST100-VII isolated in T0, and MSSP-ST70-VIII in the subsequent samplings (T1 to T4) (Table 1, Figure 1). Interestingly, both STs only differed in a silent mutation in allele *tuf* (*tuf*_2 at position T136A) and presented the exfoliatin gene *expA* (Table 1). The coexisting human (H6b) resulted a persistent carrier of a SA clone (MSSA-ST30-H). Remarkably, one dog (D6b) carried a MSSA t1451-ST398 isolate in one sampling (T3).

In case 7, neither the index human (H7a) nor any of the coexisting individuals carried the original MSSP ST21 clone in subsequent samplings (Table 1, Figure 1).

Concurrence carriage with MRCoNS and molecular characterization of isolates

Thirty-two MRCoNS isolates (28 *S. epidermidis*, 3 *S. haemolyticus* and 1 *S. succinus*) were detected along the four samplings from high rate of owners (14/16) and half of tested dogs (5/10), of which 19 (18 *S. epidermidis* and one *S. haemolyticus*) were present in concurrence with SA or SP and further investigated (Figure 1). In addition to the *S. haemolyticus* clone (MRSH-6), nine MRSE clones were observed by *Sma*I-PFGE, with clone 4 presenting two subclones (4a-4b) (Table 3).

A total of 15.6% (10/64) of SA/SP positive samples also carried a MDR MRCoNS isolate. Half of MRCoNS clones were MDR carrying a wide variety of antimicrobial resistance genes (Table 3). Interestingly, several isolates of the same clones from cases 3 (MRSE-4) and 6 (MRSE-8) exhibited different antimicrobial resistance pheno- and/or genotype to mupirocin over time (Table 3). Moreover, clone MRSE-9 in 2 individuals (H6b and D6c) presented different antimicrobial resistance profiles at the same sampling (T2). In addition to the coexistence of the *blaZ* gene in most SA/SP and concurrent MRCoNS isolates, the trimethoprim resistance *dfr*(G) gene was detected in clone MRSH-6 and MSSP-ST142-V in the same sampling (Table 3).

Half of SA/SP positive owners and 3 of the 9 positive dogs concurrently carried a MRCoNS isolate in at least one sampling. Among these, two owners ($H3a_1$ and H6a) were persistent carriers of both types of staphylococci (Figure 1, Table 3). In case 6, owner H6b and dog D6c harbored the identical MRSE-9 in sampling T2, representing a novel case of direct interspecies transmission.

Particular traits of the SA ST398 isolates

Four SA t1451-ST398 isolates from two owners (H2b, H2d) at successive samplings (case 2) and one from an unrelated household (case 6) from a dog (D6b), belonged the same SA clone (MSSA-ST398-E) based on all molecular techniques performed (Table 1).

Only these isolates exhibited inducible MLS_B resistance due to the presence of the *erm*(T) gene. PCR analysis for the presence of the cadmium resistance operon *cadDX* (recently described in physical linkage to *erm*(T) in a small MSSA ST398 plasmid, pUR3912 [43]) revealed their existence in the five isolates. All 5 isolates were tested for virulence determinants and evidenced the presence of the *scn*, *chp* and the enterotoxin gene *sec*.

Discussion

This is the first study on the dynamics of colonization of SA and SP in healthy pet-owning household members. The fact that all investigated households presented at least one individual positive for SA or SP in each of the samplings is noteworthy. In addition, the vast majority of investigated owners (14/16) and dogs (9/10) carried either SA or SP in at least one sampling, results that evidence a real flow of both bacterial species within pet-owning household settings.

Based on the evolution of colonization of SA over time in the different index cases it seems that the original source of SA was the human and that dogs serve as sporadic sources that may play a role in re-colonization or maintenance. The household with an

identical SA clone in owners and dog along the whole year (case 3) might be explained by the persistent carriage of both index persons. Interestingly, in this household, SA isolates (clone MSSA-ST45-F) with three different *spa* types in serial samplings (t073→t116→t026→t073→t073) were recovered from one of the owners ($H3a_1$). The identical PFGE band profile in these SA isolates suggests evolution of its *spa* repeat composition rather than the presence of different clones. In this line, mutations over time within the same SA clone have been previously detected in SA longitudinal studies [45-48].

The presence of a novel sporadic case of direct MDR SP interspecies transmission in one dog and one owner enhances the interspecies transmission potential of SP between in-contact individuals. The fact that SP was maintained along the whole sample year in two owners (two of the three suspected cases of zoonotic interspecies transmission) reveals that humans can be also colonized for prolonged periods of time with this bacterial species. In this respect, owner and dog from case 5 lived separately for several months between T0 and T1, which SP absence in owner in T1 matches with the idea that SP was originated in the dog. Colonization dynamics of subjects from case 6 exhibited an atypical profile, since owner seemed to be the apparent SP source. The presence in this household of two SP clones with related MLST profiles, both carrying the exfoliatin *expA* gene, is relevant, and a possible evolution of these isolates from a common ancestor might be speculated.

The presence of SP persistent owners, together with the absence of intermittent SP human carriers might point to favorable conditions in specific individuals to be colonized by SP. SP and SA were present in all carriage types (persistent, intermittent, sporadic) in dogs, with SA being in most cases sporadically detected. Our data suggest that SA may be more frequently detected in the nares of dogs than SP in nasal cavity of humans but that both bacterial species can persistently colonize non natural hosts, at least in those cases with reiterate direct contact. In addition, a single owner was positive for both bacterial species, what contrasts with the elevated number of positive dogs from which SA and SP was recovered (4/9). This observation reflects the ability of these animals to be potential "vectors" for staphylococcal acquisition.

The absence of SP in the SA-persistent owners, as well as the lack of SA in the SP-persistent owners may be relevant for the nasal persistence of the other coagulase positive staphylococcal species in humans. In contrast, two of the three persistently colonized dogs were sporadic nasal carriers of the other coagulase positive staphylococci.

While SA normally colonizes the nasal cavity of humans [1], SP seems to be more variably located in dogs, and perineum and mouth have been recently addressed as the body-sites with most SP recovery rates [2, 49]. A recent study focused on the dynamics of SP in healthy dogs with multiple sampling sites, revealed that between 2 and 5 clones were present in the same animal, what is in agreement with our results. Replacement of SP clones in an individual microbiota over time has been previously reported as common in previous longitudinal studies in SP in dogs [50]. In contrast, the SA population structure was mostly maintained in the SA persistent individuals, what is in line with other longitudinal SA studies on humans [16]. Alternatively, to our knowledge, no information is available on preferential colonization sites of SA in dogs and SP in humans, what warrants further investigations.

Pandemic SA clones were observed in humans (CC45, CC121, CC30, CC398, CC5), of which CC121 and CC398 were also sporadically detected in dogs. The elevated virulence gene content of all SA clones detected, with 70% of them carrying enterotoxin gene cluster, and 50% the leukotoxin gene *lukED*, enhances the importance to control possible sources for acquisition of potential pathogenic bacteria. In addition, all SA clones but one, detected in a dog (MSSA-ST121-G), harbored any of the genes implicated in evasion of the human immune system, what responds to a human adaptation.

Two SA clone exchanges were detected in two owners. These subjects shifted from a SA t159 or t002 to a SA t1451-ST398

clone in the last two samplings. Interestingly. All 5 MSSA-ST398-E isolates (4 from 2 humans, 1 from dog) carried the MLS_B resistance gene *erm*(T) and the cadmium resistance operon *cadDX*, which has been recently described to be co-located within pUR3912. This plasmid was obtained from a MSSA ST398 of human origin recovered in our previous one-point prevalence study on pet-owning households [7, 44] and suggests a plausible clonal spread of pUR3912 among MSSA ST398 isolates. Positive owners did not have direct contact to rural areas or farm animals and the presence of the IEC genes points to a human adaptation [51]. The detection of an enterotoxin gene (*sec*) in all 5 isolates is remarkable, given that these toxin genes are normally absent among MRSA ST398 isolates. The presence of the same *Apa*I-PFGE band profile between unrelated individuals suggests a clonal distribution. *Sma*I-PFGE resulted highly discriminative among the other SA clones, and unrelated individuals with SA with identical *spa* type and/or ST exhibited different band patterns.

Concurrence of SA and MRCoNS has been previously reported to have a negative association due to competition for the same ecological niche [52, 53]. Moreover, *S. epidermidis* is considered to "protect" the host from SA colonization [53]. Half of SA/SP positive owners also presented MRCoNS in at least one sampling, what reflects a conceivable punctual coexistence of such bacteria. Further, the persistent concurrence of SA/SP and (MDR) MRSE colonization observed in two owners (12.5% of investigated humans) shows a persistent source for acquisition of antimicrobial resistance genes in SA/SP in these individuals.

The detection of a MRSE sporadic case of interspecies transmission (which direction remains unknown) that involved isolates with different antimicrobial resistance profiles evidences transmissibility potential and quick gene pool mobilization ability of this bacterial species. Further, the differential evolution of mupirocin resistance in some isolates of the same clone deserves continued surveillance. In contrast, stability on the antimicrobial resistance profile was observed in all SA/SP clones over time. Although the acceptance of exogenous genetic material between staphylococci does seem to require special circumstances not yet properly understood [31], transmission of the *mec*A gene between MRCoNS and SA has been previously assumed [28, 29, 31]. The presence of mupirocin resistance genetic determinants in 42% (8/19) of MRCoNS isolates represents a potential risk for acquisition of the *mupA* gene during SA/SP intended decolonization with mupirocin-based treatments.

Conclusion

This study gains knowledge in the ecology of SA, SP and concomitant MRCoNS in humans and in-contact dogs and represents a first approach to understand the role that dogs may play in the epidemiology of SA and SP in the human host over time. The apparent adaptability of SP to colonize dog owners who do not carry SA, as well as great predisposition of these animals to be SA carriers should be taken into account in subsequent developments of infection control measurements. Moreover, the human-associated MSSA ST398 is shown to be transferred and maintained within household members. Further longitudinal studies on SA and SP with a larger cohort in humans and in-contact dogs are essential for confident assertions of the implication of pet-owning as increased factor to acquire and maintain potentially pathogenic bacteria.

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Table 1. Number of isolates detected and molecular characteristics of the SA and SP clones investigated.

Case	Clone[a]		Isolation data									
	SA	SP	Individual	T0	T1	T2	T3	T4	No isolates	Representative isolate	Resistance genes detected[b]	Relevant virulence genes detected[c]
1	MSSA-ST1654-A		H1a	+	+	+	+	+	6	C3494	*blaZ*	[*seg, sei, sem, sen, seu*], (*chp*, *sak*)
			D1a	+								
	MSSA-ST2619-B		D1a				+		1	C4897	*blaZ*	*lukED, etb*, (*seg, sei, sem, sen*), *sec*, (*scn*, *sak*)
2	MSSA-ST121-C		H2a	+	+	+	+	+	8	C3931	*blaZ*, *tet*(K)	*lukED*, *etb*, [*seg, sei, sem, sen*], *sak*
			D2a	+								
			H2b		+							
			H2c		+							
		MRSP-ST71-I	D2a	+					1	C3930	*blaZ*, *mec*A, *tet*(K), [*erm*(B), *aphA3*, *sat4*, *aadE*], *aacA/aphD*, *dfr*(G)	*lukS/F-I*, *siet*, *se-int*
	MSSA-ST5-D		H2d	+	+				3	C3935	*blaZ*	*lukED*, [*seg, sei, sem, sen, seo, seu*], (*chp, sak*)
			H2e	+								
		MSSP-ST77-II	D2a		+	+	+		4	C5102	*blaZ*, *tet*(K), *tet*(M), *erm*(A), *erm*(B), *aacA/aphD*, [*aphA3-sat4-aadE*], cat_{pC221}	*lukS/F-I*, *siet*, *se-int*
			H2c		+							
	MSSA-ST398-E		H2c				+	+	4	C5587	*blaZ*, (*erm*(T)[c], *cadDX*)	*sec*, (*scn, chp*)
			H2d				+	+				
3	MSSA-ST45-F		$H3a_1$	+	+	+	+	+	15	C2919	*blaZ*	[*seg, sei, sem, sen, seo*], (*scn, chp, sak*)
			$H3a_2$	+	+	+	+	+				
			D3a	+	+	+	+	+				
		MSSP-ST6-III	D3a		+				1	C3953	*blaZ*	*lukS/F-I*, *siet*, *se-int*
4	MSSA-ST121-G		D4a	+					1	C2727	-	*lukED*
		MSSP-ST42-IV	D4a		+	+	+		3	C5552	*blaZ*	*lukS/F-I*, *siet*, *se-int*
5		MSSP-ST142-V	H5a	+					3	C2915	*blaZ*, *tet*(K), *tet*(M), *erm*(B)[c], [*aphA3*, *sat4*, *aadE*], *dfr*(G), cat_{pC221}	*lukS/F-I*, *siet*, *se-int*
			D5a	+	+							
		MSSP-ST185-VI	H5a			+	+	+	6	C5578	*blaZ*	*lukS/F-I*, *siet*, *se-int*
			D5a			+	+	+				
6		MSSP-ST100-VII	H6a	+					3	C3469	*blaZ*	*lukS/F-I*, *siet*, *se-int, expA*
			D6b				+	+				
		MSSP-ST70-VIII	H6a		+	+	+	+	9	C5562	*blaZ*	*lukS/F-I*, *siet*, *se-int, expA*, (sec_{canine}, *sel*), *sea*
			D6b			+						
			D6c			+		+				
			D6d			+						

			D6e			+						
	MSSA-ST30-H		H6b		+	+	+	+	4	C5559	*blaZ*	(*seg*, *sei*, *sem*, *sen*, *seo* ,*seu*), *sak*
	MSSA-ST398-E		D6b				+		1	C5086	*blaZ*, (*erm*(T)[c], *cadDX*)	*sec*, (*scn*, *chp*)
7		MSSP-ST21-IX	H7a	+					1	C3917	*blaZ*	*lukS/F-I*, *siet*, *se-int*, *expA*
	MSSA-ST30-I		H7b	+	+	+	+	+	5	C3916	*blaZ*	*tst*, [*seg*, *sei*, *sem*, *sen*, *seu*], *sea*, (*chp*, *sak*)
	MSSA-ST5-J		H7c	+	+				2	C3918	*blaZ*, *erm*(C)[c]	*lukED*, (*scn*, *chp*, *sak*)
Total			23	17	17	16	17	14	81			

MSSA, methicillin-susceptible *S. aureus*; MSSP, methicillin-susceptible *S. pseudintermedius*. [a] Clones were designated as follows: bacterial species (MSSA or MSSP)-MLST-PFGE profile. [b] Genes physically linked are displayed in square brackets and those possibly linked based on bibliography are shown in brackets. [c] Inducible clindamycin resistance.

Table 2. Different *spa* types present among the *S. aureus* clones per household during the sample period (T0-T4), positive individuals and correspondent clones.

Case	*spa*	repeats	Clone	Sampling	Individual/s
1	t021	15-12-16-02-16-02-25-17-24	MSSA-ST1654-A	T0, T1, T2, T3, T4	H1a, D1a^{T0}
	t159	14-44-13-12-17-17-23-18-17	MSSA-ST2619-B	T3	D1a
2	t159	14-44-13-12-17-17-23-18-17	MSSA-ST121-C	T0, T1, T2, T3, T4	H2a, H2b^{T1}, H2c^{T1}, D2a^{T0}
	t002	26-23-17-34-17-20-17-12-17-16	MSSA-ST5-D	T0, T1	H2d , H2e^{T0}
	t1451	08-16-02-25-34-25	MSSA-ST398-E	T3, T4	H2b, H2d
3	t073	08-16-02-16-13- -17-34-16-34	MSSA-ST45-F	T0, T1, T2, T3, T4	H3a$_1^{T0, T3, T4}$, H3a$_2$, D3a
	t116	08-16-02-16-13-13-17-34-16-34	MSSA-ST45-F	T1	H3a$_1$
	t026	08-16- - - - - - - -34	MSSA-ST45-F	T2	H3a$_1$
4	t151	26-17-20-17-16	MSSA-ST121-G	T0	D4a
6	t1071	26-23-12-23-02-34-34	MSSA-ST30-H	T1, T2, T3, T4	H6b
	t1451	08-16-02-25-34-25	MSSA-ST398-E	T3	D6b
7	t012	15-12-16-02-16-02-25-17-24-24	MSSA-ST30-I	T0, T1, T2, T3, T4	H7b
	t002	26-23-17-34-17-20-17-12-17-16	MSSA-ST5-J	T0, T1, T2	H7$_C$

When an individual was not positive for the specific *spa* type in all indicated samplings the positive sampling is indicated in superscript. Boldface and dashes added to facilitate comparison of *spa* repeats in the different isolates of clone MSSA-ST45-F.

Table 3. Characteristics of the MRCoNS recovered in the SA/SP positive individuals per household and the concurrent SA or SP clone.

Case	Clone	Individual	Sampling	Isolate	Antimicrobial resistance profile		Concomitant SA/SP clone	
					Phenotype of resistance[a]	Resistance genes detected	SA	SP
1	MRSE-1	D1a	T3	C4812	P-O-F-E	*blaZ*, *mec*A, *mphC*, *msrA/msrB*	MSSA-ST2619-B	
2	MRSE-2	H2a	T2	C4807	P-O-F-E-M	*blaZ*, *mec*A, *mphC*, *msrA/msrB*, *mupA*	MSSA-ST121-C	
	MRSE-3	H2d	T4	C5588	P-O-F	*blaZ*, *mec*A	MSSA-ST398-E	
3	MRSE-4a	$H3a_1$	T1	C3956	P-O-F	*blaZ*, *mec*A	MSSA-ST45-F	
	MRSE-4b	$H3a_1$	T3	C5097	P-O-F-M	*blaZ*, *mec*A, *mupA*	MSSA-ST45-F	
	MRSE-4b	$H3a_2$	T2	C4798	P-O-F-M	*blaZ*, *mec*A, *mupA*	MSSA-ST45-F	
	MRSE-5	$H3a_2$	T1	C3958	P-O-F-T-E-To	*blaZ*, *mec*A, *tet*(K), *tet*(M), *mphC*, *msrA/msrB*, *aadD*	MSSA-ST45-F	
	MRSE-5	$H3a_1$	T2	C4796	P-O-F-T-E-To	*blaZ*, *mec*A, *tet*(K), *tet*(M), *mphC*, *msrA/msrB*, *aadD*	MSSA-ST45-F	
	MRSE-5	$H3a_1$	T4	C5573	P-O-F-T-E-To	*blaZ*, *mec*A, *tet*(K), *tet*(M), *mphC*, *msrA/msrB*, *aadD*	MSSA-ST45-F	
5	MRSH-6	D5a	T1	C3960	P-O-F-E-X-Fu[b]	*blaZ*, *mec*A, *mphC*, *msrA/msrB*, *dfr*(A), *dfr*(G)		MSSP-ST142-V
	MRSE-7	D5a	T4	C5577	P-O-F	*blaZ*, *mec*A		MSSP-ST185-VI
6	MRSE-8	H6a	T1	C3945	P-O-F-G-K-M-Fu[b]	*blaZ*, *mec*A, *aacA/aphD*, *mupA*		MSSP-ST70-VIIIa
	MRSE-8	H6a	T2	C4791	P-O-F-G-K-M-Fu[b]	*blaZ*, *mec*A, *aacA/aphD*, *mupA*		MSSP-ST70-VIIIb
	MRSE-8	H6a	T3	C5093	P-O-F-G-K-M-Fu[b]	*blaZ*, *mec*A, *aacA/aphD*, *mupA*		MSSP-ST70-VIIIb
	MRSE-8	H6a	T4	C5564[c]	P-O-F-G-K-Fu[b]	*blaZ*, *mec*A, *aacA/aphD*, *mupA*		MSSP-ST70-VIIIc
	MRSE-8	H6b	T4	C5558	P-O-F-G-K-M-Fu[b]	*blaZ*, *mec*A, *aacA/aphD*, *mupA*		MSSP-ST70-VIIIc
	MRSE-9	H6b	T2	C4789	P-O-F-G-To-K	*mec*A, *aacA/aphD*	MSSA-ST30-H	
	MRSE-9	D6c	T2	C4787	P-O-F-T	*blaZ*, *mec*A, *tet*(K), *tet*(M)		MSSP-ST70-VIIIb
7	MRSE-10	H7b	T4	C5581	P-O-F-E	*blaZ*, *mec*A, *mphC*, *msrA/msrB*	MSSA-ST30-J	

MRSE, methicillin-resistant *S. epidermidis*; MRSH, methicillin-resistant *S. haemolyticus*. [a] P, penicillin; O, oxacillin; F, cefoxitin; E, erythromycin; M, mupirocin; Cp, ciprofloxacin; To, tobramycin; X, co-trimoxazol; Fu, fusidic acid; G, gentamicin; K, kanamycin; T, tetracycline. [b] Fusidic acid resistance genes *fusB* and *fusC* were not detected. [c] isolate C5564 was mupirocin susceptible regardless the presence of the *mupA* gene.

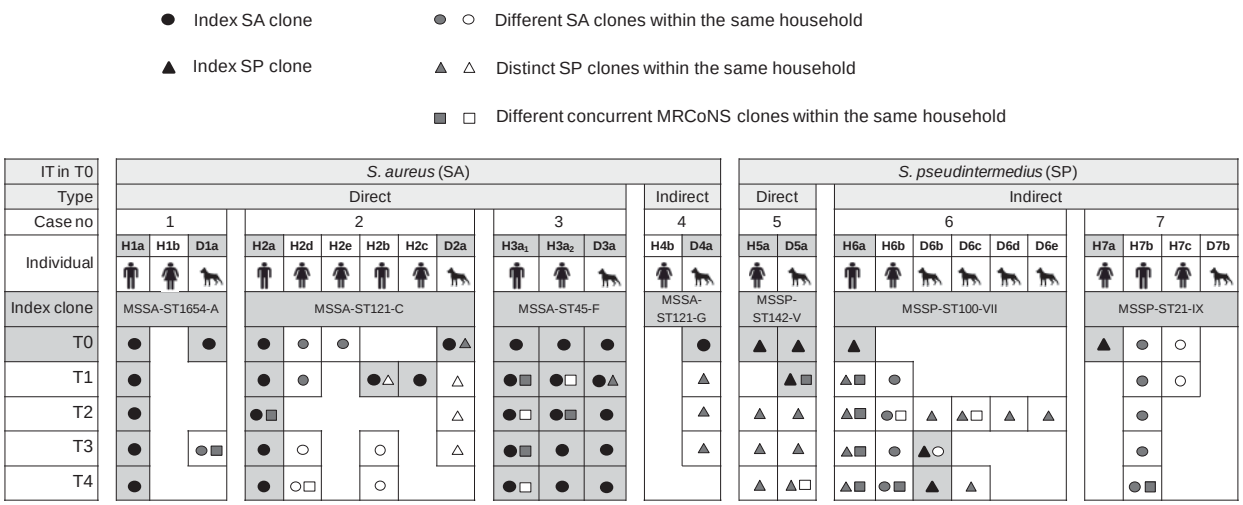

Figure 1. Schematic representation of the dynamics of colonization of the seven households investigated. MRCoNS, methicillin-resistant coagulase negative staphylococci. IT, bacterial species responsible for interspecies transmission. Type, type of interspecies transmission (Direct, Indirect). Case no, number of the households investigated in this study. Individuals are named H (for human) or D (dog) followed by the case number (1 to 7) and a lower-case letter to differentiate subjects per household; if necessary, a lower script number was added. Nomenclature of individuals involved in index cases (T0) is displayed with gray background. T0 to T4, the different samplings. Index clone, *S. aureus* or *S. pseudintermedius* clones responsible for interspecies transmission in index cases (T0).

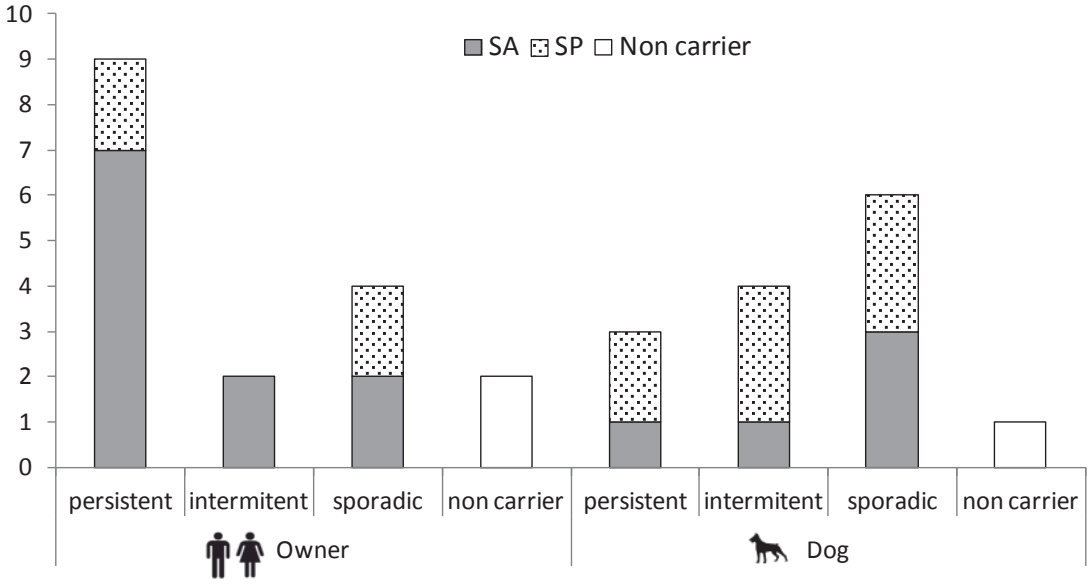

Figure 2. Number of investigated individuals (owners and dogs) with different *S. aureus* (SA) and *S. pseudintermedius* (SP) carriage status over the sample year (T0-T4). It should be noted that the sum is higher than the otal number of isolates given that individuals could belong to 2 distinct carriage types depending on bacterial species.

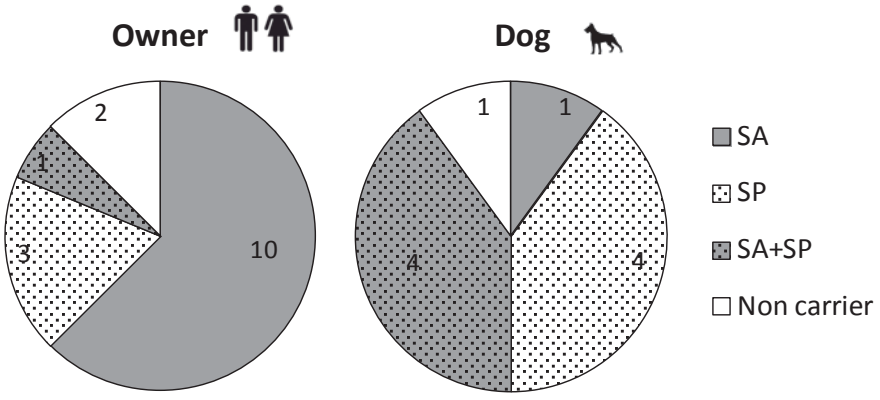

Figure 3. Distribution of investigated individuals (owners, dogs) related to the SA and/or SP carriage as a summary of the sampling year (T0-T4).

Chapter 2

S. aureus and *S. pseudintermedius* from pound dogs

CHAPTER 2. *S. aureus* AND *S. pseudintermedius* FROM POUND DOGS

The ecology of *S. aureus* and *S. pseudintermedius* in animals has gained attention in the last years due to the spread of LA-MRSA in livestock (EFSA, 2009b, García-Alvarez et al., 2011), and the emergence of MDR MRSP in dogs, specially MRSP ST71 in Europe and MRSP ST68 in North America (Perreten et al., 2010; van Duijkeren et al., 2011b). It has been recently suggested that LA-MRSA ST398 may have originated in humans as MSSA ST398 (Price et al., 2012), what has enhanced the interest for methicillin-susceptible strains of different origins. The objective of this chapter was to determine the nasal *S. aureus* and *S. pseudintermedius* rates in kennel dogs and to characterize the recovered isolates. We sought to assess the possible implication of these animals in the evolution of successful *S. aureus* and *S. pseudintermedius* lineages.

Ninety-eight kennel dogs were screened for nasal CoPS carriage. All isolates were characterized by different molecular techniques, antimicrobial resistance profile and virulence genes content. Clonal relatedness of isolates was also investigated by SmaI or ApaI/PFGE.

Twenty-two dogs carried *S. pseudintermedius* (22.4%). Eight MRSP (8% of animals) were detected from unrelated animals. Seven isolates were MRSP ST71 and represented its first description in Spain at that time. All strains were MDR and all MRSP ST71 revealed closely related PFGE patterns. PCR mapping and sequencing revealed the presence of the aminoglycoside and streptothricin resistance gene cluster *aadE-sat4-aphA3* in physical linkage to the MLS_B *erm*(B) [designated *erm*(B)-Tn*5405*-like elements] in all MRSP strains. A nucleotide substitution in a *gyrA* region outside the QRDR of the ciprofloxacin resistant strains that had been reported to show higher MIC values to enrofloxacin revealed not increased MIC values among the ciprofloxacin resistant isolates. Sixteen MSSP (15.3% of dogs) and one MS *S. schleiferi* subspecies *coagulans* were recovered. In contrast to MRSP, high clonal diversity was revealed among MSSP, and remarkably, 75% of MSSP exhibited novel STs. In total, 75% of MSSP showed resistance to at least one antimicrobial, with tetracycline resistance as the most common trait (56.3%, versus 100% in our MRSP strains). Only 2 MSSP (12.5%) harboured the resistance gene cluster and the *erm*(B) gene, in not apparent association. All *S. pseudintermedius* isolates investigated (MRSP and MSSP) harboured the *lukS/F-I*, *seit* and *se-int* virulence genes, while only MSSP (31.3%) exhibited the *expA* or *expB* genes.

Twenty-four MSSA (24.5% of animals) were obtained while no MRSA was recovered. Thirteen *spa*-types and 12 STs were detected among MSSA. Both livestock and human associated lineages were observed (ST398, ST133, ST1, ST5, ST146, ST188, ST15, ST78, ST45, ST217, and the novel ST1655 and ST2329), being related to the rural or urban origin of the animals. Interestingly, MSSA ST398 (with *spa* types t034, t108 and t5883) was the predominant lineage (29.2%). Although all MSSA ST398 positive dogs were unrelated and were sampled in 3 distinct periods, all showed closely related PFGE profiles. Fifty-percent of MSSA isolates showed resistance to at least one of the tested antimicrobials, with remarkably low MSSA penicillin resistance (20.8%). MSSA isolates ST398, ST133, ST1 and ST2329 were susceptible to all antimicrobials and were the only ones lacking the IEC genes. High diversity of enterotoxin genes was detected among non-ST398/ST133 MSSA isolates.

The detection of MRSP ST71 evidences the presence of this MDR clone in Spain. MRSP and MSSP reveal differential genetic characteristics. Incidence of traditionally human or farm-animal associated *S. aureus* lineages evidences the ability of dogs to carry *S. aureus* lineages of in-contact environments. Moreover, MSSA ST398 predominance in tested dogs points to a broader host spectrum for this MSSA sub-lineage than considered.

These studies have led to one paper published and another submitted in international peer-reviewed journals.

1.1. PAPER 3.

Gómez-Sanz E, Torres C, Lozano C, Sáenz Y, Zarazaga M. Detection and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in healthy dogs in La Rioja, Spain. Comp Immunol Microbiol Infect Dis. 2011;34(5):447-53.

1.2. PAPER 4.

Gómez-Sanz E, Torres C, Benito D, Lozano C, Zarazaga M. Animal and human *Staphylococcus aureus* associated lineages – with ST398 predominance – and high rate of *S. pseudintermedius* novel lineages in Spanish kennel dogs. Vet. Microbiol (advanced stage of revision).

Paper 3

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Detection and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in healthy dogs in La Rioja, Spain☆

Elena Gómez-Sanz[a], Carmen Torres[a,b], Carmen Lozano[a], Yolanda Sáenz[b], Myriam Zarazaga[a,*]

[a] *Área Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain*
[b] *Área de Microbiología Molecular, Centro de Investigación Biomédica de La Rioja (CIBIR), Logroño, Spain*

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ABSTRACT

The objective was to identify the methicillin-resistant coagulase-positive staphylococci (MRCoPS) nasal carriage rate of healthy dogs in La Rioja (Spain) and to characterize the recovered isolates by different molecular techniques. Nasal samples from 196 dogs were obtained (98 household-dogs, 98 pound-dogs). Isolates were identified and characterized by *spa*-, SCC*mec*- and MLST-typing, *Sma*I-PFGE, antimicrobial susceptibility, determination of antimicrobial resistance and toxin genes profiling. *S. pseudintermedius* was the only species recovered. Nine methicillin-resistant *S. pseudintermedius* (MRSP) were obtained from 9 of 196 sampled dogs (8% pound-dogs, 1% household-dogs). MRSP isolates were typed (MLST/PFGE/*spa*/SCC*mec*) as: ST71/A/t02/II–III (7 isolates), ST92/C/t06/V (1 isolate), and ST26/B/non-typable/non-typable (1 isolate). All MRSP were resistant to [resistance gene/number isolates]: β-lactams [*mecA* + *blaZ*/9], tetracycline [*tet*(K)/7, *tet*(M)/2], macrolides and lincosamides [*erm*(B)/9], aminoglycosides [*aacA-aphD* + *aadE* + *aphA-3*/9], and co-trimoxazol [*dfr*(G)/9]. Eight MRSP isolates showed also resistance to fluoroquinolones and amino acid changes in GyrA [Ser84Leu + Glu714Lys, 7 isolates; Ser84Leu, 1 isolate] and GrlA [Ser80Ile, 8 isolates] proteins were detected. The remaining isolate was chloramphenicol resistant and harboured cat_{pC221} gene. All MRSP isolates harboured the *aadE-sat4-aphA-3* multiresistance-gene-cluster linked to *erm*(B) gene as well as the *siet*, *si-ent* and *lukS/F-I* toxin genes. MRSP is a moderately common (4.6%) colonizer of healthy dogs in Spain. A major MRSP lineage (ST71) was detected and its future evolution should be tracked.

1. Introduction

Staphylococcus pseudintermedius is a coagulase-positive *Staphylococcus* (CoPS) species, first described in 2005 [1]. This species belongs to the *S. intermedius* group (SIG) which compiles three related species: *S. intermedius*, *S. pseudintermedius*, and *S. delphini*. Isolates formerly identified as *S. intermedius* by phenotypic characteristics have been reclassified based on molecular techniques, and it has been proposed to report all strains belonging to the SIG from dogs as *S. pseudintermedius*, unless genomic investigations prove that the strain belongs to another of these related species [3]. *S. pseudintermedius* is the most prevalent CoPS inhabitant of the skin and mucosa of dogs and cats, and is a common opportunistic pathogen causing skin and soft tissue infections in these animals [2,3]. Additionally, the prevalence of methicillin-resistant *S. pseudintermedius* (MRSP) has increased in the last few years [3–5]. MRSP

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* Corresponding author at: Área Bioquímica y Biología Molecular, Universidad de La Rioja, Madre de Dios 51, 26006 Logroño, Spain. Tel.: +34 941 299751; fax: +34 941 299721.
E-mail address: myriam.zarazaga@unirioja.es (M. Zarazaga).

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isolates are usually multidrug-resistant [2,6], leaving few therapeutic options when they are implicated in infections.

Although the zoonotic potential of this microorganism is not as high as that of *S. aureus*, *S. pseudintermedius*, including MRSP infections [6], has been isolated from people in contact with infected pets [7] and from human infections [8]. Since phenotypic discrimination of CoPS species is difficult, the real prevalence of *S. pseudintermedius* might have been underestimated being misidentified with other CoPS, especially *S. intermedius* or *S. aureus*, in routine laboratory diagnostics [4].

MRSP belonging to sequence type (ST) 71 is the predominant and spreading clonal lineage in Europe [2,3], although it has also been detected in North America and China [2,9]. Nevertheless, MRSP has not been so far detected in Spain. The first clinical human infection caused by this multidrug-resistant ST71-MRSP lineage has been recently described in Switzerland [6], emphasizing its zoonotic potential.

In addition to the traditional *S. aureus* toxins (leukocidins, haemolysins, and pyrogenic-toxin super antigens), some specific virulence factors of *S. intermedius* and/or *S. pseudintermedius*, including an analog of the Panton-Valentine Leukocidin (PVL), the leukotoxin LukI, the exfoliative toxins SIET and EXI, and some enterotoxins (SI–ENT, SEC_{CANINE}), are of relevant importance on the pathogenic potential of these isolates [10,11].

The present study was focussed on determination of the methicillin-resistant CoPS (MRCoPS) nasal carriage rate of healthy dogs in Spain, identification of isolates, determination of their antimicrobial resistance profiles, their potential virulence properties and clonal relationship, and characterization of the recovered isolates by different molecular approaches.

2. Materials and methods

2.1. Isolation of MRCoPS and identification of S. pseudintermedius

One-hundred-ninety-six healthy dogs (aged from 1 to 13) were screened for nasal carriage of MRCoPS in La Rioja (Northern Spain) between March 2009 and June 2010. Ninety-eight swabs from household-dogs were provided by distinct owners on voluntary bases and 98 from pound-dogs were taken in one shelter house in seven sampling periods. This dog pound hosts up to 50 stray dogs captured in diverse locations and a mean of 3000 dogs are euthanized per year. Animals stay in individual cages for a maximum period of 10 days. Tested dogs were captured in different locations, at different periods, and were hosted in different cages prior sacrifice, and therefore it can be estimated that they are not directly related animals. None of the animals was under antimicrobial treatment at the time of sampling. Swabs were inoculated into Brain-Heart-Infusion broth (BHI, Difco) supplemented with 6.5% NaCl and incubated at 37 °C for 24 h. One-hundred microliters were seeded on Oxacillin-Resistant-Staphylococcal-Agar-Base (ORSAB, OXOID) plates supplemented with 2 mg/L of oxacillin, and incubated at 35 °C for 24–48 h. One presumptive MRCoPS colony per plate from each positive sample was selected and further studied. Identification of isolates was based on colony morphology, Gram staining, and catalase and DNase activities. A multiplex PCR was employed to differentiate *S. aureus* and S. *(pseud)intermedius* [12]. Discrimination between *S. intermedius* and *S. pseudintermedius* was conducted by PCR-RFLP of *pta* gene with *Mbo*I endonuclease [13]. The presence of *mecA* gene was investigated by PCR [14].

2.2. Molecular typing and clonal relatedness of isolates

The recovered isolates were characterized by Multi-Locus-Sequence-Typing (MLST), *spa*-typing, and Pulse-Field-Gel-Electrophoresis (PFGE) with *Sma*I macrorestriction enzyme. MLST was carried out by amplification and subsequent sequencing of 5 housekeeping genes (*pta*, *cpn60*, *tuf*, 16S rRNA and *agrD*), following by assignment of sequence types according to a scheme previously proposed [15]. *Spa*-typing was performed by amplification and sequence analysis of the polymorphic region of *spa* gene of *S. pseudintermedius*, with assessment of repeats and types according to those previously proposed [16]. PFGE with *Sma*I was conducted following the Harmony protocol [17] and using electrophoresis conditions as recommended [2]. Staphylococcal-Cassette-Chromosome-*mec* (SCC*mec*) types I–VII, and SCC*mec*V (5C2&5), were determined by multiplex PCRs [2,18,19]. Additionally, the recently described SCC*mec*II–III and SCC*mec*VII-241 in MRSP were studied as recommended [2].

2.3. Antimicrobial susceptibility testing

Susceptibility to 16 antimicrobial agents was performed by the agar disk-diffusion method. Antimicrobials tested were as follows (μg/disk): oxacillin (1), cefoxitin (30) erythromycin (15), clindamycin (2), gentamicin (10), kanamycin (30), streptomycin (10U), tobramycin (10), tetracycline (30), trimethoprim-sulfamethoxazole (1.25 + 23.75), chloramphenicol (30), ciprofloxacin (5), mupirocin (200), fusidic acid (10), vancomycin (30), and linezolid (30). The disk-diffusion method and breakpoints recommended by CLSI [20] were employed for all antimicrobials except for streptomycin and fusidic acid, where the methods and breakpoints recommended by the Société Française de Microbiologie (http://www.sfm.asso.fr) were used.

The double-disk diffusion test (D-test) was conducted for all isolates to detect inducible clindamycin resistance. Resistance to oxacillin, cefoxitin, and ciprofloxacin was also determined by the broth microdilution method [20].

2.4. Antimicrobial resistance genes profile

The presence of 30 antimicrobial resistance genes was investigated by PCR: *mecA*, *blaZ*, *tet*(K), *tet*(M), *tet*(L), *tet*(O), *erm*(A), *erm*(B), *erm*(C), *erm*(T), *erm*(Y), *mph*(C), *msr*(A), *msr*(B), *lnu*(A), *vga*(C), *aacA-aphD*, *aphA-3*, *aadE*, *aadD*, *aadA*, *str*, *sat4*, *dfr*(A), *dfr*(D), *dfr*(G), *dfr*(K), cat_{pC221}, *fexA*, and *cfr* [14,21]. Positive controls from the collection of the University of La Rioja were included in each reaction.

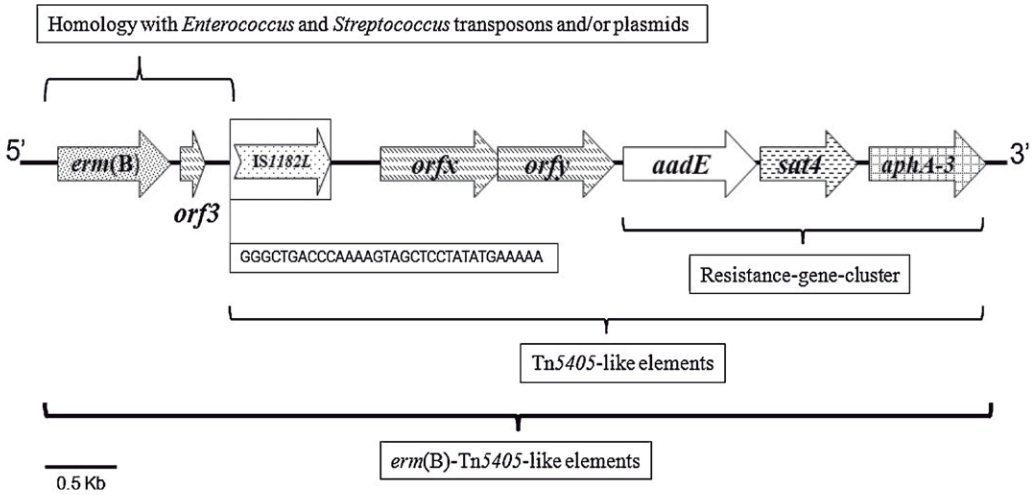

Fig. 1. Physical map of *erm*(B)-Tn*5405*-like elements of the representative ST71-MRSP strain investigated (C2597). The position and orientation of the antimicrobial resistance genes [*erm*(B), *aadE*, *sat4*, *aphA-3*], *orfx* (nucleotidyltransferase domain protein), *orfy* (putative methyltransferase), and truncated transposase are indicated by arrows showing the direction of transcription. IS*1182L* truncated element is shown as a box with the arrow indicated the transposase gene. The 33-bp inverted repeat of IS*1182L* is shown within a box below the IS*1182L* element. The nucleotide sequence of the 7.1 kb region of this *erm*(B)-Tn*5405*-like element has been submitted to GenBank under the accession no. JF909978.

Mutations in the Quinolone-Resistance-Determining-Region (QRDR) of *gyrA* gene were determined by PCR and sequencing [22]. Additionally, another set of primers was designed [gyrA(678/714)-fw TCGGTACAGCCCAAGCGTCAC and gyrA(678/714)-rv TCACACCCATACCGCCACGG (according to GenBank accession number AM262972)] to amplify a DNA region outside *gyrA* QRDR in which two point mutations in the resultant proteins (Thr678Ala and Glu714Lys) have been reported to come out in higher MICs (>16 mg/L) to enrofloxacin among *S. pseudintermedius* strains [23]. Primers to amplify the QRDR of *grlA* gene were designed based on the GenBank available sequence with accession number AM262969 [grlA(80/84)-fw AAAACAGTTGGGGACGT and grlA(80/84)-rv GATGCGTTACACTGAAGCGA].

2.5. Determination of antimicrobial resistance genes linkage

The potential physical linkage of the aminoglycoside resistance genes *aadE* and *aphA-3*, as well as the streptothricin resistance gene *sat4*, was investigated on all recovered isolates by PCR and sequencing [24]. Likewise, the complete sequence of this antimicrobial multiresistance-gene-cluster was investigated on a representative strain (C2597, ST71), applying a primer-walking strategy [25] with subsequent sequencing of amplicons. Additionally, the potential inclusion of this cluster into a previously described transposon structure, Tn*5405* [26], as well as its physical linkage to *erm*(B) gene [24,25], were in the same way studied on the representative C2597 strain (Fig. 1).

2.6. Characterization of mecA regulatory elements

In order to gain insight into the regulatory elements of *mecA* expression, as well as in the genetic variability of these elements among distinct MRSP lineages, the presence and sequence analysis of promoter region of *mecA* gene, its regulatory genes *mecR1* (sensor/signal transducer) and *mecI* (*mecA* transcription represor), were investigated by PCR and sequencing [27,28].

2.7. Virulence genes profiling

PCR-based determination of the leukocidin genes *lukS/F-PV*, *lukM*, *lukE/D*, and *lukS/F-I* was conducted in all isolates [14,29]. Isolates were also screened by PCR for the presence of *hla*, *hlb*, *hld*, *hlg*, and *hlg-v* haemolysin genes; and *eta*, *etb*, *etd*, *siet*, and *exi* exfoliative genes [11,12,14]. Likewise, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, *seu*, sec_{canine}, and *si-ent* enterotoxin genes, and the toxic-shock syndrome 1 toxin gene *tst* were investigated by PCR [14,30,31].

3. Results

3.1. Identification and molecular typing of MRCoPS

Nine of the 196 samples were positive for MRCoPS (4.6%) and all of them were identified as MRSP. Eight MRSP were recovered from pound-dogs (8% of the 98 tested samples) and one from a household-dog (1%). Seven of the 9 MRSP were typed as ST71, *spa*-type t02, harboured the SCC*mec*II–III, and showed PFGE pattern A (Table 1). Among them, five closely related PFGE subtypes were detected (no of isolates): A1 (2), A2 (2), A3 (1), A4 (1), and A5 (1) (Fig. 2). One isolate was typed as ST26, PFGE pattern B, and *spa* and SCC*mec* non-typable employing the techniques described above; however, this isolate presented the recombinase genes *ccrC2*. The remaining MRSP isolate, obtained from a household-dog, was typed as ST92, PFGE pattern C, *spa*-type t06, and SCC*mec*V. This isolate harboured both the *ccrC2* and *ccrC8* and, thus, it likely belongs to SCC*mec*V (5C2&5) (Table 1).

Table 1
Molecular typing, antimicrobial resistance profiles, and clonal diversity of the 9 MRSP isolates studied.

Isolate	MLST-PFGE-*spa*-type	SCC*mec*	Resistance phenotype[a]	Resistance genes detected	Amino acid changes:		
					In-QRDR		Outside-QRDR
					GrlA	GyrA	GyrA[b]
C2382	ST71-A1-t02	II–III	OXA-FOX-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(K) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	E714K
C2597	ST71-A1-t02	II–III	OXA-FOX-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(K) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	E714K
C2602	ST71-A2-t02	II–III	OXA-FOX-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(K) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	E714K
C2604	ST71-A2-t02	II–III	OXA-FOX-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(K) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	E714K
C2383	ST71-A3-t02	II–III	OXA-FOX-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(K) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	E714K
C2601	ST71-A4-t02	II–III	OXA-FOX-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(K) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	E714K
C2603	ST71-A5-t02	II–III	OXA-FOX-TET-ERY-CLI[d]-GEN-TOB-KAN-STR-SXT-CHL	*mecA* + *blaZ* + *tet*(M) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G) + cat_{pC221}	Wild	Wild	I729T
C2607	ST26-B-NT[c]	NT[c]	OXA-FOX-TET-ERY-CLI[d]-GEN-TOB-KAN-STR-SXT-CHL	*mecA* + *blaZ* + *tet*(M) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G) + cat_{pC221}	Wild	Wild	I729T
C2381[e]	ST92-C-t06	V[f]	OXA-FOX-TET-ERY-CLI[d]-GEN-TOB-KAN-STR-SXT-CIP	*mecA* + *blaZ* + *tet*(M) + *erm*(B) + *aacA-aphD* + *aphA-3* + *aadE* + *sat4* + *dfr*(G)	S80I	S84L	Wild

[a] OXA, oxacillin; FOX, cefoxitin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; CIP, ciprofloxacin.
[b] An extra silent nucleotide mutation (C2196T) was detected using the described primers in all strains.
[c] NT; non-typable.
[d] Inducible resistance to lincosamides (iMLS$_B$).
[e] Isolate obtained from a household-dog.
[f] This isolate harboured both the *ccrC2* and *ccrC8* and thus it is expected to belong to SCC*mecV* (5C2&5).

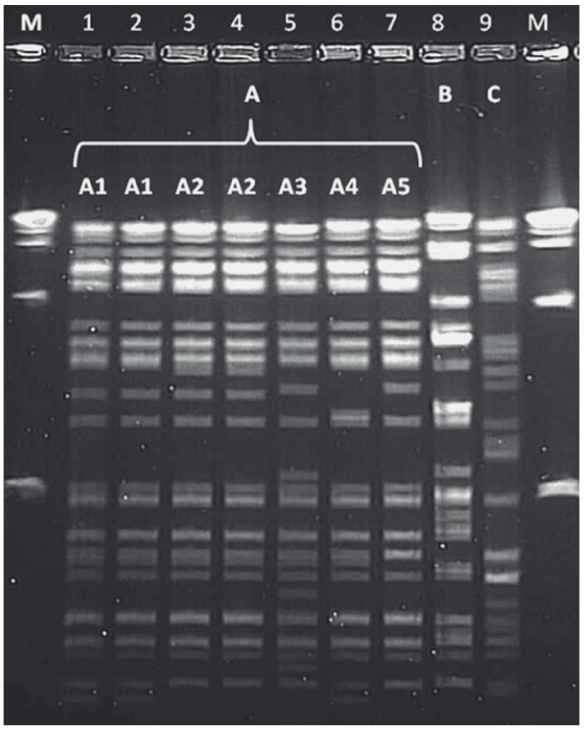

Fig. 2. PFGE patterns of chromosomal DNA digested with *Sma*I of MRSP strains isolated from healthy dogs in this study. Three major types were obtained being correlated with the sequence type of the isolates: A (ST71), B (ST26), C (ST92). Differentiated subtypes were designated using numerals (A1, C2382 and C2597; A2, C2602 and C2604; A3 C2383; A4, C2601; A5, C2603). M (Lambda Ladder PFG Marker).

3.2. Antimicrobial resistance pheno- and genotype

All 9 MRSP isolates were resistant to oxacillin with MIC of >64 mg/L. The cefoxitin MICs appear to be related to the genetic characteristic of the MRSP strains (7 isolates ST71-SCC*mec*II–III: 8 mg/L; 1 isolate ST26-SCC*mec*NT: 16 mg/L; 1 isolate ST92-SCC*mec*V: 32 mg/L). All MRSP were multidrug-resistant including six distinct families of antimicrobials (β-lactams, tetracyclines, macrolide–lincosamide–streptomycin/spectinomycin, aminoglycosides, potentiated sulphonamides, and either fluroquinolones or amphenicols), and harboured numerous resistance genes. Complete characterization of 9 MRSP isolates is shown in Table 1.

All MRSP ST71 and the ST92 isolate were resistant to ciprofloxacin and presented an amino acid change in the QRDR of GrlA (Ser80Ile) and GyrA (Ser84Leu). Sequence analysis of the outside-QRDR of GyrA revealed another amino acid change (Glu714Lys) in all ST71, whilst no additional mutation was observed in strain ST92. No differences in their ciprofloxacin MIC values were found (32 mg/L).

3.3. Linkage of antimicrobial resistance genes

The *aadE-sat4-aphA-3* multiresistance-gene-cluster was identified in all MRSP isolates. Primer-walking strategy on strain C2597 (ST71) revealed the inclusion of this cluster in Tn*5405*-like elements and the absence of the

IS*1182R* downstream of the *aphA-3* gene. This isolate presented a truncated transposase within the IS*1182L*. Furthermore, a physical linkage between these Tn*5405*-like elements and *erm*(B) gene was revealed upstream of the IS*1182L*. This strain carried two additional *orf* (*orfx* and *orfy*) as part of the Tn*5405*-like elements right downstream of the IS*1182L*. The putative coded protein ORFX contains a conserved nucleotidyltransferase domain which is present in some aminoglycoside-modifying enzymes (Fig. 1).

3.4. Characterization of mecA regulatory elements

Sequence analysis of the *mecA* operon on ST71-MRSP evidenced 100% similarity to MRSP strain KM1381 (GenBank AM904732) [23], which also presented the SCC*mec*II–III, harbouring the whole *mecA* regulatory elements. The amplified *mecA* gene region of our ST26-MRSP strain (SCC*mec* non-typable) was identical to that of ST71-MRSP strains; however, several attempts to amplify its *mecA* promoter region did not yield any positive results, suggesting mutations in the annealing sites of the primers used. This strain was found to be negative for the presence of its regulatory elements (*mecR1-mecI*). Alternatively, both the *mecA* gene and *mec* promoter region of ST92-MRSP [SCC*mec*V (5C2&5)] were identical to those of ST71-MRSP, although only amplification of the membrane spanning part of *mecR1* was obtained, showing 100% similarity to the same region of ST71 isolates.

3.5. Presence of virulence genes

All MRSP isolates, independent of their genetic background, harboured both *S. intermedius* leukocidin genes *lukS-I* and *lukF-I*, the exfoliatin gene *siet*, and the enterotoxin gene *si-ent*. The enterotoxin gene sec_{canine} as well as the *S. pseudintermedius* exfoliatin gene *exi*, were absent in all MRSP isolates. ST71-MRSP isolates were negative for the rest of toxin genes investigated, whereas MRSP ST26 and ST92 also harboured the haemolysin gene *hlg-v*.

4. Discussion

In the present study, a 1% MRSP nasal carriage rate among healthy household-dogs was observed. Previous studies have reported a variable prevalence of MRSP carriage, with rates of 0–4.5% in healthy dogs in the community [3,4]. However, a somewhat worrisome 8% occurrence among pound-dogs was detected in our study. There is very scarce data on the MRSP in animal shelters. One publication reported a MRSP occurrence of 16.7% among individuals from a dog rescue in Hong Kong [32]. However, no information is given on the living conditions or potential animal contact of tested dogs. The difference in MRSP prevalence among our two dog tested communities is remarkable, suggesting origin and animal lifestyle as a potential risk factor for the MRSP prevalence. This data might unveil a potential MRSP reservoir population and could be a health threat of special relevance for people in contact or adopting dogs from shelter houses. No MRSA was detected in our study, what is in accordance to other reports with very low rates of MRSA among healthy dogs (<1%) [4].

Seven of the 9 recovered MRSP were ST71 and showed closely related PFGE patterns. This is the first description of this emerging multidrug-resistant clonal lineage in Spain. All ST71-MRSP presented the recently described SCC*mec*II–III which has been reported in most ST71 isolates so far [2,5,23]. Given the small size of this reorganized SCC*mec* element (25 kb), future investigations are required to track both its specificity and its potential transmission capacity, not only to other MRSP lineages but also to other staphylococci. Our ST71-MRSP showed identical molecular typing characteristics to those previously described (ST71-t02-SCC*mec*II–III) [2,5,16], emphasising their capacity for clonal spread.

The remaining MRSP isolate from a pound-dog was typed as ST26. To date, this MRSP lineage has been previously described in Japan and in the USA [13,33]; however, to our knowledge, this is the first time it has been detected in Europe. Interestingly, neither the *spa* nor the SCC*mec* were typable, suggesting the presence of novel characteristics.

Whilst the presence of SCC*mec*V (5C2&5) was firstly described in *S. aureus* [18], it has already been reported in most ST68 and also in ST115 MRSP lineages [2,34]. In the present study, this SCC*mec*V type was found in the ST92-MRSP obtained from a household-dog. This spread evidences the presence of this cassette within distinct lineages and staphylococcal species and lineages.

All our MRSP were multidrug-resistant. Resistance profiles of MRSP ST26 and ST92 differed from those of ST71 in their tetracycline resistance gene [*tet*(M) versus *tet*(K)], and in that they showed clindamycin inducible expression of *erm*(B). Additionally, the ST26 strain was susceptible to fluoroquinolones but showed resistance to chloramphenicol harbouring the cat_{pC221} gene. It is remarkable that none of our ST71-MRSP were resistant to chloramphenicol since high rates (57–100%) of resistance to this antimicrobial agent have been described among ST71-MRSP isolates [2,5]. The homology in the antimicrobial resistance pheno- and genotype of MRSP (ST71 and non-ST71) described to-date is noteworthy [2,3,5,6], suggesting a potential species-specific tendency to acquire particular antimicrobial resistance genes.

Despite this emerging multidrug-resistance profile, determination of antimicrobial resistance gene linkages had not been previously conducted on MRSP. In this context, the presence of the *aadE-sat4-aphA-3* cluster in all our MRSP, which confers resistance to aminoglycosides and streptothricin, and which has also been described in other staphylococci, enterococci and streptococci [24,35,36], emphasizes the ubiquity of this cluster and the resistance gene acquisition capacities of *S. pseudintermedius*. Furthermore, MRSP C2597 carried this multiresistance-gene-cluster within Tn*5405*-like elements, which were linked to *erm*(B) gene. This association had been reported in other *Staphylococcus* and *Enterococcus* species [18,25,35], with different genetic structures, which suggests that this genetic mosaic might have originated from different Gram-positive bacteria by transference and further genetic reorganization. It is not known whether this element is carried on a plasmid or within the chromosome. Nevertheless, based on the recently deposited complete genome sequence in the GenBank database (CP002478)

of a *S. pseudintermedius* isolate (strain ED99), it is known that ED99 harbours a fragment of 99% similarity to the *erm*(B)-Tn*5405*-like elements described in this report. This finding strongly suggests that this element is carried on the chromosome and that a vertical transmission of this multidrug-resistant genetic structure has occurred. This would also explain the high rate of multidrug-resistant MRSP isolates described so far carrying these four resistance genes (*erm*(B), *aadE*, *sat4*, *aphA-3*) [2,3,5,6], and enhances the ability of this species to maintain resistance determinants in its genome. The presence of the putative protein ORFX containing a nucleotidyltransferase domain needs further investigation.

Seven of the 8 ciprofloxacin-resistant MRSP isolates presented amino acid substitutions within the QRDR of GyrA and GlrA, and an extra amino acid change within the outside-QRDR region of GyrA protein (Glu714Lys). However, the remaining fluoroquinolone-resistant MRSP isolate did not present this mutation, showing it to be, at least, not essential for this resistance. Further investigations are required to elucidate its biological significance.

Lack of correspondence between β-lactam resistance phenotypes and *mecA* expression levels have been recently reported among MRSP isolates of different genetic lineages, ST71 among others [33]. Although, in the present study, the *mecA* expression levels were not investigated, it is important to underline the presence/absence of *mecA* regulatory elements relative to the phenotype of resistance to β-lactams in our MRSP strains. It has been suggested that a tight repression of *mecA* gene expression in the absence of β-lactams could contribute to the apparent fitness of successful strains in *S. aureus* [37], which might be also the case in the successfully spread of ST71-MRSP lineage.

It is of interest that regardless of the fact that all 196 sampled dogs were healthy, all MRSP isolates, independent of their genetic background, harboured the PVL analogous leukocidin genes *lukS/F-I*, the exfoliative gene *siet*, and the enterotoxin gene *si-ent*. The presence of these genes could play an important role in the severity of infections caused by these commensal bacteria when becoming opportunistic pathogens.

MRSP prevalence has been shown to be different among the two communities tested, with a remarkable higher occurrence among pound-dogs (8% versus 1%), and a major MRSP lineage detected (ST71/A/t02/II–III), suggesting the success and emerging spread of this multidrug-resistant clone. The presence of multiple antimicrobial resistance genes, the coinheritance of some of them, and the detection of virulence determinants, leads us to consider this species as an important reservoir of antimicrobial resistance, with the corresponding difficulties implied in the treatment infections caused by this microorganism, but also as a virulent opportunistic pathogen. Given the close contact of companion animals to humans, especially pet owners and veterinary personnel, and given to the recent description of this species in human infections, future investigations are warranted to elucidate its human health implications as a zoonotic microorganism.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

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Annex to paper 3

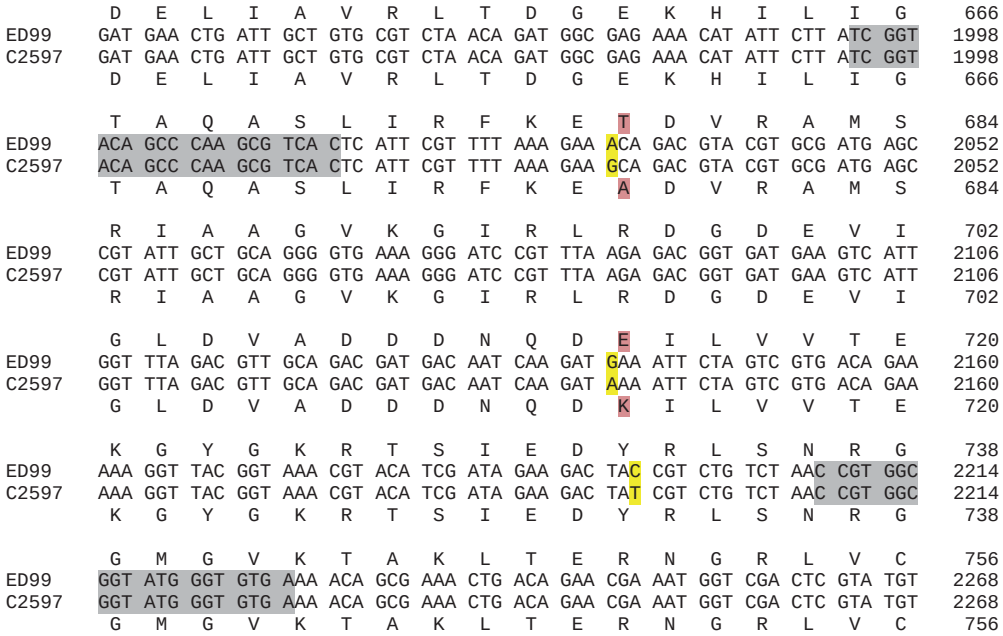

Figure S1. DNA and amino acid alignment and comparison of a segment of the *gyrA* gene in MSSP ED99 (fluoroquinolone susceptible) and MRSP C2597 (fluoroquinolone resistant). Stretch of primers designed and employed is indicated in gray. Nucleotide substitutions in yellow and amino acid substitutions in pink.

Paper 4

nimal and human *Staphylococcus aureus* associated lineages – with ST398 predominance – and high rate of *S. pseudintermedius* novel lineages in Spanish kennel dogs

Elena Gómez-Sanz, Carmen Torres, Daniel Benito, Carmen Lozano, Myriam Zarazaga[#]

Área Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain

Methicillin-susceptible *Staphylococcus aureus* (MSSA) and *S. pseudintermedius* (MSSP) are gaining interest to track the evolution of emerging methicillin-resistant strains in animals and humans. We investigated the nasal carriage by MS coagulase-positive staphylococci (MSCoPS) in 98 healthy kennel-dogs and characterized the recovered isolates by *spa*, *agr*, MLST and SmaI/ApaI-PFGE. Antimicrobial resistance and virulence profiles were investigated. Presence of the human-associated Immune-Evasion-Cluster (IEC) genes was analyzed in MSSA. Twenty-four MSSA (24.5% of animals), 16 MSSP (15.3%) and one MS *S. schleiferi* subspecies *coagulans* were obtained. Thirteen *spa*-types and 12 sequence-types (STs) were detected among MSSA, with ST398 predominance (29.2%). MSSA isolates were enclosed within 6 clonal complexes (% of isolates): CC5 (33.3), CC398 (29.2), CC88 (16.6), CC45 (8.3), CC133 (4.2), and CC22 (4.2). High clonal diversity was observed among MSSP, and 14 STs (10 of them new) were detected. Fifty-percent of MSSA and 75% of MSSP isolates showed resistance to at least one of the tested antimicrobials, with low MSSA penicillin resistance (20.8%) and high MSSP tetracycline resistance (56.3%). MSSA isolates ST398, ST133, ST1 and ST2329$_{[new]}$ were susceptible to all antimicrobials and were the only ones lacking the *scn*, *chp* and/or *sak* IEC genes. High diversity of enterotoxin genes was detected among non-ST398/ST133 MSSA isolates. MSSP showed a more homogeneous virulence genes profile. Presence of traditionally human and farm-animal associated *S. aureus* lineages evidences the ability of dogs to carry *S. aureus* lineages of in-contact environments. Continue surveillance on MSSA and MSSP in animals is needed.

Keywords: MSSA ST398; MSSP; human-associated lineages; animal-associated lineages; kennel dogs; Spain.

1. Introduction

Staphylococcus aureus and *Staphylococcus pseudintermedius* are coagulase positive staphylococci (CoPS) that can be found in the skin and mucous of dogs. *S. aureus* is recovered from these animals (<20%), but its presence seems to be associated with human contact (anthropozoonotic origin) (Boost et al., 2008; Hanselman et al., 2009; Haenni et al., 2012; Walther et al., 2012; Gómez-Sanz et al., 2013). In contrast, dogs represent the main reservoir of *S. pseudintermedius*, with variable carriage rates depending on the body-site (Bannoehr and Guardabassi, 2012). Humans can be subsequently exposed to different CoPS through dog contact, with plausible implications in human health. (van Duijkeren et al., 2011).

The ecology of *S. aureus* and *S. pseudintermedius* in animals has gained attention in the last years due to the spread of livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) strains, such as ST398 (EFSA, 2009), and the emergence of multidrug-resistant methicillin-resistant *S. pseudintermedius* (MDR-MRSP) in dogs, specially MRSP-ST71 in Europe and MRSP-ST68 in North America (Perreten et al., 2010; Gómez-Sanz et al., 2011; van Duijkeren et al., 2011). It has been recently suggested that MRSA ST398 may have originated in humans as methicillin-susceptible *S. aureus* (MSSA) ST398 (Price et al., 2012), what has enhanced the interest for methicillin susceptible (MS) strains of different origins. However, very scarce data are available on the presence and predominant lineages of MSSA and methicillin-susceptible *S. pseudintermedius* (MSSP) in dogs and its possible implication in the evolution of successful lineages (Black et al., 2009; Vincze et al., 2012; Gómez-Sanz et al., 2013).

The phage-encoded Immune Evasion Cluster (IEC) encloses a group of genes coding for relevant factors implicated in the *S. aureus* evasion of the human immune system, which can also carry the enterotoxin genes *sea* and *sep*. This prophage is integrated within the citotoxin *hlb* gene and it is considered a human-specific element. Elevated IEC carriage rates are typically exhibited in human *S. aureus* isolates, in comparison to those obtained from animals (van Wamel et al., 2006).

A previous report on methicillin-resistant CoPS (MRCoPS) (Gómez-Sanz et al., 2011) in pound- and household-dogs in Spain detected 8% and 1% of dogs, respectively, positive for MRSP. The objective of the current study was to investigate the methicillin-susceptible CoPS (MSCoPS) nasal carriage rate obtained from the same group of pound-dogs and to perform the molecular characterization and clonal relatedness of recovered isolates. We sought to unveil whether dogs can be reservoirs of MSSA and/or MSSP of emerging MRSA and/or MRSP lineages.

1. Materials and methods

2.1 Origin of samples

Ninety-eight apparently healthy dogs coming from a dog pound were sampled for MSCoPS nasal carriage between June-October 2009. Tested dogs were swabbed in 7 sampling periods (S1 to S7). This kennel hosted up to 50 stray dogs captured in diverse locations (surrounding La Rioja region, Northern Spain), and a mean of 3000 dogs are euthanized per year. When possible, animals stay in individual cages for a maximum average period of 10 days. Animals tested were captured in 50 different locations [82 in rural areas (<25,000 inhabitants) and 16 dogs in urban areas (>150,000 inhabitants)], and none were under antimicrobial treatment at sampling.

2.2 Isolation and identification

Samples were inoculated into Brain-Heart-Infusion broth (BHI, Difco) supplemented with 6.5% NaCl and incubated at 37ºC for 24h. Seventy microliters were inoculated on Manitol-Salt-Agar (MSA; BD) plates for the isolation of MSCoPS and were incubated at 35ºC for 24-48h. Up to two colonies with different appearance were subcultured on BHI agar (Difco) and further studied. Preliminary identification of isolates was based on colony morphology, Gram staining, and catalase and DNase activities. A species-specific multiplex PCR was performed to identify *S. aureus* and *S. (pseud)intermedius* isolates (Lautz et al., 2006). Discrimination between *S. intermedius* and *S. pseudintermedius* was conducted by restriction fragment length polymorphism of the *pta* gene (Bannoehr and Guardabassi, 2012). MSCoPS isolate/s negative for *S. aureus* or *S. (pseud)intermedius* were identified by amplification and sequencing of the 16S rRNA gene (Lane, 2002). Only methicillin-susceptible isolates were further investigated in this study.

2.3 Molecular typing of isolates

All *S. aureus* and *S. pseudintermedius* isolates were subjected to *spa* typing as previously described (http://www.spaserver.ridom.de; Moodley et al., 2009), and *S. aureus* sequences were analyzed using Ridom Staph-Type software version 2.0.21 (Ridom GmbH). Grouping analysis of *S. aureus spa*-types was performed using the Based Upon Repeat Pattern (BURP) with a cost value of 8. Multi-Locus-Sequence-Typing (MLST) and subsequent assignment of clonal complexes (CC) was undergone as recommended for *S. aureus* isolates (www.mlst.net; http://eburst.mlst.net). MLST on *S. pseudintermedius* was performed on five housekeeping genes (*pta*, *cpn60*, *tuf*, 16S rDNA and *agrD*), as previously described (Bannoehr et al., 2007).

2.4 Pulsed-Field-Gel-Electrophoresis (PFGE)

The genetic relatedness of all *S. aureus* and *S. pseudintermedius* isolates obtained was investigated by PFGE of total DNA digested with SmaI or ApaI macrorestriction enzymes (Murchan et al., 2003). *S. aureus* non-typeable with SmaI were subjected to ApaI-PFGE and run for 20h at 6V/cm using pulsed time ramping from 2 to 5 s. SmaI-digested plugs from *S. pseudintermedius* isolates were run for 24 h at 5.6V/cm using pulse times from 2 to 5 s (Perreten et al., 2010). *S. aureus* band patterns were visually analyzed following Tenover's criteria. PFGE *S. pseudintermedius* profiles were analyzed using GelCompar software package (Applied Maths, Kortrijk, Belgium). The Unweighted Pair Group Method using Arithmetic averages (UPGMA) based on Dice coefficient was implemented (maximum position tolerance of 2.5%). PFGE clusters were defined as those formed a 77% cut-offs. *S. aureus* clones and *S. pseudintermedius* clusters were distinguished by capital- and lower-case Arabic letters, respectively. *S. aureus* subclones (up to three bands difference) or the different subtypes (unique band profiles) within a *S. pseudintermedius* cluster were indicated with the major lettering type followed by a number.

2.5 Antimicrobial susceptibility testing and detection of resistance genes

Susceptibility testing to 17 antimicrobial agents was performed in all isolates by agar disk-diffusion

method (CLSI, 2012). Antimicrobials tested were as follows (µg/disk): penicillin (10U), oxacillin (1), cefoxitin (30) erythromycin (15), clindamycin (2), gentamicin (10), kanamycin (30), streptomycin (10U), tobramycin (10), tetracycline (30), trimethoprim-sulfamethoxazole (1.25+23.75), chloramphenicol (30), ciprofloxacin (5), mupirocin (200), fusidic acid (10), vancomycin (30), and linezolid (30). For streptomycin and fusidic acid, methods and breakpoints employed were those recommended by the Société Française de Microbiologie (http://www.sfm.asso.fr). Inducible or constitutive clindamycin resistance was determined by the double-disk diffusion test (D-test) (CLSI, 2012).

Presence of antimicrobial resistance genes was investigated by PCR (Gómez-Sanz et al., 2010; Gómez-Sanz et al., 2011). The multidrug resistance-gene-cluster *aadE-sat4-aphA3* and its potential physical linkage to *erm*(B) gene [*erm*(B)-Tn*5405*-like element] were investigated in resistant isolates (Gómez-Sanz et al., 2013).

2.6 Toxin gene profiling

PCR-based determination of the leukocidin genes *lukS/F-PV*, *lukM*, and *lukE/D* was conducted on all isolates (Jarraud et al., 2002). They were likewise screened for the *hla*, *hlb*, *hld*, *hlg* and *hlgv* haemolysin genes and the *eta*, *etb* and *etd* exfoliative genes (Jarraud et al., 2002). Presence of eighteen enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) and the toxic shock syndrome toxin gene *tst* were tested by PCR (Jarraud et al, 2002; Hwang et al., 2007). In addition, discrimination of *sec* and *sec* canine variant (sec_{canine}) was implemented by PCR and sequencing (Gómez-Sanz et al., 2013). Overlapping PCRs were performed to detect the enterotoxin gene *egc* operon (*seg* + *sei* + *sem* + *sen* + *seo*), the egc_{like} (*egc* + *seu*) or other *egc* variants.

The leukocidin genes *lukS/F-I* (Futagawa-Saito et al., 2004a), the exfoliative genes *siet*, *expA* and *expB* (Lautz et al., 2006; Futagawa-Saito et al., 2009; Iyori et al., 2011) and the enterotoxin gene *se-int* (Futagawa-Saito et al., 2004b) were tested by PCR on all *S. pseudintermedius* isolates.

S. aureus were tested by PCR for the human-specific IEC genes encoding the chemotaxis inhibitory protein (*chp*), staphylococcal complement inhibitor (*scn*) and staphylokinase (*sak*), enclosed within prophage φ3 (van Wamel et al., 2006).

2. Results

3.1 Isolates recovered, species identification and molecular typing

Twenty-four MSSA (from 24 dogs, 24.5%) and 16 MSSP isolates (from 15 dogs, 15.3%) were recovered. In addition, one MS *S. schleiferi* subsp. *coagulans* isolate was obtained (1.0%). Five animals concurrently carried two distinct MSCoPS isolates. In total, 41 MSCoPS were recovered from 36 dogs. Dogs from urban areas presented higher *S. aureus* occurrence (43.8%) than those of rural areas (20.7%), while those from rural areas exhibited higher *S. pseudintermedius* nasal prevalence (17% versus 6.3%).

Twelve sequence-types (STs) (ST1655 and ST2329 new, 16.7% of STs) were detected, with ST398 as the major lineage, comprising 29.2% of isolates (Table 1). All MSSA isolates were enclosed within 6 clonal complexes: CC5 (8 isolates), CC398 (7), CC88 (4), CC45 (2), CC133 (1), CC22 (1) and one singleton (1).

Thirteen *spa*-types were detected among the 24 MSSA isolates, two of them being new (t8764 and t8765) (Table 1). Five *spa*-types were grouped in two separate clusters, i) Cluster 1, which enclosed all ST398/CC398 isolates, comprised *spa*-types t034, t5883 and t108, with t034 as predicted *spa* founder (CC034), ii) Cluster 2, which comprised all ST45/CC45 isolates, enclosed *spa*-types t040 and t015 (Figure 1). The remaining *spa*-types (61.5%) were singletons. *agr*_I was predominant (54.2% of isolates), followed by *agr*_III (25%) and *agr*_II (16.7%), while *agr*_IV was only detected in the MSSA isolate with novel *spa* and ST (4.2%).

As for MSSP, fourteen distinct STs were detected among the 16 MSSP, among which, 10 STs presented novel alleles or allele combinations (Table 2; Table 3). Novel STs were distributed in 12 of the 16 isolates (75% of MSSP recovered). The novel ST189 (isolate C3463) was a single-locus-variant (SLV) of the European clonally-spread MRSP lineage ST71, diverging in the *agrD* allele. Only 3 *S. pseudintermedius* isolates were *spa*-typeable (t02, t05, t43) (Table 2).

Four dogs concurrently carried two distinct MSCoPS species (3 of them presented a MSSA

ST398 together with a MSSP (ST181 or ST190) or the MS *S. schleiferi* subsp. *coagulans*). (Table 1, Table 2). In addition, one animal harbored two distinct *S. pseudintermedius* strains (MSSP ST185 and MSSP ST190).

3.2. Analysis of the clonal relatedness of isolates

Fourteen distinct SmaI-PFGE band profiles, enclosed within 9 major clones (B-J), were detected among the 17 non-ST398 MSSA isolates (Figure 2). Only MSSA isolates exhibiting t186 showed the same SmaI-PFGE pattern. The 7 ST398 isolates were SmaI non-typeable. ApaI-PFGE revealed that all ST398 isolates presented identical or closely related band patterns (clone A) (Figure 2).

A high variety of *S. pseudintermedius* profiles was revealed (Figure 3). Cluster analysis generated 6 major groups (a–f) with 15 individual patterns. Group c was prominent with 7 distinct profiles. Strains with novel and previously detected STs were homogenously distributed in the different branches. Both *S. pseudintermedius* strains that belonged to the novel ST183 were grouped separately (c5 and g). All *S. pseudintermedius* strains clustered together with a similarity coefficient of 69%, whereas the *S. schleiferi* subsp. *coagulans* strain (C3510, pattern g) formed a more distant branch (62.5% similarity). Different *agr* allotypes were present in strains of the same clusters.

3.3. Antimicrobial resistance profile

The following antimicrobial resistances were detected among MSSA isolates (% of isolates/resistance gene detected): penicillin (20.8/*blaZ*), erythromycin-clindamycin [20.8/*erm*(C) or *erm*(A)], tetracycline [4.2/*tet*(K)] and streptomycin (4.2/*str*). Twelve MSSA (50%) were susceptible to all antimicrobials tested (Table 1).

MSSP isolates were resistant to the following antimicrobials (% of isolates/resistance gene detected): penicillin (56.3/*blaZ*), tetracycline [56.3/*tet*(M) + *tet*(L) or *tet*(M)], erythromycin-clindamycin [12.5/*erm*(B)], streptomycin (12.5/*aadE*), kanamycin (12.5/*aphA3*), and chloramphenicol (12.5/cat_{pC221}). Four MSSP isolates (25%) were susceptible to all tested antimicrobials (Table 2). Two isolates harbored the resistance gene cluster *aadE-sat4-aphA3*, but not in apparent association with the *erm*(B) gene. Two isolates (12.5% of isolates) that shared identical phenotypic and genotypic characteristics were the only ones that exhibited a multidrug-resistance (MDR) profile (resistance to more than 3 antimicrobial families).

The single MS *S. schleiferi* subsp. *coagulans* isolate was susceptible to all antimicrobial agents tested.

3.4. Presence of virulence genes

All MSSA isolates but those that belonged to ST398, ST133, ST1 and the novel ST2329 exhibited the human-associated *scn*, *chp* and/or *sak* IEC genes (54.2% isolates) (Table 1). Different haemolysin gene combinations were observed among all MSSA isolates, with MSSA/ST398 the only ones that presented an integral copy of the *hlb* gene (Table 1). The leukocidin *lukE/D* gene was detected in 15 MSSA isolates (62.5%). Thirteen isolates (54.2%) harbored enterotoxin gene or gene combinations, with 7 presenting the *egc*, egc_{like} or other *egc* variant (29.2% of isolates). MSSA isolates of the lineages ST398, ST133 and some CC5 were the only ones that lacked all investigated enterotoxin genes. The *sea* and *sep* genes were present in one IEC-negative ST1 MSSA isolate and in both ST5 MSSA isolates, which also harbored the *scn*, *chp* and *sak* genes.

All MSSP isolates harbored the leukocidin *lukS/F-I*, exfoliatin *siet* and enterotoxin *se-int* genes. Three isolates (18.8%) exhibited the exfoliatin *expB* gene, 2 the *expA* gene, and one the normally-clustered enterotoxin genes sec_{canine} and *sel* (Table 2).

4. Discussion

High MSSA nasal carriage (24.5%) is revealed among the investigated dog population. It should be noted that no MRSA was detected in the former study focused on MRCoPS in the same group of animals (Gómez-Sanz et al., 2011); hence, this value represents the overall *S. aureus* nasal occurrence. *S. aureus* values are notoriously higher than previous reports among dogs with direct human contact (1.8-14%) (Boost et al., 2008; Hanselman et al., 2009; Walther et al., 2012; Gómez-Sanz et al., 2013). However, very scarce data is available on the occurrence of *S. aureus* in pound dogs, especially MSSA, and on possible risk factors for *S. aureus* acquisition (Loeffler et al., 2010; Gingrich et al., 2011; Kasprowicz et al., 2011). Although very limited dogs came from urban areas, the high rate detected among this group

(43.8%) is relevant, and might respond to increased likelihood of exposure of these animals to the human microbiota in comparison to those from non-urban areas.

This is the first report on *S. aureus* ST398 in dogs in Spain, detected in 7.1% of animals sampled. Moreover, the predominance of MSSA of the lineage ST398 (29.2% of isolates) is outstanding, and represents the first epidemiological study detecting MSSA/ST398 in dogs. Few data are published on *S. aureus* ST398 in dogs, which reported MRSA (Witte et al., 2007; Nienhoff et al., ,2009; Reischl et al., 2009; Floras et al., 2010; Zhang et al., 2011; Fessler et al., 2012; Haenni et al., 2012). To our knowledge, a single MSSA/ST398 canine strain was included in a recent comparative genetic analysis of a convenience sample collection of human and canine isolates (Vinzce et al., 2012).

All MSSA/ST398 isolates presented closely or identical PFGE profiles. This might suggest cross-contamination by these bacteria at the shelter, but the presence of 3 distinct *spa*-types and the fact that animals were tested at 3 different periods, point to a real spread of this lineage among our tested dog population. Low discriminatory power of the ApaI enzyme might be also speculated. Although all but one MSSA/ST398 isolates were obtained from dogs captured in rural areas, the potential contact to livestock remains unknown. The presence of two *spa*-types typically found in porcine isolates (t034 and t108) (EFSA, 2009), and the absence of any of the genes encoded by the IEC cluster (found in all MSSA/ST398 from humans to date) may suggest an animal origin. However, loss of these genes as a consequence of host adaptation from a human source is also possible.

MSSA ST133/CC133 is a well-known small ruminant associated lineage (Guinane et al., 2010), and it has been recently detected as a predominant *S. aureus* lineage in donkey (Gharsa et al., 2012). Although our single positive animal came from a small village, the possible contact to farm animals could not be determined. Again, the absence of the IEC genes supports the hypothesis of an animal origin.

CC5 was the major MSSA clonal complex detected (33.3%), and enclosed great diversity of STs. This clonal complex represents the greatest group formed with eBURST v3 (as of 04/03/2013). Since the results from this algorithm are dynamic, it should not be surprising that STs within traditionally independent clonal complexes, such as ST1/CC1 or ST15/CC15, are now enclosed within CC5, and shows the rapid dynamic of connections among different *S. aureus* lineages. The absence of *spa* BURP clustering among our CC5 isolates reveals that the evolution of *S. aureus* ST associations diverges from those observed among *spa*-types. *S. aureus*, especially MRSA, of the lineage ST1 is a typical community-associated lineage, but its presence in different animal species (horses, pigs, cattle, dogs) is also frequent (Pantosti, 2012). Absence of the IEC genes among our MSSA/ST1 isolates suggests an animal origin, assumption supported by the rural origin of these animals.

Our MSSA isolates of the lineage CC88 presented identical phenotypic and genotypic profiles. It is important to remark that these isolates share typical characteristics of those detected in community-associated (CA-)MRSA of the same lineage, in special with CA-MRSA ST78, which normally carries the *sec* and *sel* enterotoxin genes, and the MLS_B resistance gene *erm*(A), in addition to the *blaZ* (Monecke et al., 2011). Since 3 of the 4 positive dogs were rescued in urban areas, a feasible human origin is expected; however, given that isolates were obtained in the same sampling, cross-contamination cannot be discarded.

The CC45 *S. aureus* lineage, detected in 2 isolates with clustered *spa*-types, was recently detected in a household dog-owner pair in Spain (Gómez-Sanz et al., 2013), and in a genetic comparative study of humans and dogs in Germany (Vinzce et al., 2012). Since this is one of the greatest and most common CC lineages, their detection in dogs should not be unexpected. Alternatively, regardless *S. aureus* CC22 is a widespread clonal group, and it is traditionally one of the major lineages detected in dogs (Monecke et al., 2011; Vinzce et al., 2012), only one animal resulted positive.

It is interesting to underline that all isolates that belonged to traditionally well-known human-associated lineages (all but ST398, ST133, ST1, $ST2329_{[new]}$) presented the *scn*, *chp* and/or the *sak* genes. Further molecular surveillance in *S. aureus* from animals and humans is needed to uncover whether the presence/absence of these cluster might be indicative of its plausible origin.

High association was observed between absence of the IEC genes and antimicrobial susceptibility. Moreover, the low rate of *S. aureus* penicillin resistance (20.8%) is outstanding, since

over 90% of human *S. aureus* isolates are resistant to penicillin, showing the *blaZ* gene (Lowy et al., 2003). Absence of antimicrobial pressure among the tested population might justify the lack of antimicrobial resistance.

Slightly lower virulence genes diversity was found among our *S. aureus* isolates in comparison with a recent report in household-dogs in the same region (Gómez-Sanz et al., 2013). Additionally, while MSSA/ST398 and ST133 lacked all enterotoxin genes tested, 81.3% of non-ST398/ST133 isolates were positive for at least one pyrogenic toxin superantigen gene. It is important to remark that these toxins are involved in food poisonings diseases.

Our results give evidence that dogs can be *S. aureus* carriers of not only typical human-associated lineages but also of common lineages detected among farm animals. However, the real origin of the isolates remains unknown and the cohort investigated was small for conclusive assertions. Hence, further epidemiological and longitudinal studies on *S. aureus* in dogs from diverse environments are mandatory.

The low MSSP carriage rate (15.3%) obtained among our dog population may be due to the body-site tested, since mouth and perineum have been reported as the most common sites for *S. pseudintermedius* (Bannoehr and Guardabassi, 2012). Alternatively, the unexpected low nasal occurrence observed among dogs from urban areas (6.3%) contrasts with the high MSSA rate detected among this group of animals, whose implications in the low MSSP recovery cannot be ruled out. It should be noted that 8 MRSP strains (7 ST71, 1 ST26) were detected in the former study on MRCoPS among this dog population (Gómez-Sanz et al., 2011). Although MR detection was out of the scope of this study, a total of 22 animals (22.5%) were positive for *S. pseudintermedius*, what shows a more approximate occurrence value to the estimated average for nasal *S. pseudintermedius* in dogs (31%) (Bannoehr and Guardabassi, 2012).

Great diversity of *S. pseudintermedius* lineages and PFGE patterns was observed among our isolates. High clonal variety of *S. pseudintermedius* is a common characteristic among *mec*A-negative isolates, and evidences the diverse genetic background of this bacterial species (Black et al., 2009; Walther et al., 2012; Gómez-Sanz et al., 2013). On the other hand, the elevated rate of novel MSSP STs (62.5%) is remarkably higher than recent reports on *S. pseudintermedius* from household dogs (<43%) (Walther et al., 2012; Gómez-Sanz et al., 2013). These results propose that not-commonly-tested dog groups may be reservoirs of *S. pseudintermedius* novel lineages, what warrants further surveillance. Since very limited successful MR lineages are currently geographically spread (Perreten et al., 2010), the presence of a SLV of the European clonally-spread MRSP lineage ST71 in one MSSP isolate is notable. Although *spa*-typing seems to be a good molecular method to investigate MRSP, MSSP are commonly *spa* non-typeable (Moodley et al., 2009). Hence, it is remarkable that 3 of our MSSP achieved amplification of the *spa* region. Whether these MSSP strains are potential acceptors of SCC*mec* elements remains unknown.

MSSP isolates were more resistant to the antimicrobials tested than MSSA, with 75% of *S. pseudintermedius* showing resistance to at least one antimicrobial. However, the MDR occurrence observed (only in both identical isolates) remarkably differs from the MDR value (39%) recently detected among MSSP from household dogs (Gómez-Sanz et al., 2013). Again, these relatively low resistance values suggest low antimicrobials exposure, what otherwise correlates to the rural origin of the isolates. Alternatively, tetracycline resistance is common among *S. pseudintermedius* isolates, what is in line with the high resistance values observed among our MSSP (56.3%). Since they seem to prefer transposon-born resistance genes, such as *tet*(M) gene, rather than plasmid borne *tet*(K) or *tet*(L), the presence of *tet*(L) in 37.5% of isolates is significant, and evidences its gene acquisition capacities (Kadlec et al., 2012).

Our *S. pseudintermedius* isolates showed a more homogeneous virulence profile than *S. aureus*. High frequency of the *lukS/F-I*, *siet* and *se-int* genes has been previously reported among MSSP of healthy dogs, showing ubiquity of virulence properties among commensal *S. pseudintermedius* (Gómez-Sanz et al., 2013). Interestingly, the exfoliatin *expA* and *expB* genes were more prevalent than previous descriptions on healthy animals (Iyori et al., 2011; Gómez-Sanz et al., 2013).

The *S. schleiferi* subspecies *coagulans* rate observed is in line with other reports on CoPS

in dogs (Hanselman et al., 2009; Walther et al., 2012), and shows that, although these animals are considered reservoirs for this bacterium, its incidence is low.

5. Conclusions

High MSSA and low MSSP nasal carriage is revealed among our kennel dogs tested. The MSSA/ST398 predominance among a sampled dog population is a novel observation, and suggests a more extended host spectrum of this sub-lineage than considered. Presence of traditionally human and farm-animal associated lineages evidences the high capacity of dogs to acquire the predominant *S. aureus* lineages of in-contact environments. The high rate of novel MSSP STs proposes that not commonly tested dog groups may be sources of *S. pseudintermedius* novel lineages. Continue surveillance on MSCoPS in animals is required to track the evolution of current successful lineages, their possible role in the dissemination of clones well-adapted to other hosts and to assess potential unforeseen resistance and virulence reservoirs.

Nucleotide sequence accession numbers

Novel *S. pseudintermedius* alleles sequences obtained in this study have been deposited in the GenBank/EMBL databases with accession numbers JX416710 (*pta*_30), JX416711 (*cpn60*_42), and HE970632 (*agr*_11).

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Conflicts of interest

None.

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Table 1. Molecular characterization, antimicrobial resistance and virulence profile of the 24 methicillin-susceptible *S. aureus* isolates investigated.

Dog ID[a]	Origin[b] (sampling)	Isolate	CC[c]	MLST	*spa*	*agr*	PFGE SmaI (ApaI)	Antimicrobial resistance profile		Virulence genes detected									
								Phenotype[d]	Genes detected	IEC[e]	Leukocidin and haemolysins								Pyrogenic Toxin Superantigen Genes[f]
										scn	*chp*	*sak*	*lukE/D*	*hla*	*hlb*	*hld*	*hlg*	*hlgv*	
7	R (S1)	C3471	CC398	ST398	t034	I	NT[g] (A1)							+	+	+	+		
9	U (S1)	C3473		ST398	t034	I	NT (A2)							+	+	+	+		
95	R (S7)	C3509		ST398	t034	I	NT (A3)							+	+	+	+		
96	R (S7)	C3487		ST398	t034	I	NT (A3)							+	+	+	+		
64	R (S5)	C3490		ST398	t5883	I	NT (A3)							+	+	+	+		
67	R (S5)	C3491		ST398	t5883	I	NT (A1)							+	+	+	+		
63	R (S5)	C3489		ST398	t108	I	NT (A4)							+	+	+	+		
8	R (S1)	C3472	CC133	ST133	t1166	I	B						+	+		+		+	
15	U (S1)	C3475	-	ST2329[h]	t8765[i]	IV	C						+	+		+		+	[*sei-sem-sen-seu*], *ser*, *seh*
24	R (S2)	C3476	CC5	ST1	t177	III	D1						+	+		+		+	*seh*
25	R (S2)	C3477		ST1	t177	III	D2						+	+		+		+	*sei*, *sen*, *seu*, *sea*
31	R (S2)	C3478		ST5	t002	II	E1	ERI-CLi	*erm*(C)	+	+	+	+	+		+		+	[*seg-sei-sem-sen-seo-seu*], *sep*
86	R (S7)	C3485		ST5	t002	II	E2	TET	*tet*(K)	+	+	+	+	+		+		+	[*seg-sei-sem-sen-seo-seu*], (*sed-sej-ser*), *sep*
98	R (S7)	C2738		ST146	t002	II	E3			+		+	+	+		+		+	[*seg-sei-sem-sen-seo*]
73	R (S6)	C2734		ST188	t189	I	F1	PEN	*blaZ*	+	+	+	+	+		+		+	
87	U (S7)	C2737		ST1655[h]	t189	I	F2	PEN	*blaZ*	+		+	+	+		+		+	
12	R (S1)	C3474		ST15	t084	II	G	STR	*str*	+	+		+	+		+		+	
68	R (S5)	C3480	CC88	ST78	t186	III	H	ERI-CLi	*erm*(A)	+	+	+	+	+		+		+	(*sec2-sel*), *sen*
69	U (S5)	C2733		ST78	t186	III	H	ERI-CLi	*erm*(A)	+		+	+	+		+		+	(*sec-sel*)
70	U (S5)	C3481		ST78	t186	III	H	ERI-CLi	*erm*(A)	+		+	+	+		+		+	(*sec-sel*)
71	U (S5)	C3482		ST78	t186	III	H	ERI-CLi	*erm*(A)	+		+	+	+		+		+	(*sec-sel*)
90	U (S7)	C3479	CC45	ST45	t040	I	I1	PEN	*blaZ*	+	+	+		+		+	+	+	[*seg-sei-sem-sen-seo-seu*]

32	R (S3)	C3486		ST45	t015	I	I2	PEN	*blaZ*	+	+	+	+	+		+	+		[*seg-sei-sem-sen-seo-seu*]
82	R (S6)	C2735	CC22	ST217	**t8764**[i]	I	J	PEN	*blaZ*	+	+	+		+		+	+		[*seg-sei-sem-sen-seo*]

The novel *spa* types and STs are shown in bold letters.

[a] Dog 67 concomitantly carried a MSSP ST181; Dog 86 a MSSP ST184; Dog 95 a MSSP ST190, and Dog 96 a MS *S. schleiferi* subsp *coagulans*.

[b] U, urban area; R, rural area. Sampling: the 7 sampling period are indicated as S1 to S7.

[c] Clonal complex.

[d] ERY, erythromycin; CLIi, inducible resistance to clindamycin; TET, tetracycline; PEN, penicillin; STR, streptomycin.

[e] Inmune Evasion Cluster.

[f] Genes within squared brackets are those proved to be physically linked, while those within brackets are those expected to be linked based on bibliography.

[g] Non-typeable.

[h] The novel ST1655 revealed a novel *aroE* allele, named 240, and exhibited the following allele combination: *arcC*_3, *aroE*_240, *glpF*_1, *gmk*_8, *pta*_1, *tpi*_1, *yqiL*_1. It is a single-locus-variant of the ancestor ST188. The novel ST2329 presented a novel *arcC* allele, named 252, and showed the following allele combination: *arcC*_252, *aroE*_291, *glpF*_6, *gmk*_2, *pta*_7, *tpi*_32, *yqiL*_2.

[i] The novel *spa* type t8765 presented the following repeats: r04-r20-r17-r31-r24-r17-r23-r50-r17, while those detected in the novel t8764 were as follows: r26-r23-r25-r17-r25-r17-r25-r16-r28.

Table 2. Molecular characterization, antimicrobial resistance and virulence profile of the 16 methicillin-susceptible *S. pseudintermedius* isolates investigated.

Dog ID[a]	Origin[b] (sampling)	Isolate	MLST	*spa*	PFGE cluster	Antimicrobial resistance profile[c]		Virulence genes detected[c]					
								Leukocidins	Exfoliatins			Pyrogenic Toxin Superantigen Genes	
						Phenotype[d]	Genes detected	*lukS/F-I*	*siet*	*expA*	*expB*	*seint*	Others
58	U (S4)	C3464	ST20	NT[e]	a3			+	+			+	
91	R (S7)	C3466	ST41	NT	c4	PEN-TET	*blaZ*, *tet*(M), *tet*(L)	+	+		+	+	
79	R (S6)	C2723	ST44	NT	c1	PEN	*blaZ*	+	+	+		+	(*sec*$_{canine}$-*sel*)
18	R (S1)	C3460	ST160	NT	a4	PEN	*blaZ*	+	+		+	+	
67	R (S5)	C2720	ST181	NT	d	PEN	*blaZ*	+	+			+	
75	R (S6)	C2721	ST182	NT	c5	PEN-TET	*blaZ*, *tet*(M)	+	+			+	
26	R (S2)	C3462	ST183	NT	c6			+	+			+	
76	R (S6)	C2722	ST183	NT	f			+	+			+	
86	R (S7)	C2724	ST184	t05	c2			+	+			+	
92	R (S7)	C2725	ST185	t43	b2	PEN-TET	*blaZ*, *tet*(M), *tet*(L)	+	+	+		+	
1	R (S1)	C3458	ST186	t02	a2	TET	*tet*(M), *tet*(L)	+	+			+	
3	R (S1)	C3459	ST187	NT	a1	TET	*tet*(M), *tet*(L)	+	+			+	
23	R (S2)	C3461	ST188	NT	b1	PEN-TET	*blaZ*, *tet*(M), *tet*(L)	+	+			+	
51	R (S4)	C3463	ST189	NT	e	TET	*tet*(M), *tet*(L)	+	+		+	+	
92	R (S7)	C3467	ST190	NT	c3	PEN-TET-ERI-CLIi-KAN-STR-CLO	*blaZ*, *tet*(M), *erm*(B), [*aadE-sat4-aphA3*], *cat*$_{pC221}$	+	+			+	
95	R (S7)	C2726	ST190	NT	c3	PEN-TET-ERI-CLIi-KAN-STR-CLO	*blaZ*, *tet*(M), *erm*(B), [*aadE-sat4-aphA3*], *cat*$_{pC221}$	+	+			+	

The novel STs are shown in bold letters.

[a] Dog 67 concomitantly carried a MSSA ST398, Dog 86 a MSSA ST5, Dog 95 a MSSA ST398, and Dog 92 two distinct *S. pseudintermedius* isolates (a MSSP ST185 and a MSSP ST190).

[b] U, urban area; R, rural area. Sampling: the 7 sampling period are indicated as S1 to S7.

[c] Genes within squared brackets are those proved to be physically linked, while those within brackets are those expected to be linked based on bibliography.

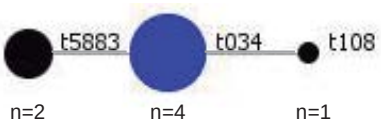
[d] PEN, penicillin; TET, tetracycline; ERY, erythromycin; CLIi, inducible resistance to clindamycin; KAN, kanamycin; STR, streptomycin; CHL, cloramphenicol. [e] Non-typeable.

Table 3. Characteristics of the sequence types detected in the 16 methicillin-susceptible *S. pseudintermedius* recovered in this study.

Isolate/s	MLST	*16S rRNA*	*tuf*	*cpn60*	*pta*	*agrD*		Novel characteristics
						allele	allotype	
C3464	ST20	1	1	2	1	1	III	-
C3466	ST41	1	1	13	1	2	II	-
C2723	ST44	1	1	2	1	3	IV	-
C3460	ST160	1	1	13	4	1	III	-
C2720	ST181	1	1	7	4	2	II	allele combination
C2721	ST182	1	2	2	30	1	III	allele *pta*_30 (JX416710)[a]
C2722, C3462	ST183	1	1	42	4	3	IV	allele *cpn60*_42 (JX416711)[a]
C2724	ST184	1	2	11	1	2	II	allele combination
C2725	ST185	1	1	6	1	2	II	allele combination
C3458	ST186	1	1	2	2	11	IV	allele *agr*_11 (HE970632)[a]
C3459	ST187	1	1	2	4	3	IV	allele combination
C3461	ST188	1	1	26	1	1	III	allele combination
C3463	ST189[b]	1	1	9	2	3	IV	allele combination
C2726, C3467	ST190	1	2	3	4	1	III	allele combination

Sequences types and alleles in gray shaded are those with novel characteristics.
[a] GenBank/EMBL accession numbers.
[b] ST189 is a single-locus-variant of the European clonally-spread MRSP lineage ST71. They diverge in the *agrD* allele (ST71, *agrD*_1, allotype_III).

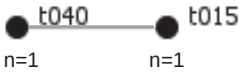
A. Cluster 1



ST398/CC398

t5883	r08	r16	r02	r25				r24	r25
t034	r08	r16	r02	r25	r02	r25	r34	r24	r25
t108	r07		r02	r25	r02	r25	r34	r24	r25

B. Cluster 2



ST45/CC45

t040	r09		r02	r16		r13	r17	r34	r16	r34
t015	r08	r16	r02	r16	r34	r13	r17	r34	r16	r34

Figure 1. Characteristics of the *spa*-types clustered using BURP clustering (Ridom Staph-Type software version 2.0.21), with a cost value of 8. Identities between the different *spa* repeats, number of isolates enclosed and the ST and CC lineage of isolates are indicated. A) Cluster 1 representation and repeats of the *spa*-types grouped. This cluster enclosed the 7 methicillin-susceptible *S. aureus* (MSSA) ST398/CC398 isolates obtained. B) Cluster 2 display and characteristics of the *spa*-types grouped. This cluster enclosed both MSSA ST45/CC45 isolates.

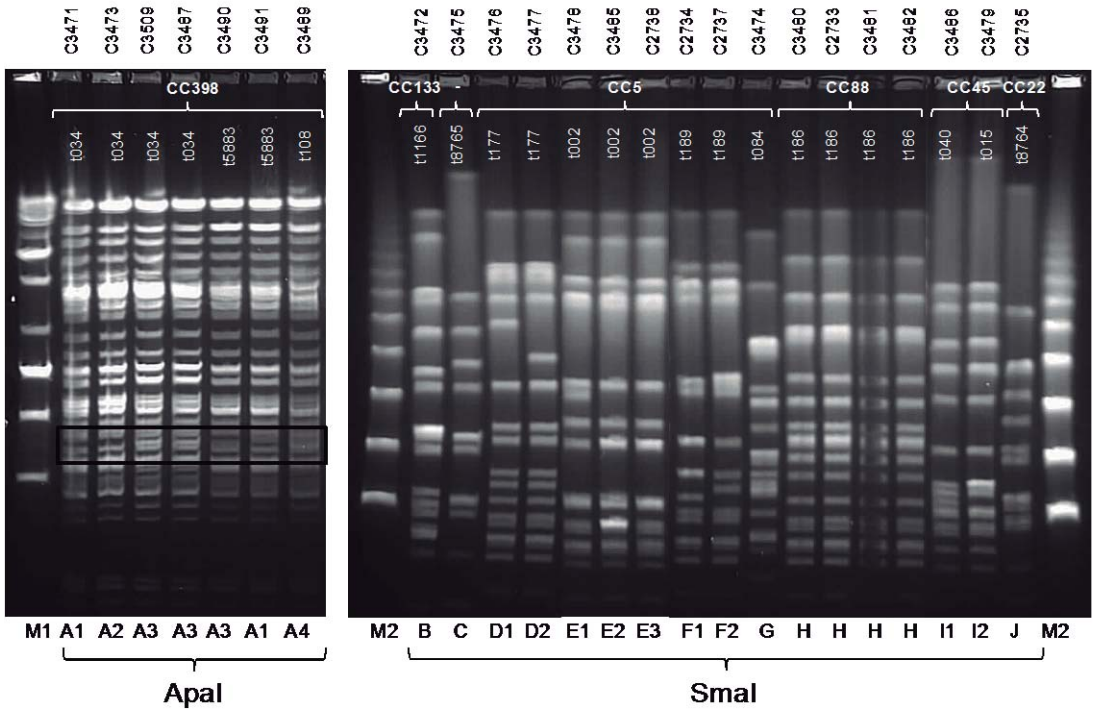


Figure 2. PFGE band patterns of genomic DNA digested with ApaI (7 ST398 isolates) or SmaI (17 non-ST398 isolates) enzymes of all methicillin-susceptible *S. aureus* isolates obtained in this study. Isolates identification and lineage (*spa*-type and clonal complex) are indicated on each band pattern. Arabic letters (A to J) indicate the different PFGE clones while the numbers are used to distinguish closely related patterns. M1, low range ladder marker; M2, lambda ladder marker (New England Biolabs).

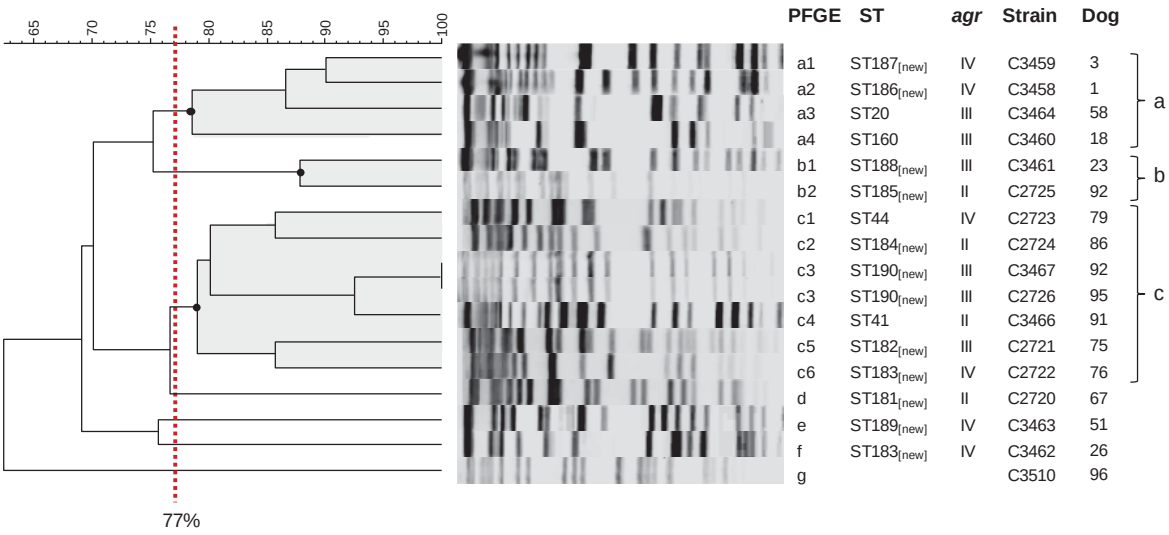


Figure 3. Dendrogram displaying the clonal relatedness of genomic DNA digested with SmaI macrorestriction enzyme after PFGE of the 16 methicillin-susceptible *S. pseudintermedius* isolates and the single *S. schleiferi* subsp. *coagulans* obtained in this study. A 77% cut-off similarity was applied to differentiate 7 major clusters, named A to G. Numbers following the name are used to distinguish the different PFGE patterns of clustered isolates. A total of 15 *S. pseudintermedius* distinct band profiles were identified. The ST and *agr* allotype of all isolates is also indicated. Isolates and dog identification are also shown.

Annex to chapter 1 and 2

Table S3. Comparative distribution of *S. aureus* genetic lineages detected among household owners and their pets (**paper 1**) and those detected in kennel dogs (**chapter 2**).

***S. aureus* CC/ST-*spa* in household individuals (n=36)**	***S. aureus* *spa*-ST/CC in kennel dogs (n=24)**
CC398/ST398-t571 (1 owner)	CC398/ST398-t034 (**4 dogs**)
CC45/ST45-t073 (3 owners, **1 dog, 1 cat**)	CC398/ST398-t5883 (**2 dogs**)
CC45/ST45-t015 (1 owner)	CC398/ST398-t108 (**1 dog**)
CC5/ST5-t002 (3 owners**, 1 cat**)	CC45/ST45-t040 (**1 dog**)
CC5/ST146-t002 (1 owner)	CC45/ST45-t015 (**1 dog**)
CC8 (CC5)/ST8-t5173 (1 owner)	CC5/ST5-t002 (**2 dogs**)
CC8 (CC5)/**ST2176**-t711 (**1 cat**)	CC5/ST146-t002 (**1 dog**)
CC8 (CC5)/ST72-t148 (1 owner)	**CC15 (CC5)**/ST188-t189 (**1 dog**)
CC9 (CC5)/ST109-t209 (1 owner, **1 dog**)	**CC15 (CC5)**/**ST1655**-t189 (**1 dog**)
CC121/ST121-t159 (3 owners, **1 dog**)	**CC15 (CC5)**/ST15-t084 (**1 dog**)
CC121/ST121-t151 (**1 dog**)	CC22/ST217-t8764 (**1 dog**)
CC121/ST121-t2155 (1 owner)	CC133/ST133-t1166 (**1 dog**)
CC121/ST95-t1077 (1 owner)	CC1/ST1-t177 (**2 dogs**)
CC121/**ST2177**-t1077 (1 owner)	CC88/ST78-**t186** (**4 dogs**)
CC30/ST30-t440 (1 owner)	singleton/**ST2329**-**t8765** (**1 dog**)
CC30/ST30-t037 (1 owner)	
CC30/ST30-t012 (1 owner)	
CC30/ST34-t3711 (1 owner)	
CC30/ST34-t3916 (1 owner)	
CC22/**ST1733**-t7901 (2 owners)	
CC7/ST7-t091 (1 owner)	
singleton/**ST1654** -t021 (1 owner, **1 dog**)	
singleton/**ST2175**-t054 (1 owner)	

Novel STs and *spa* types detected in this thesis are indicated in blue colour. Individuals underlined are those involved in the direct cases of interspecies transmission. CCs coloured in green are those recently reclassified into CC5, which is showed within brackets. Different colour backgrounds indicate the common STs observed in both populations tested.

Table S4. Comparative distribution of *S. pseudintermedius* genetic lineages detected among household owners and their pets (**paper 1**) and those detected in kennel dogs (**chapter 2**).

ST-*spa* in household individuals (n=18)	**ST-*spa* in kennel dogs (n=24)**
ST71-t02 (**1 dog**)	ST71-t02 (**7 dog**)
ST92-t06 (**1 dog**)	ST26-NT (**1 dog**)
ST7-NT (**2 dogs**)	ST20-NT (**1 dog**)
ST21-t37 (1 owner)	ST41-NT (**1 dog**)
ST29-NT (**1 dog**)	ST44-NT (**1 dog**)
ST33-NT (**1 dog**)	ST160-NT (**1 dog**)
ST42-NT (**2 dogs**)	**ST181**-NT (**1 dog**)
ST77-NT (**1 dog**)	**ST182**-NT (**1 dog**)
ST100-NT (1 owner)	**ST183**-NT (**2 dogs**)
ST141-NT (**1 dog**)	**ST184**-t05 (**1 dog**)
ST142-NT (1 owner, **1 dog**, 1 cat)	**ST185**-t43 (**1 dog**)
ST146-NT (**1 dog**)	**ST186**-t02 (**1 dog**)
ST154-NT (**1 dog**)	**ST187**-NT (**1 dog**)
ST155-NT (**1 dog**)	**ST188**-NT (**1 dog**)
	ST189-NT (**1 dog**)
	ST190-NT (**2 dogs**)

Novel STs detected are indicated in blue colour. Lineages underlined in red are MRSP. The coloured background indicates the common ST observed in both populations tested.

Table S5. Comparative distribution of the antimicrobial resistance profile (AMR) of *S. aureus* isolates recovered from household individuals (owners, dogs, cats) (**paper 1**) and kennel dogs (**chapter 2**).

Antimicrobial	**% AMR** [no and resistance gene detected]	
	Isolates from household individuals (n=36)	**Isolates from Kennel dogs (n=24)**
Penicillin	91.2 [33 *blaZ*]	20.8 [5 *blaZ*]
Oxacillin/cefoxitin	2.8 [1 *mecA*]	-
Tetracycline	5.6 [2, *tet*(K)]	4.2 [1 *tet*(K)]
Streptomycin	5.6 [2 *str*]	4.2 [1 *str*]
Erythromycin/Clindamycin	13.9 [2 *erm*(A), 2 *erm*(C), 1 *erm*(T)]	20.8 [4 *erm*(A), 1 *erm*(C)]
Erythromcyin only	2.8 [1 *msrA*/*msrB*+*mphC*]	-
Kanamycin	2.8 [1 *aphA3*]	-
Ciprofloxacin	2.8 [1 $GrlA^{S80Y}$ + $GyrA^{S84L}$]	-
Susceptible to all tested agents	8.3 [3]	50 [12]

Gray background indicates higher value.

Table S6. Comparative distribution of the antimicrobial resistance profile of *S. pseudintermedius* isolates recovered from household individuals (owners, dogs, cats) (**paper 1**) and kennel dogs (**chapter 2**).

Antimicrobial	**% AMR** [number and resistance gene detected]	
	Isolates from household individuals (n=18)	**Isolates from Kennel dogs (n=24)**
Penicillin	94.4 [17 *blaZ*]	70.8 [17 *blaZ*]
Oxacillin/cefoxitin	11.1 [2 *mec*A]	33.3 [8 *mec*A]
Tetracycline	44.4 [5 *tet*(M), 1 *tet*(K), 2 *tet*(K)+*tet*(M)]	70.8 [7 *tet*(K), 4 *tet*(M), 6 t*et*(M)+*tet*(L)]
Streptomycin	38.9 [7 *aadE*]	41.7 [10 *aadE*]
Erythromycin/Clindamycin	38.9 [6 *erm*(B), 1 *erm*(A)+*erm*(B)]	41.7 [10 *erm*(B)]
Kanamycin	38.9 [7 *aphA3*]	41.7 [10 *aphA3*]
streptothricin	38.9 [7 *sat4*]	41.7 [10 *sat4*]
Gentamycin/tobramycin	16.7 [3 *aacA-aphD*]	33.3 [8 *aacA-aphD*]
Trimethoprim	22.2 [4 *dfr*(G)]	33.3 [8 *dfr*(G)]
Chloramphenicol	27.8 [5 cat_{pC221}]	12.5 [3 cat_{pC221}]
Ciprofloxacin	11.1 [1 $GrlA^{S80I}$ + $GyrA^{S84L+E714K}$, 1 $GrlA^{S80I}$ + $GyrA^{S84L}$]	29.2 [7 $GrlA^{S80I}$ + $GyrA^{S84L+E714K}$]
Susceptible to all tested agents	5.6 [1]	16.7 [4]

Gray background indicates higher value.

UNUSUAL RESISTANCE PHENOTYPE

It is interesting to remark that all MRSP ST71 (all of which carried the SCC*mec* II-III), and only them, exhibited an unusual inducible cefoxitin resistance phenotype by oxacillin disk when tested 15-20 mm apart (D-shape; **Fig. S2**). Double-disk diffusion test (oxacillin and other β-lactam) with 11 β-lactams revealed that this phenomenon was not observed with any of the tested agents. Additionally, these MRSP ST71 isolates showed heterogeneous resistance to cefoxitin segregating highly resistant subpopulations. Sequential passes (up to 100, one per day) of MRSP ST71 isolates on non selective agar (free-antibiotic medium) were carried out to determine the stability of this inducible phenotype. From the 60th streak on, this observable trend became to be instable in most strains. On the other hand, the highly resistant colonies growing inside the cefoxitin inhibition halo maintained their highly resistance phenotype after numerous sequential passages (**Fig. S2**).

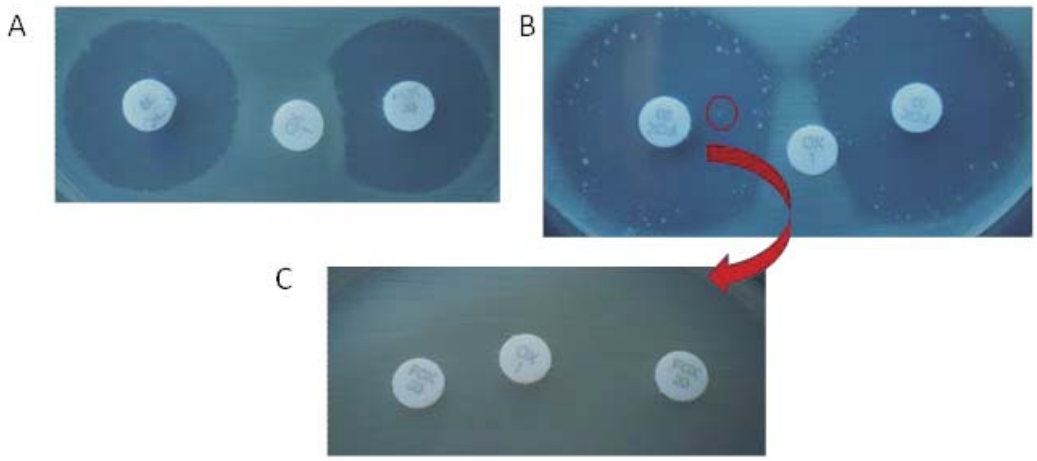


Figure S2. A) Cefoxitin inducible resistance expression of MRSP ST71 isolates by disk-diffusion agar method. B) Cefoxitin inducible resistance and heterogeneous resistance expression of MRSP ST71 isolates by disk-diffusion agar method. C) Highly cefoxitin resistant subpopulation after isolation and continuous subculture.

It is difficult to unveil the potential mechanism of the regulatory elements involved in this novel phenotype, given that multiple factors may be involved [β-lactamase regulatory elements (*blaR1-blaI*); the two-component system VraSR; autolysins, etc]. Whether this phenotype may be promoted by the SCC*mec* II-II element remains also unknown. This inducible cefoxitin resistance phenotype might be of great relevance to (i) detect *mec*A-mediated β-lactam resistance by cefoxitin disk-difusion test among MRSP ST71 and/or (ii) to detect the presence of SCC*mec* II-III. However, the

number of strains examined in this report had no epidemiological burden. Further studies with a great number of isolates are needed to unveil whether this is a punctual phenomenon, a SCC*mec*-specific characteristic or whether this could represent a lineage-specific profile. Additionally, hetero-resistance is a common trait among staphylococcal strains that possess *mec*A gene. Phenotypic stability on highly resistant subpopulations would suggest the selection of mutations or rearrangements, while instability would be more characteristic of induction by additional levels of regulation. Nevertheless, the nature of the mutational event(s) that leads to high-level β-lactam resistance is still only a matter of speculation.

Chapter 3

MRSA from slaughter pigs and CoPS from hospitalized equines

CHAPTER 3. SARM AND SPRM FROM SLAUGHTER PIGS AND HOSPITALIZED EQUINES.

Although Spain accounts for one of the highest rates of pig production holdings in Europe, no data was available on MRSA ST398 in pigs at slaughter in this country. Scarce data was also reported on the possible influence of pig age on the colonization of MRSA ST398 (Smith et al., 2008). Further, there has been an increase in the presence of MRSA ST398 in hospitalized horses in different countries (Couto et al., 2012; Haenni et al., 2010; Sieber et al., 2011;; van Duijkeren et al. 2011a), although its detection in Spain was so far undocumented. The objective of this chapter was to detect and characterize MRSA isolates obtained from slaughter pigs of two distinct ages and to characterize all CoPS recovered from hospitalized equines in a Veterinary Laboratory.

Nasal swabs from 53 finishing-pigs and 53 suckling-piglets were collected at two different abattoirs during September 2008/March 2009. MRSA isolates were characterized by different molecular techniques, antimicrobial resistance profile and virulence genes content. Clonal relatedness of isolates was investigated by ApaI/PFGE.

MRSA isolates were recovered from 11 finishing-pigs (20.7%, with 14 isolates) and 26 suckling-piglets (49%, 30 isolates). Forty MRSA (91% of isolates) belonged to lineage ST398 and exhibited four related *spa* types (t011, t108, t1197 and t2346). Interestingly, the remaining four isolates (9%) – all from finishing-pigs - presented *spa* type t3992 and were ascribed to a new ST, designated ST1379, which corresponds to a SLV of the bovine associated ST97 (traditionally enclosed within CC97). PFGE cluster analysis revealed 5 major groups with up to nine individual profiles. Relatively low genomic relatedness between MRSA from finishing-pigs and suckling-piglets was observed. A great variety of resistance gene patterns was detected, with all isolates being resistant to tetracycline. Interestingly, four strains from finishing-pigs harboured the recently described for the first time in staphylococci MLS_B resistance *erm*(T) gene. In general, finishing-pigs exhibited resistance to more antimicrobials, and 71.4% of them were MDR, in comparison to the 37% detected among suckling-piglets. Low virulence gene content was detected among MRSA ST398 while MRSA ST1379 harboured *eta*, *lukE/D*, and *hlg-2* toxin genes.

Between 2005 and 2011, thirty-nine samples from hospitalized equines were analyzed in the Infectious Diseases Laboratory of the Faculty of Veterinary of the University of Zaragoza. Eight potential CoPS (7 unrelated horses, one donkey) were recovered and further investigated.

Six *S. aureus* were detected and 4 of them were MRSA ST398. One MRSA ST398 strain was isolated in 2005 and represents the earliest MRSA ST398 reported in Spain to date. All MRSA were MDR and all were resistant to tetracycline and aminoglycosides. PCR mapping revealed that three MRSA ST398 carried the recently described *dfrK*-transposon Tn*559* within their chromosomal *radC* gene. The two MSSA were typed as ST1 and ST1660, and were only penicillin resistant. Although positive horses were unrelated and recovered in different years, all MRSA ST398 showed closely related PFGE patterns. The 2 remaining isolates were *S. pseudintermedius*; one was *mec*A-positive (SCC*mec* V) and belonged to the American clonally spread ST68 lineage. This strain was MDR and harboured the *erm*(B)-Tn*5405*-like element. Surprisingly, this *mec*A-positive MSSP ST68 strain presented a clear susceptible phenotype to oxacillin and cefoxitin, regardless of the presence of an integral copy of the *mec*A gene and promoter. The remaining isolate was MSSP, recovered from a donkey, and was typed as ST184, ST recently described in a dog reported in **chapter 2**.

MRSA ST398 is spread among slaughter swine and age of animals seems to be an important factor to consider when designing and comparing studies. Our results reveal the presence of MRSA ST398 in horses in Spain and the first *S. pseudintermedius* ST68 in this animal species. Due to the close contact between horses and owners, and the persistent occupational contact of pigs and workers, potential public health implications on humans in contact to these animals are conceivable.

These studies have led to one published paper and one, at present, in advanced stage of revision in international peer-reviewed journals.

3.1. PAPER 5.

Gómez-Sanz E, Torres C, Lozano C, Fernández-Pérez R, Aspiroz C, Ruiz-Larrea F, Zarazaga M. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. Foodborne Pathog Dis. 2010;7(10):1269-77.

3.2. PAPER 6.

Gómez-Sanz E, Simón C, Ortega C, Gómez P, Lozano C, Zarazaga M, Torres C. First detection of methicillin-resistant *Staphylococcus aureus* ST398 and *Staphylococcus pseudintermedius* ST68 from hospitalized equines in Spain. Zoonosis and Publich Health (accepted).

Paper 5

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Original Article

Detection, Molecular Characterization, and Clonal Diversity of Methicillin-Resistant *Staphylococcus aureus* CC398 and CC97 in Spanish Slaughter Pigs of Different Age Groups

Elena Gómez-Sanz,[1] Carmen Torres,[1] Carmen Lozano,[1] Rocío Fernández-Pérez,[1] Carmen Aspiroz,[2] Fernanda Ruiz-Larrea,[1] and Myriam Zarazaga[1]

Abstract

The objective of this study was to determine the frequency of nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in slaughter pigs, to characterize the recovered isolates, and to investigate their genomic relatedness. Nasal swabs were collected from 53 finishing-pigs (F-pigs) and 53 suckling-piglets (S-piglets) at two different abattoirs in La Rioja (Northern Spain) coming from six production holdings. MRSA isolates were characterized by *spa*−, *agr*−, SCC*mec*−, and multilocus sequence typing, pulsed-field gel electrophoresis (PFGE)-*Apa*I, toxin gene profiling, antimicrobial susceptibility, and determination of antimicrobial resistance genes. MRSA isolates were recovered from 11 F-pigs (14 isolates) and 26 S-piglets (30 isolates). Forty of the 44 MRSA presented the *spa*-types t011, t108, t1197, and t2346, which corresponded to the sequence type ST398 and to the clonal complex CC398. Interestingly, the remaining four isolates from F-pigs presented the *spa*-type t3992, and they were ascribed to a new sequence type named ST1379 (a single-locus variant of ST97), which was included in clonal complex CC97. Five PFGE-*Apa*I clusters with up to nine individual patterns detected among our MRSA and low genomic relatedness was observed between F-pig and S-piglet isolates. All MRSA were positive for *hla*, *hld*, and *hlg* hemolysin genes. ST1379 isolates harbored *eta*, *lukE/D*, and *hlg-2* toxin genes, whereas ST398 isolates were positive for *hlb*. A great variety of distinct resistance gene patterns were observed, most of them coming from F-pig isolates. MRSA virulence properties seem to be dependent of the isolate clonal lineage. This study showed that slaughter pigs are frequently colonized by MRSA CC398; moreover, the detection of strains belonging to CC97 underlines that other lineages are also able to spread in livestock. Further studies should assess the risk of CC398 and non-CC398 MRSA to enter the food chain as well as the human health implications.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common human pathogen causing important therapeutical problems, being also a recently emerging problem in veterinary medicine. A livestock-associated MRSA (LA-MRSA) of sequence type ST398, which belongs to the clonal complex CC398, is emerging in recent years in farm animals and subsequently in humans (Armand-Lefevre *et al.*, 2005; EFSA, 2009a; Aspiroz *et al.*, 2010; Vanderhaeghen *et al.*, 2010; Weese and van Duijkeren, 2010). Pigs are considered the reservoir of this MRSA lineage; and some reports have regarded pig-contact as a risk factor for increased human MRSA nasal carriage and consequent infection. MRSA of lineage CC398 is not especially pathogenic for pigs; nevertheless, several studies have described them as causing infections to these animals (Kadlec *et al.*, 2009; Meemken *et al.*, 2009). In addition, CC398-MRSA strains are increasingly reported not only in pigs but also in other farm animals such as cattle, poultry, and horses, as well as sporadic cases in pets (Witte *et al.*, 2007; EFSA, 2009a; Nienhoff *et al.*, 2009; Weese and van Duijkeren, 2010; Mulders *et al.*, 2010; Vanderhaeghen *et al.*, 2010). Moreover, human infections caused by CC398-MRSA are being increasingly described (Witte *et al.*, 2007; Welinder-Olsson *et al.*, 2008; Weese and van Duijkeren, 2010; Aspiroz *et al.*, 2010). The entrance of this lineage in meat products for human consumption has also been described (EFSA, 2009a; Lozano *et al.*, 2009). Nevertheless, little is known about the possible influence of age on the dynamic of colonization of ST398 strains, not only in pigs but also in other animal species (Vanderhaeghen *et al.*, 2010). Alternatively, the presence of non-CC398 lineages in livestock-associated strains is raising

[1]Departamento de Agricultura y Alimentación, Universidad de La Rioja, Logroño, Spain.
[2]Servicio Microbiología, Hospital Royo Villanova, Zaragoza, Spain.

interest, as other potential pathogenic lineages may have been overlooked in terms of animal and human health implications. In these means, recent studies have reported ST9 (non-CC398) as the most prevalent MRSA lineage in pigs in China (Wagenaar *et al.*, 2009). Likewise, a recent European survey carried out in pig farms detected MRSA non-CC398 in 7 of the 26 analyzed countries (EFSA, 2009b). MRSA ST97 and other related STs (belonging to CC97), which are traditionally closely associated to cattle, have been recently described in both healthy and diseased pigs in some European countries (EFSA, 2009b; Meemken *et al.*, 2009; Battisti *et al.*, 2010).

There is current concern about LA-MRSA in Spain, considering that this country accounts for a high rate of pig production holdings; nevertheless, data on the prevalence in pigs are still scarce (EFSA, 2009a, 2009b). The aim of this study was to investigate the carriage rates of MRSA in pigs of two distinct age groups at slaughter in Spain, to characterize the recovered isolates using distinct molecular techniques, and to determine the clonal diversity of the studied isolates.

Materials and Methods

Origin of samples

Individual nasal swabs were collected from 106 healthy pigs in La Rioja (Northern Spain) between September 2008 and March 2009. Samples corresponded to 53 finishing-pigs (F-pigs) and 53 suckling-piglets (S-piglets). Sampling was implemented on four different occasions (two for F-pigs and two for S-piglets) at two abattoirs, one for each age-group, with a slaughtering capacity of about 300 pigs per day. Tested animals came from six different farrow-to-finish holdings (Table 1).

Bacterial isolates and polymerase chain reaction screening of samples

Swabs were inoculated into Brain-Heart-Infusion broth supplemented with 6.5% NaCl and incubated at 37°C for 24 h; an aliquot of 100 μL was then streaked on Oxacillin-Resistant-Staphylococcal-Agar-Base (Oxoid) plates supplemented with 2 μg/mL of oxacillin (Oxoid) and incubated at 37°C for 24–48 h. Up to three blue presumptive MRSA colonies were selected from each positive sample and preserved for further studies. Identification of *S. aureus* was based on colony morphology, Gram staining, and catalase and DNase activities. MRSA identification was confirmed by detection of *nuc* (*S. aureus* specific) and *mecA* (methicillin-resistant staphylococci specific) genes by multiplex polymerase chain reaction (PCR), as previously recommended (CRL-AR, 2009).

Table 1. Methicillin-Resistant *Staphylococcus aureus* Detection in Finishing-Pigs and Suckling-Piglets From Six Different Farrow-to-Finish Holdings

Animal type	*Holding ID*	*No. of studied animals*	*No. (%) of animals with MRSA carriage*	*No. of studied MRSA isolates*
F-pigs	F-1	32	5 (15.6)	7
	F-2[a]	11	3 (27.3)	4
	F-3	10	3 (30)	3
Total		53	11 (20.7)	14
S-piglets	F-2[a]	19	7 (36.8)	10
	F-4	17	9 (52.9)	10
	F-5	13	10 (76.9)	10
	F-6	4	0	–
Total		53	26 (49)	30

[a]Same holding of origin.
MRSA, methicillin-resistant *Staphylococcus aureus*; F-pigs, finishing-pigs; S-piglets, suckling-piglets.

PFGE of MRSA strains

Due to the non-typeability of ST398 strains using *SmaI* enzyme (caused by a restriction/methylation system leading to protection from *SmaI* digestion), strain relatedness was analyzed by PFGE of total DNA restricted with *ApaI* enzyme applying the HARMONY protocol guidelines (Murchan *et al.*, 2003). The switching times of electrophoresis for *ApaI* digests were those implemented by Kadlec *et al.* (2009). PFGE patterns were analyzed using GelCompar software package (Applied Maths). To determine the percentage of similarity necessary for strain discrimination, reproducibility studies were conducted (López *et al.*, 2008), analyzing by duplicate the macrorestriction patterns of 5 MRSA ST398/CC398 isolates. PFGE groups were defined by those formed at 89% cut-offs. The similarities between profiles were calculated using the Dice coefficient, with a maximum position tolerance of 1.2%, and the Unweighted Pair Group Method using Arithmetic averages.

Molecular typing of MRSA strains

All MRSA isolates were characterized by *spa*-typing, determination of staphylococcal cassette chromosome *mec* (SCC*mec*), and *agr* allotype by specific PCRs (http://spaserver.ridom.de; Shopsin *et al.*, 2003; IWG-SCC, 2009). Multi-locus sequence typing (MLST) was performed as described (www.mlst.net) on one representative strain per PFGE pattern and *spa*-type as well as in those isolates that presented an uncommon *spa*-type.

Toxin gene profiling

The presence of the genes encoding the Panton-Valentine-Leukocidin (PVL) toxin (*lukS/F-PV*), LukM-LukF-PV (*lukM*), and LukE-LukD (*lukE/D*) leukocidins were tested by PCR (Lina *et al.*, 1999; Jarraud *et al.*, 2002). Likewise, MRSA isolates were also screened by PCR for genetic determinants of hemolysins (*hla*, *hlb*, *hld*, *hlg*, and *hlgv*), exfoliative toxins (*eta* and *etb*), 18 enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*), as well as for the toxic-shock syndrome 1 toxin (*tst*) (Jarraud *et al.*, 2002; Hwang *et al.*, 2007).

Antimicrobial susceptibility testing and detection of resistance genes

Antimicrobial susceptibility testing to 16 antimicrobial agents (Fig. 1) was performed by disk-diffusion agar (CLSI, 2009). Streptomycin, fusidic acid, and mupirocin breakpoints were considered as recommended by the Societé Française de Microbiologie (www.sfm.asso.fr). Double-disk diffusion test (D-test) was implemented in all

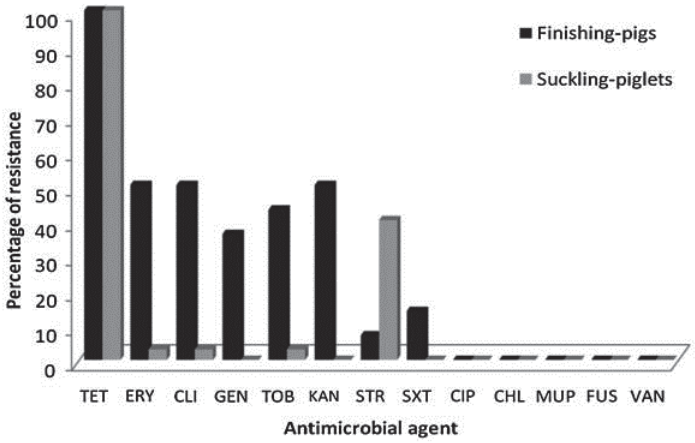

FIG. 1. Comparison of non-beta-lactam antimicrobial resistance patterns of MRSA isolates coming from finishing-pigs versus those isolated from suckling-piglets. TET, tetracycline; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; SXT, trimethoprim-sulphametoxazole; CIP, ciprofloxacin; CHL, chloramphenicol; MUP, mupirocin; FUS, fusidic acid; VAN, vancomycin; MRSA, methicillin-resistant *Staphylococcus aureus*.

isolates to detect inducible clindamycin resistance. Detection of antimicrobial resistance genes was investigated by specific PCRs and in some cases by sequencing (Table 2). Positive and negative controls from the collection of the University of Rioja were used.

Results

Detection and isolation of MRSA

MRSA were detected in 11 of 53 F-pigs (21%) and in 26 of 53 S-piglets (49%) corresponding to 5 of 6 of the sampled production holdings. Forty-four MRSA isolates (14 of F-pigs and 30 of S-piglets) were recovered from the MRSA-positive animals and were further characterized (Table 1).

Molecular typing of studied strains

Genetic relatedness by PFGE after *Apa*I digestion of the chromosomal DNA of our MRSA strains yielded a relatively wide range of fragment profiles. Cluster analysis generated five major clusters (A–E), with nine individual patterns. The resulting dendrogram, its correlation with the other typing methods implemented, and the origin of the studied isolates are shown in Figure 2.

Three *spa*-types were identified among the 14 MRSA isolates from F-pigs (number of isolates): t011 (5), t108 (5), and t3992 (4) (Table 3). The *spa*-types t011 and t108 are characteristic of the CC398 lineage (EFSA, 2009b; Vanderhaeghen *et al.*, 2010), as was confirmed by MLST on six selected strains (one per PFGE type and *spa*-type) (Fig. 2). MLST analysis of the four isolates with *spa*-type t3992 revealed a new allelic profile, now classified as ST1379. This new ST is a single-locus-variant of ST97, presenting a single nucleotide mutation (C110A) at *tpi* locus, resulting in a nonsynonymous amino acid change (Ala37Asp). Consequently, ST1379 belongs to the clonal complex CC97. In this regard, 7 of 11 (63.6%) MRSA-positive F-pigs were colonized by CC398-MRSA, and the remaining ones (36.3%) carried MRSA non-CC398. MRSA recovered from F-pigs were typed as either SCC*mec*IVa (five isolates) or SCC*mec*V (nine isolates). SCC*mec*IVa was identified in four of five isolates *spa*-type t011 and in one of six isolates *spa*-type t108 (Table 3).

Twenty-eight of the 30 MRSA isolates recovered from S-piglets presented the *spa*-type t011, one isolate was typed as t2346, and the remaining one was typed as t1197. These three *spa*-types are associated with CC398, as confirmed by MLST analysis of four selected strains. All isolates were associated with SCC*mec*V except for the MRSA t1197 strain, which harbored SCC*mec*IVa (Table 3). All 44 MRSA studied revealed *agr* type I.

Toxin gene profile and antimicrobial resistance pheno- and genotypes

All MRSA isolates were negative for the PVL toxin genes (*lukS/F*-PV) as well as for *luk*M, *etb etd*, *tst*, and all tested enterotoxin genes. All strains, independently of their clonal complex, were positive for *hla*, *hld*, and *hlg* genes. However, only ST1379/CC97 harbored the *eta*, *lukE/D*, and *hlgv* genes, whereas all ST398/CC398 isolates were positive for the *hlb* gene.

As for their antimicrobial resistance phenotypic characteristics, all MRSA strains showed resistance to beta-lactams and tetracycline, and different profiles were exhibited for the rest of the tested antimicrobial agents, which were dependent of the age-group (Fig. 1). Multi-resistance patterns (resistance to at least three classes of antimicrobial agents, in addition to beta-lactams) were exhibited in six MRSA strains from F-pig origin and only in one isolate from S-piglet origin.

MRSA isolates from F-pigs harbored more antimicrobial resistance genes than those of S-piglets and also exhibited a greater variability in the antimicrobial genotype profiles (Table 3). All 44 MRSA isolates carried the *mecA* and *blaZ* genes related to beta-lactam resistance. All isolates were resistant to tetracycline, and *tet*(K), *tet*(M), and/or *tet*(L) genes were present in different combinations. Among the eight macrolide-lincosamide-resistant strains, seven came from F-pigs and just one from S-piglets, and they harbored *erm*(A), *erm*(C), *erm*(T), and/or *msr*(A) genes in diverse combinations. All these isolates showed constitutive expression of resistance to macrolide-lincosamide-streptogramin B (MLS_B), as shown by D-test. Gentamicin resistance was only detected in five isolates obtained from F-pigs, all of which carried the *aacA/aphD* gene. Kanamycin and tobramycin resistance was exhibited in six isolates from F-pigs, being mediated by the *aadD* gene (associated with *aphA3* in one strain). Twelve MRSA strains (11 from S-piglets and 1 from F-pig) showed streptomycin resistance, and the *str* with/without *aadA* gene were identified. Two strains from F-pig origin were resistant to SXT, and both revealed the same combination of genes: *dfrA* + *dfrG* + *dfrK*.

Table 2. Oligonucleotides Used for Polymerase Chain Reaction Detection of Antimicrobial Resistance Genes

Gene	*Primer name*	*Sequence (5′ → 3′)*	*Amplicon size (bp)*	*Reference*[a]
mecA	mecA-1	GGGATCATAGCGTCATTATTC	527	*CRL-AR (2009)*
	mecA-2	AACGATTGTGACACGATAGCC		
blaZ	blaZ-F	CAGTTCACATGCCAAAGAG	772	*Schnellmann et al. (2006)*
	blaZ-R	TACACTCTTGGCGGTTTC		
tet(K)	tet(K)-1	TTAGGTGAAGGGTTAGGTCC	697	*Aarestrup et al. (2000)*
	tet(K)-2	GCAAACTCATTCCAGAAGCA		
tet(M)	tet(M)-1	GTTAAATAGTGTTCTTGGAG	576	*Aarestrup et al. (2000)*
	tet(M)-2	CTAAGATATGGCTCTAACAA		
tet(L)	tet(L)-1	CATTTGGTCTTATTGGATCG	456	*Aarestrup et al. (2000)*
	tet(L)-2	ATTACACTTCCGATTTCGG		
tet(O)	tet(O)-1	GATGGCATACAGGCACAGAC	615	*Aarestrup et al. (2000)*
	tet(O)-2	CAATATCACCAGAGCAGGCT		
erm(A)	ermA-1	TCTAAAAAGCATGTAAAAGAA	645	*Sutcliffe et al. (1996)*
	ermA-2	CTTCGATAGTTTATTAATATTAG		
erm(B)	ermB-1	GAAAAGTACTCAACCAAATA	639	*Sutcliffe et al. (1996)*
	ermB-2	AGTAACGGTACTTAAATTGTTTA		
erm(C)	ermC-1	TCAAAACATAATATAGATAAA	642	*Sutcliffe et al. (1996)*
	ermC-2	GCTAATATTGTTTAAATCGTCAAT		
erm(T)	ermT-1	CCGCCATTGAAATAGATCCT	200	*This study (FN390947)*
	ermT-2	TTCTGTAGCTGTGCTTTCAAAAA		
erm(Y)	ermY-1	AGGCCCCTTTTAAAGACGAAGGCA	320	*This study (AB014481)*
	ermY-2	GGCGCGATTGTTCATTTTAAGGCCC		
mph(C)	mphC-F1	ATGACTCGACATAATGAAAT	900	*Schnellmann et al. (2006)*
	mphC-R1	CTACTCTTTCATACCTAACTC		
vga(C)	vgaC-1	ACGAGGGGACAATCACGCCG	156	*This study (FN377602)*
	vgaC-2	TTGGTCGTCGGTTCGTCTGC		
msr(A)	msrA-1	GGCACAATAAGAGTGTTTAAAGG	940	*Lina et al. (1999)*
	msrA-2	AAGTTATATCATGAATAGATTGTCCTGTT		
msr(B)	msrB-1	TATGATATCCATAATAATTATCCAATC	595	*Lina et al. (1999)*
	msrB-2	AAGTTATATCATGAATAGATTGTCCTGTT		
lnu(A/A′)	linA-1	GGTGGCTGGGGGGTAGATGTATTAACTGG	323	*Lina et al. (1999)*
	linA-2	GCTTCTTTTGAAATACATGGTATTTTTCGATC		
lsa(B)	lsaB-1	TGCCGAAGCCATGTACCGTCC	396	*This study (AJ579365)*
	lsaB-2	CGGTTAGACCAACCAGCCGAACG		
aacA-aphD	aacA-aphD-1	CCAAGAGCAATAAGGGCATA	220	*Van de Klundert and Vliegenthart (1993)*
	aacA-aphD-2	CACTATCATAACCACTACCG		
aphA3	aphA3-1	GCCGATGTGGATTGCGAAAA	292	*Van de Klundert and Vliegenthart (1993)*
	aphA3-2	GCTTGATCCCCAGTAAGTCA		
aadD	aadD-1	GCAAGGACCGACAACATTTC	165	*Van de Klundert and Vliegenthart (1993)*
	aadD-2	TGGCACAGATGGTCATAACC		
aadE	aadE-1	ACTGGCTTAATCAATTTGGG	597	*Clark et al. (1999)*
	aadE-2	GCCTTTCCGCCACCTCACCG		
aadA	aadA-1	TGATTTGCTGGTTACGGTGAC	284	*Clark et al. (1999)*
	aadA-2	CGCTATGTTCTCTTGCTTTTG		
str	str-pS194-F	TATTGCTCTCGAGGGTTC	646	*Schnellmann et al. (2006)*
	str-pS194-R	CTTTCTATATCCATTCATCTC		
dfrA	dfrA-F	CCTTGGCACTTACCAAATG	374	*Schnellmann et al. (2006)*
	dfrA-R	CTGAAGATTCGACTTCCC		
dfrD	dfrD-F	TTCTTTAATTGTTGCGATGG	582	*Schnellmann et al. (2006)*
	dfrD-R	TTAACGAATTCTCTCATATATATG		
dfrG	dfrG-1	TCGGAAGAGCCTTACCTGACAGAA	323	*This study (AB205645)*
	dfrG-2	CCCTTTTTGGGCAAATACCTCATTCCA		
dfrK	dfrK-1	GAGAATCCCAGAGGATTGGG	423	*This study (FM207105)*
	dfrK-2	CAAGAAGCTTTTCGCTCATAAA		

[a]GenBank accession number is indicated within brackets.

Discussion

A high rate of MRSA detection among the production holdings tested was observed in this study. A recent European survey showed that the prevalence of breeding holdings positive for MRSA was 27%, and the prevalence found in Spain was 50% (EFSA, 2009b). However, that survey was conducted at farm level on dust samples, and MRSA prevalence might be different due to the sampling procedure.

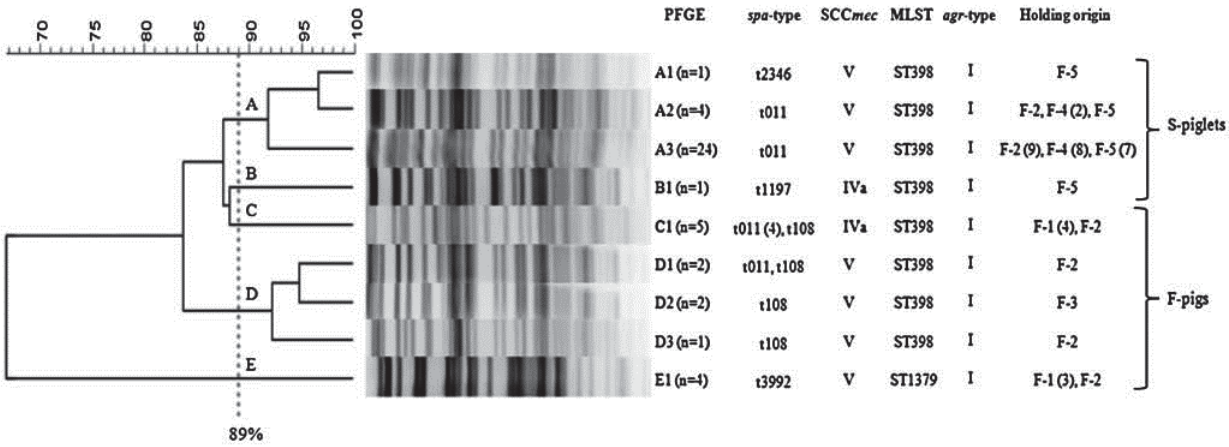

FIG. 2. Dendrogram showing the relatedness between *Apa*I macrorestriction fragment profiles generated after PFGE from the MRSA ST398/CC398 and ST1379/CC97 isolates studied. A cut-off at 89% similarity was used to distinguish the five major clusters (labeled A to E), and 9 individual patterns were obtained (labeled A-E plus number). The *spa*-type, SCC*mec* cassette, MLST, *agr*-type, holdings of origin and animal type associated with each *Apa*I fingerprint are also indicated. The numbers in brackets after the different fragment profiles indicate the number of isolates that exhibited the respective fragment type. PFGE, pulsed-field gel electrophoresis; SCC*mec*, staphylococcal cassette chromosome *mec*; MLST, multilocus sequence typing.

Additionally, a high occurrence of MRSA carriers within slaughter pigs is detected in the present study (21% F-pigs, 49% S-piglets). Very few studies have so far pointed animal age as a possible factor that might influence LA-MRSA prevalence, which have obtained diverse results (Vanderhaeghen *et al.*, 2010). Therefore, further investigations should try to unveil this possible influence of age, using a larger number of animals.

Most of our MRSA isolates belong to the CC398 (overall prevalence of 91%) with the remaining strains being ST1379/

TABLE 3. CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES RECOVERED FROM FINISHING-PIGS AND SUCKING-PIGLETS IN THIS STUDY

Animal type (No. isolates)	*PFGE type (No. isolates)*[a]	*Spa-type/SCCmec (No. isolates)*[a]	*Resistance phenotype*	*Resistance genes detected*
F-pigs (14)	C1	t011/IVa	TET-ERY-CLI-GEN-TOB-KAN	*tet*(K), *tet*(M), *tet*(L), *erm*(T), *aacA-aphD*, *aadD*
	C1	t011/IVa	TET-ERY-CLI-GEN-TOB-KAN	*tet*(M), *tet*(L), *erm*(T), *aacA-aphD*, *aadD*
	C1	t011/IVa	TET-ERY-CLI-GEN-TOB-KAN	*tet*(K), *tet*(M), *tet*(L), *erm*(T), *msr*(A), *aacA-aphD*, *aadD*
	C1	t011/IVa	TET-GEN-TOB-KAN	*tet*(M), *aacA-aphD*, *aadD*
	D1	t011/V	TET-SXT	*tet*(M), *tet*(L), *dfrA*, *dfrG*, *dfrK*
	C1	t108/IVa	TET-ERY-CLI-GEN-TOB-KAN	*tet*(K), *tet*(M), *tet*(L), *erm*(C), *erm*(T), *msr*(A), *aacA-aphD*, *aadD*, *aphA3*
	D2 (2)	t108/V	TET	*tet*(M)
	D1	t108/V	TET-SXT	*tet*(K), *tet*(M), *tet*(L), *dfrA*, *dfrG*, *dfrK*
	D3	t108/V	TET-ERY-CLI	*tet*(K), *tet*(M), *erm*(C), *erm*(T), *msr*(A)
	E1 (2)	t3992[b]/V	TET	*tet*(K), *tet*(M)
	E1	t3992[b]/V	TET-ERY-CLI-TOB-KAN	*tet*(K), *tet*(M), *tet*(L), *erm*(C), *erm*(T), *aadD*
	E1	t3992[b]/V	TET-ERY-CLI-KAN-STR	*tet*(K), *tet*(M), *erm*(C), *erm*(T), *aphA3*, *str*
S-piglets (30)	A2 (2), A3 (8)	t011/V	TET	*tet*(K)
	A1, A2 (2), A3 (6)	t011 (8), t2346/V	TET	*tet*(K), *tet*(M)
	A3 (6)	t011/V	TET-STR	*tet*(K), *tet*(M), *str*
	A3 (4)	t011/V	TET-STR	*tet*(K), *tet*(M), *aadA*, *str*
	B1	t1197/IVa	TET-ERY-CLI-TOB-STR	*tet*(M), *erm*(C), *erm*(T), *aadD*, *str*

[a]Number of isolates when more than one is included.
[b]*spa*-type presented in the 4 ST1379/CC97 isolates.
TET, tetracycline; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; STR, streptomycin; SXT, trimethoprim-sulphametoxazole.
PFGE, pulsed-field gel electrophoresis; SCC*mec*, staphylococcal cassette chromosome *mec*.

CC97. In the European study (EFSA, 2009b), 92% of MRSA were CC398, whereas 8% of strains were non-CC398, with ST1/CC1 and ST97/CC97 being the most frequently recovered. It is important to remark that in the European survey, MRSA CC97 was not detected among MRSA isolated from Spain.

The lineage CC97 is traditionally closely associated with cattle, and MRSA belonging to this clonal complex have been detected worldwide, being in most cases responsible for bovine mastitis (Smith *et al.*, 2005; Aires de Sousa *et al.*, 2007; Sung *et al.*, 2008; EFSA, 2009b). Moreover, methicillin-susceptible *S. aureus* (MSSA) and MRSA CC97 have also been reported on human clinical isolates obtained in different countries (Feil *et al.*, 2003; Chung *et al.*, 2004; Ellington *et al.*, 2008; Sung *et al.*, 2008). In these human infections, no information on the possible animal contact was given. Additionally, the first description of CC97 in pigs was reported on MSSA, causing a swine infection in France (Armand-Lefevre *et al.*, 2005), and recently the first detection of CC97-MRSA on clinical isolates in pigs in Germany has been published (Meemken *et al.*, 2009) and from healthy slaughter pigs in Italy (Battisti *et al.*, 2010). Further, a recent publication detected one CC97-MRSA on a rat living on a pig farm in Denmark (van de Giessen *et al.*, 2009).

As far as we know, no contact between pigs and dairy cattle along the breeding or production chain occurred in our study. Moreover, CC97-MRSA strains came from two different farrow-to-finish holdings and were sampled at different periods. Due to the traditional close association of this clonal lineage with mastitis in cattle as well as its presence in human infections, epidemiological studies on the potential ways of interspecies transmission as well as investigations of the human health implications of the non-CC398 MRSA detected should be definitively addressed.

Data analysis of PFGE macrorestriction fragments showed remarkable clustering results given that groups seem to cluster according to their age group and in line with their SCC*mec* cassette. Several recent reports have shown a closer genomic background within CC398 isolates with the same SCC*mec* type (SCC*mec*IVa or SCC*mec*V) after digestion with *Cfr9*I enzyme, which also suggest that CC398-MSSA strains could have acquired the SCC*mec* cassette at different stages and then evolve and diversify (Argudín *et al.*, 2010; van Wamel *et al.*, 2010). This is the first study showing this association when *Apa*I enzyme was used. Further, this enzyme also allowed direct comparisons of CC398 and non-CC398 *S. aureus* isolates. It is interesting to remark that the same *spa*-types were present in different clusters and sub-clusters detected by PFGE. Our results point to the suggestion that distinct lineages within CC398 could be better adapted to different animal ages. In this sense, future investigations addressing this observation will be performed.

In our study, the multi-resistance genotype is generally associated with the presence of SCC*mec*IVa, differing from typical community-acquired-MRSA-SCC*mec*IVa (Vanderhaeghen *et al.*, 2010). On the other hand, the only multi-resistant MRSA isolates carrying SCC*mec*V were two CC97 strains. There are only two studies reporting the SCC*mec* cassette on CC97 isolates, which detected SCC*mec*IVa and SCC*mec*V, respectively (Chung *et al.*, 2004; Ellington *et al.*, 2008). Likewise, this is the first report describing the antimicrobial genetic patterns of CC97 strains.

None of our isolates carried the PVL-encoding genes. PVL-positive CC398-MRSA isolates are uncommon; however, since its first finding from humans with no exposure to animal husbandry in China (Yu *et al.*, 2008), its detection has been described on several occasions, always in isolates causing human disease (van Belkum *et al.*, 2008; Welinder-Olsson *et al.*, 2008). There is no report on PVL-positive CC398-MRSA isolated from livestock so far, differentiating these LA-MRSA strains obtained from animals from typical community-acquired-MRSA, which are characteristic for the presence of these toxin genes. On the other hand, PVL-positive CC97-MRSA isolates have not yet been reported.

All MRSA studied showed a low number of virulence determinants. Interestingly, the presence of *eta* and *lukE/D* genes as well as *hlgv* was homogeneous in the four CC97-MRSA isolates. LukED is closely associated with bovine mastitis caused by *S. aureus* and classically seldom detected in pigs (Yamada *et al.*, 2005; Battisti *et al.*, 2010), which emphasizes the possible bovine origin of these isolates. In addition, a great prevalence of these genes has been reported among clinical human *S. aureus* isolates (Vandenesch *et al.*, 2003), which remarks the potentially higher pathogenic properties of our CC97 isolates comparing with those CC398 recovered.

As for the enterotoxin genes tested, very few data are available on the presence of these genes in CC398-MRSA strains of animal origin (Kadlec *et al.*, 2009; Vanderhaeghen *et al.*, 2010). Regarding CC97-MRSA isolates, a previous report showed the presence of few of these genes in ST97-MRSA obtained from clinical human isolates (Ellington *et al.*, 2008). However, to the best of our knowledge, this is the first report that addresses the presence or absence of these genes in CC97-MRSA of animal origin. Although in our study the presence of these genes was negative, in-depth virulence studies should be conducted on these lineages to elucidate the potential enterotoxigenic properties of these strains, due to the potential impact on human health.

All MRSA strains studied in this report (either CC398 or CC97) showed resistance to tetracycline, which is a common trait among *S. aureus* of animal origin. The resistance gene *tet*(L) has been rarely detected in MRSA isolates (Kadlec *et al.*, 2009). Hence, it is remarkable that 7 out of 14 strains from F-pigs harbored this gene. Alternatively, none of the MRSA strains from S-piglets carried *tet*(L) gene. This underlines that the phenotype of resistance seems to be associated not only to the animal age but also to the presence of particular genes.

Published data on MRSA from livestock have reported erythromycin-clindamycin resistance (Witte *et al.*, 2007; Kadlec *et al.*, 2009; Battisti *et al.*, 2010; Mulders *et al.*, 2010). Nevertheless, due to the absence of D-test analysis, there are no data on the prevalence of either constitutive or inducible resistance until now. Consequently, the constitutive expression of MLS_B resistance revealed in our study is noteworthy, as it may be of special interest for treatment of infections caused by these organisms. It is interesting to remark that all MLS_B resistant isolates harbored the *erm*(T) gene. This gene has been recently detected for the first time in staphylococci, being located in a plasmid (pKKS2187) also carrying *tet*(L) and *dfrK* genes (Kadlec and Schwarz, 2010). Nevertheless, in our isolates, these genes were not detected by PCR, suggesting either another mediator or a mechanism of recombination and subsequent loss of the plasmid. This finding will be

addressed in future investigations. Further, this is the first description of *erm*(T) in non-CC398 *S. aureus* isolates.

As for the two SXT-resistant MRSA isolates, the aforementioned *dfrK* gene was identified, which also harbored *tet*(L) gene. This is in accordance with Kadlec and Schwarz (2009), who recently described this gene among porcine CC398-MRSA and demonstrated the physical linkage of both genes in a plasmid (pKKS25).

In addition, within the streptomycin resistance genes, and to the best of our knowledge, the *str*-positive isolates as well as the *aadA* gene have not been reported in CC398-MRSA strains earlier.

This report evidences a high capacity of CC398 lineage to acquire and maintain resistant determinants, given that for most resistance properties more than one resistance gene was present, especially in F-pigs. This observation is in line with CC97 resistance patterns detected, underlining that two of four strains were multi-resistant.

Conclusions

To our knowledge, this is the first comprehensive molecular characterized study on MRSA in pigs at slaughter in Spain and the first detection of CC97-MRSA in Spain. The resistance gene acquisition capacities of all isolates studied (CC398 and CC97) is of particular significance, because both clonal lineages can colonize various animal and human hosts. PFGE macrorestriction fragments also show the usefulness of PFGE-*Apa*I analysis for distribution of CC398-MRSA isolates into groups and subgroups as well as for direct comparison with non-CC398 strains. Epidemiological studies on the circulating lineages in diverse animal ecosystems should be conducted, to elucidate the transmission routes of MRSA lineages among animals and humans as well as their rates of colonization and infection. Similarly, age of animals must be considered when designing and comparing studies, to estimate the most relevant animal ages in terms of animal and human implications, on which comprehensive studies on MRSA should be addressed.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Carmen Torres, Ph.D.
Departamento de Agricultura y Alimentación
Universidad de La Rioja
Madre de Dios 51
26006 Logroño
Spain

E-mail: carmen.torres@unirioja.es

Annex to paper 5

Table S7. Antimicrobial resistance genes detected and type of positive animal.

Antimicrobial	AMR gene/gene combination	Type of animal	CC
Tetracycline	*tet*(K)+*tet*(M)+***tet*(L)** (4)	Adult (4)	CC398, CC97
	tet(K)+*tet*(M) (23)	Piglet (19), Adult (4)	CC398, CC97
	tet(M)+***tet*(L)** (3)	Adult (3)	CC398, CC97
	tet(K) (10)	Piglet (10)	CC398
	tet(M) (4)	Adult (3), Piglet (1)	CC398
Macrolides/Lincosamides	***erm*(T)** (4)	Adult (4)	CC398
	erm(C)+*vga*(A) (4)	Adult (3), Piglet (1)	CC398
Gentamicin/Tobramycin	***aacA-aphD*** (5)	Adult (5)	CC398
/Kanamycin	***aadD*** (6)	Adult (6)	CC398, CC97
	aphA3 (1)	Adult (1)	CC97
Streptomycin	*str* (7)	Piglet (6), Adult (1)	CC398, CC97
	str+*aadA* (5)	Piglet (5)	CC398
Trimethoprim/sulphametoxazol	***dfrA+dfrG+dfrK*** (2)	Adult (2)	CC398

In grey background those genes/gene combination only detected among MRSA isolates recovered from adult swine. The AMR genes only detected in MRSA from adult swine are highlighted.

Paper 6

First detection of methicillin-resistant *Staphylococcus aureus* ST398 and *Staphylococcus pseudintermedius* ST68 from hospitalized equines in Spain

Elena Gómez-Sanz[1], Carmen Simón[2], Carmelo Ortega[2], Paula Gómez[1], Carmen Lozano[1], Myriam Zarazaga[1], Carmen Torres[1]

[1]Area Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain; [2]Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain.

Impact

- The first description of methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 in horses in Spain and one of the possibly earliest MRSA ST398 isolates in this country is reported. First detection on *Staphylococcus pseudintermedius* ST68 in horses.
- The identification of *dfrK* gene within the chromosomally located transposon Tn*559* in MRSA ST398 from horses is a novel observation.
- A *mec*A-positive *S. pseudintermedius* ST68 isolate showed a susceptible phenotype to oxacillin. This finding enhances the need for *mec*A gene testing in routine analysis to avoid treatment failures and for a better evaluation on antimicrobial resistance prevalence.

Abstract

Eight coagulase positive staphylococci from equines with different pathologies obtained between 2005 and 2011, were investigated. Isolates were characterized by different molecular techniques (*spa*-, *agr*-, MLST typing) and clonal relatedness of strains was investigated by ApaI and SmaI PFGE. Antimicrobial resistance and virulence profiles were determined. Six isolates were identified as *Staphylococcus aureus*, and two as *Staphylococcus pseudintermedius*. Of these, four isolates were methicillin-resistant *S. aureus* (MRSA) ST398 and one *S. pseudintermedius* was *mec*A-positive and typed as ST68. One MRSA ST398 strain was isolated in 2005 and might be one of the earliest MRSA ST398 descriptions in Spain. All 5 *mec*A-positive strains were multidrug-resistant and were isolated from hospitalized equines. Three MRSA ST398 strains carried the recently described Tn*559*. The *mec*A-positive *S. pseudintermedius* ST68 strain was also multidrug-resistant and harbored the *erm*(B)-Tn*5405*-like element. This ST68 strain presented a clear susceptible phenotype to oxacillin and cefoxitin regardless of the presence of the *mec*A gene, what enhances the need for testing the presence of this gene in routine analysis to avoid treatment failures. These data reflect the extended antimicrobial resistance gene acquisition capacities of both bacterial species and evidence their pathogenic properties. The first detection of MRSA ST398 and *S. pseudintermedius* ST68 in horses in Spain is reported.

Key words: MRSA ST398, *mec*A-positive *S. pseudintermedius* ST68, Tn*559*, equidaes, Spain

Introduction

Staphylococcus aureus and *Staphylococcus pseudintermedius* are coagulase positive staphylococci (CoPS) isolated from different animal species and commonly implicated in opportunistic infections (Ruscher et al., 2009; Couto et al., 2011; Sieber et al., 2011; van Duijkeren et al., 2011a; Pantosti, 2012; Gómez-Sanz et al., 2013). Methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) are emerging pathogens of different animal species, including humans (Ruscher et al., 2009; Kadlec et al., 2010a; Perreten et al., 2010; Couto et al., 2011; Lozano et al., 2011a; Sieber et al., 2011; van Duijkeren et al., 2011a; Couto et al., 2012; Pantosti, 2012). Pigs are the major host for MRSA of the Multilocus

Sequence Type (MLST) ST398 with a worldwide distribution; and cases of human infection due to MRSA of this genetic lineage are steadily increasing (Gómez-Sanz et al., 2010; Lozano et al., 2011a; Lozano et al., 2011b; Pantosti, 2012). MRSP is mainly isolated from dogs, with MRSP ST71 and ST68 as the predominant lineages detected in Europe and North America, respectively (Ruscher et al., 2009; Perreten et al., 2010; Gómez-Sanz et al., 2011; van Duijkeren et al., 2011a). In recent years, there has been an increase in the occurrence of MRSA ST398 in hospitalized horses in different countries, especially in Europe (van de Eede et al., 2009; Haenni et al., 2010; Sieber et al., 2011; van Duijkeren et al. 2011a; Couto et al., 2012), and the first suspected horse-to-human transmission of MRSA ST398 causing a human infection has been recently described (van Duijkeren et al., 2011b). In contrast, *S. pseudintermedius* and MRSP have been only detected in equidaes at rare occasions (Ruscher et al., 2009; De Martino et al., 2010; Haenni et al., 2010), and even though cases of human infection have been detected, narrow host adaptability is expected for this bacterial species (van Duijkeren et al., 2011a).

Given that molecular characteristics of *S. aureus* and *S. pseudintermedius* in equidaes is poorly understood, and due to the lack of information on this subject in Spain, the objective of this study was to identify and characterize the CoPS isolated from clinical samples of hospitalized equines in Spain.

Materials and methods

Origin of isolates and characteristics of animals

Between 2005 and 2011, 39 samples from hospitalized equines showing different pathologies were received and analyzed in the Infectious Diseases Laboratory of the Veterinary Faculty of the University of Zaragoza (Spain). Eight potential CoPS (from 7 horses and one donkey) were recovered from these samples (Table 1). Most CoPS positive samples came from infections related to the skin or mucosa. Six of the 8 animals (equines 1, 3, 4, 6-8) were under antimicrobial treatment previous to sampling: five of them received penicillin plus gentamicin and the other one trimethoprim-sulfametoxazole (horse 7).

Bacterial identification

Identification of isolates was performed by conventional methods (Gram-staining, catalase and oxidase test, and DNase production). Identification of *S. aureus* and *S. pseudintermedius* was confirmed by a multiplex PCR that amplifies the *nuc* gene of *S. aureus* or *S. (pseud)intermedius* (Baron et al., 2004). Discrimination between *S. intermedius* and *S. pseudintermedius* was performed by restriction fragment length polymorphism of the *pta* gene with MboI enzyme (Bannoehr et al., 2009). Presence of the *mec*A gene was investigated by PCR in all isolates (CLR-AR, 2009).

Molecular typing of isolates

All *S. aureus* isolates were subjected to *spa* typing as previously described (http://www.spaserver.ridom.de), and sequences were analyzed using Ridom Staph-Type software version 2.0.21 (Ridom GmbH). The *agr* types were investigated by specific PCRs (Shopsin et al., 2003)). Multi-Locus-Sequence-Typing (MLST) on *S. aureus* was performed as recommended (www.mlst.net). SCC*mec* types I to V were investigated in *S. aureus* isolates by PCR of the *ccr* recombinases (1 to 5) and the *mec* gene complex type (A to C) as recommended by IWG-SCC (IWG-SCC, 2009; Kondo et al., 2007). An additional PCR was performed to differentiate different subtypes of SCC*mec* IV (a to d) (Zhang et al., 2005).

S. pseudintermedius isolates were investigated by species specific *spa* typing (Moodley et al., 2009). MLST on this bacterial species was performed on five housekeeping genes (*pta*, *cpn60*, *tuf*, 16S rDNA and *agrD*) followed by sequence comparison of the alleles with those deposited in GenBank/EMBL databases (Bannoehr et al., 2007). *S. pseudintermedius* isolates were tested for the presence of the SCC*mec* types I to V, in addition to the so far *S. pseudintermedius* specific SCC*mec* V_T and II-III (Perreten et al., 2010).

Antimicrobial resistance profile

Susceptibility testing and interpretation was done as recommended by Clinical and Laboratory Standards Institute (CLSI, 2012), except for streptomycin and fusidic acid, for which methods and breakpoints recommended by Société Française de Microbiologie were used (SFM, 2010). Antimicrobials tested were as follows (µg/disk): penicillin (10U), oxacillin (1), cefoxitin (30) erythromycin (15), clindamycin (2), gentamicin (10), kanamycin (30), streptomycin (10U), tobramycin (10), tetracycline (30), trimethoprim-sulfamethoxazole (1.25 + 23.75), trimethoprim (5), chloramphenicol (30), ciprofloxacin (5), mupirocin (200), fusidic acid (10), vancomycin (30), and linezolid (30) (CLSI, 2012).

The double-disk diffusion test (D-test) was performed to detect inducible clindamycin resistance (CLSI, 2012). Minimum Inhibitory Concentration (MIC) to oxacillin and cefoxitin was determined by agar dilution method on three independent assays using *S. aureus* ATCC29213 and *Enterococcus faecalis* ATCC29212 as reference strains and quality controls (CLSI, 2012). Presence of the following antimicrobial resistance genes was analyzed by PCR in all isolates: *mec*A, *blaZ*, *tet*(K), *tet*(M), *tet*(L), *erm*(A), *erm*(B), *erm*(C), *erm*(T), *aacA-aphD*, *aphA-3*, *aadD*, *aadE*, *aadA*, *str*, *sat4*, *dfr*(A), *dfr*(D), *dfr*(G) an *dfrK* (Gómez-Sanz et al., 2010; Gómez-Sanz et al., 2011).

The presence of the *erm*(B)-Tn*5405*-like element, which carries the macrolides-lincosamides-streptogramines B resistance gene *erm*(B), streptomycin and kanamycin/neomycin resistance determinants (*aadE* and *aphA3*, respectively), in addition to the streptothricin resistance gene *sat4*, was investigated by mapping PCRs (Gómez-Sanz et al., 2011). Mutations in the quinolone resistance determining region (QRDR) of GyrA and GrlA proteins, in addition to relevant mutations outside QRDR of GyrA, were investigated in the ciprofloxacin resistant isolate (Gómez-Sanz et al., 2011).

Determination of mecA *and* blaZ *regulatory elements*

Presence of the regulatory genes of *mec*A [*mec*I (*mec*A transcription repressor) and *mec*R1 (sensor/signal transducer)] and *bla*Z (*bla*I/*bla*R1) operons were investigated by PCR in all isolates (Black et al., 2011; Gómez-Sanz et al., 2011).

In addition, primers mecA(SP)-ups (5'-TGGAATTAACGTGGAGACGA-3') and mecA(SP)-rv (5'-TTATTCATCTATATCGTATT-3') (GenBank accession no. AM904732) were used to amplify the entire *mec*A gene in strain C5337. The *mec*A promoter region of this strain was also characterized by PCR and sequencing (Gómez-Sanz et al., 2011).

Location and genetic environment of the dfrK *gene in* S. aureus *isolates*

PFGE after linearization of plasmid DNA with S1 (Takara) and digestion of genomic DNA with ICeuI (New England Biolabs) were performed as previously described on *dfrK* positive isolates (López et al., 2012). S1-PFGE and I-CeuI-PFGE gels obtained were subjected to hybridization and detection with specific probes of the *dfrK* to determine the chromosomal DNA and/or plasmid location of this gene in the genome (López et al., 2012). The possibility that the *dfrK* gene formed part of the recently described Tn*559*, which carries the transposase genes *tnpA*, *tnpB* and *tnpC*, in addition to the *dfrK* gene, was investigated by specific PCRs of the transposase genes and *dfrK* in different combinations (Fig. 1, Table 2). Given that this transposon has been so far located integrated in the chromosomal *radC* gene (Kadlec and Schwarz 2010b; Fessler et al., 2011; Lopez et al., 2012), a specific PCR was employed to evaluate the possible integration of Tn*559* within *radC* (Table 2), with two possible amplicon sizes achieved: i) 114 bp when *radC* is intact and ii) 4,403 bp if Tn*559* is located within the *radC* gene. An additional PCR using dfrK-fw and radC_SA-rv was employed for confirmation of the integration of the Tn*559* within the *radC*.

Virulence genes profile

The presence of the leukocidin genes *lukS/F-PV*, *lukM*, and *lukE/D*, the *hla*, *hlb*, *hld*, *hlg*, and *hlgv* haemolysin genes and the exfoliative genes *eta*, *etb*, and *etd* was determined in all isolates by specific PCRs (Lina et al., 1999; Jarraud et al., 2002). The toxic shock syndrome toxin *tst* and the collagen adhesion precursor *cna* genes were likewise investigated (Witte et al., 2007; Jarraud et al., 2002). PCR-based

determination of 18 enterotoxin genes was performed on all isolates (Hwang et al., 2002).

In addition, the leukocidin *lukS/F-I*, the exfoliatin genes *siet, expA* and *expB,* and the enterotoxin genes *se-int* and *sec$_{canine}$* were investigated by PCR in *S. pseudintermedius* isolates (Gómez-Sanz et al., 2013).

Clonal relatedness of strains

Pulsed-Field-Gel-electrophoresis (PFGE) after digestion of genomic DNA with SmaI and ApaI macrorestriction enzymes was performed on *S. aureus* and *S. pseudintermedius* isolates as previously recommended (Gómez-Sanz et al., 2010). PFGE of ApaI or SmaI digested plugs was run for 20 h at 6V/cm using pulsed time ramping from 2 to 5s.

Results

Identification of isolates and molecular typing

Six of the 8 isolates were identified as *S. aureus* (Table 1). Four *S. aureus* were MRSA and typed t011(*spa*)-I(*agr*)-ST398(MLST)-SCC*mec*IVa. The remaining two *S. aureus* were MSSA; one showed a novel *spa* type registered as t10576 (r04-r20-r69-r31-r70-r13-r16-r16-r16), and presented ST1660. The other MSSA showed t1508 and exhibited ST1.

The remaining two isolates were *S. pseudintermedius* One isolate was *mec*A positive and typed as t06-IV-ST68-SCC*mec*V$_T$ (5C2&5). The other isolate, recovered from a donkey, was a MSSP, *spa* non-typeable and presented the ST184.

Characteristics of β-lactam resistance and presence of regulatory elements

In addition to MRSA isolates, both MSSA were penicillin resistant (harbouring the *blaZ* gene) and all amplified the *bla*I/*bla*R regulatory elements. The four MRSA ST398 isolates showed oxacillin MIC values of 16-32 μg/mL, while cefoxitin MICs were 16 μg/mL. Both MSSA isolates exhibited oxacillin and cefoxitin MICs values of 0.25-0.5 μg/mL or 2 μg/mL, respectively. The four MRSA ST398 isolates lacked the transcription repressor *mec*I gene and showed a truncated *mecR*1 gene, as expected for the presence of the SCC*mec* IVa.

Both *S. pseudintermedius* isolates were penicillin resistant harboring the *blaZ* gene and both regulatory elements (*bla*I/*bla*R). Disk-diffusion method showed that both strains were susceptible to oxacillin (inhibition zone: 21-22 mm) and cefoxitin (35-40 mm), even though one of them was *mec*A-positive (C5337). Macrodilution assays confirmed that both isolates were oxacillin (MIC=0.25 μg/mL) and cefoxitin (MIC=0.25-0.5 μg/mL) susceptible. The *mec*A-positive *S. pseudintemedius* strain C5337 also lacked the *mec*I gene and showed a truncated *mec*R, as expected for the presence of the SCC*mec*V$_T$ element.

Sequencing of the complete *mec*A gene of *S. pseudintermedius* strain C5337 and its promoter region revealed no mutations that might explain such atypical phenotype. Sequence comparison analysis of the complete *mec*A of C5337 with deposited sequences on the GenBank database evidenced complete identity to *mec*A found within the SCC*mec*V$_T$ of a MRSP ST68 strain (GenBank accession no. FJ544922) among others.

Antimicrobial resistance profile to non β-lactams

Complete antimicrobial resistance pheno- and genotype of strains is shown in Table 1. The four MRSA isolates were multidrug resistant (MDR) (resistance to at least three classes of antimicrobial agents). They presented the tetracycline resistance genes *tet*(K) and *tet*(M) and the aminoglycosides resistance gene *aacA-aphD*, while three of them were also trimethoprim resistant [*dfrK* and/or *dfr*(G)] and one showed additional resistance to streptomycin (*str*) (Table 1).

As for *S. pseudintermedius*, the multidrug resistance-gene-cluster *aadE-sat4-aphA3* and its physical linkage to *erm*(B) gene [*erm*(B)-Tn*5405*-like element] was evidenced in the *mec*A-positive ST68 strain. This isolate showed resistance to tetracyclines, aminoglycosides, macrolides-lincosamides and trimethoprim (Table 1). The MSSP isolate was tetracycline resistant and presented both *tet*(K) and *tet*(M) genes.

Genetic environment of the dfrK *gene*

The three MRSA ST398 strains that carried the *dfrK* gene harbored a complete and conserved transposon Tn*559* (Fig. 1). I-CeuI-PFGE hybridization experiments revealed that

the *dfrK* gene was only located within the chromosomal DNA. In addition, based on the PCR amplicon obtained, Tn*559* was located within the chromosomal *radC* gene. Circular forms were also detected indicating a functional activity.

Toxin gene pattern

All MRSA ST398 strains were only positive for the *cna* gene whereas both MSSA strains showed a more extensive virulence profile with haemolysins, and enterotoxins; the leukocidin gene *lukED* was detected in MSSA ST1 (Table 1).

The leukocidin *lukS/F-I*, exfoliatin *siet*, and enterotoxin *se-int* genes were present in both *S. pseudintermedius* strains.

Clonal relatedness of strains investigated

SmaI-PFGE showed a slightly higher discriminative power for the differentiation of both MSSA isolates with the implemented PFGE conditions (Fig. 2). As expected, MRSA ST398 strains were not digested by the SmaI enzyme. The four MRSA ST398 isolates presented closely related patterns by ApaI-PFGE regardless they were isolated in different years from unrelated horses (Fig. 2).

Both enzymes were useful to discriminate both *S. pseudintermedius* isolates, ApaI exhibiting more restriction sites.

Discussion

Even though MRSA ST398 has become the predominant MRSA lineage in diseased horses in Europe in the last years (Sieber et al., 2011), this is the first report on MRSA in equidaes in Spain. The first description of MRSA ST398 in Spain was reported in food samples of pig origin in 2008 (Lozano et al., 2009), and several reports have been later described on swine and people in contact with these animals (Gómez-Sanz et al., 2010; Lozano et al., 2011a; Lozano et al., 2011b). Our strain recovered in 2005 represents therefore the earliest case of MRSA ST398 in Spain to date and evidences its early capability to be present in non-porcine animal species. The horse carrying this strain came from a horse farm located very close to animal husbandries and to a slaughterhouse. Unfortunately, potential risk factors for MRSA ST398 acquisition and infection could not be established in the rest of positive animals, given that these data were not collected at sampling. One of the MSSA belonged to ST1660, a so far uncommon lineage that was described for the first time in a borderline-oxacillin-resistant *S. aureus* strain from a diseased horse in Switzerland (Sieber et al., 2011). In contrast, isolates of the lineage ST1, which have been frequently detected before, appear highly adapted to different animal species (Pantosti, 2012).

S. pseudintermedius of the sequence type ST68 is the predominant MRSP lineage in North America in dogs. In Europe, MRSP of this lineage have only been detected in Portuguese dogs (Couto et al., 2011). To our knowledge, this is the first description of *S. pseudintermedius* ST68 in horses, and suggests a less restricted geographic and animal host spectrum than expected. Background of the patient could not be determined and therefore, role of possible dog contact cannot be ruled out. Since very scarce data is so far available on the nasal or skin microbiota of donkey (Ruscher et al., 2009), the presence of *S. pseudintermedius* in one animal is remarkable. Curiously, ST184 was recently described in a MSSP of dog origin in Spain (Gómez-Sanz et al., 2012).

Although the epidemiological significance of the present data is very low due to the limited number of samples, the proportion of *mec*A-positive isolates (5/8, 62.5%) is notable. Interestingly, *mec*A-positive *S. pseudintermedius* ST68 strain C5337 was susceptible to both oxacillin and cefoxitin by both disk-diffusion and agar-dilution methods. Several reports have indicated low reliability of cefoxitin disk-diffusion tests to determine the methicillin-resistance phenotype of *S. pseudintermedius* using conventional guideline breakpoints [≤21mm resistant, ≥22 susceptible (CLSI, 2012)] (Bemis et al., 2012). Also, this study has suggested an epidemiological breakpoint for cefoxitin zone diameter of ≤30 mm for resistant and ≥31 for susceptible, after analyzing a large *S. pseudintermedius* collection (Bemis et al., 2012). Remarkably, strain C5337 remained not only susceptible to cefoxitin following the latter criteria but also to oxacillin, which is still considered a reliable

marker of methicillin-resistance in this species. The fact that neither the *mec*A gene nor its promoter region presented mutations that could explain this atypical phenotype is surprising. The detection of the entire *bla*I/*bla*R1 regulatory may be speculated to be implicated in this stringent regulation of *mec*A expression. Moreover, other factors (different mutations on surface proteins, lower permeability, etc) or additional regulatory elements might affect the *mec*A expression levels in C5337, what warrants further in-depth investigations (McCallum et al., 2010).

It is interesting to remark that all strains resistant to penicillin, gentamicin, or trimethoprim-sulfametoxazole were recovered from animals that had received previous treatment with those antimicrobials. The complete *erm*(B)-Tn*5405*-like resistance gene cluster or variants seem to be ubiquitous on MRSP strains (Perreten et al., 2010; Gómez-Sanz et al., 2011; van Duijkeren et al., 2011); this enhances its MDR pattern and increases concerns when addressing the possible risk of zoonotic transmission.

The recently described *dfrK*-carrying transposon Tn*559* has been rarely detected so far in a porcine isolate and some poultry/poultry products in staphylococci, all of the lineage ST398 (Fessler et al., 2011; Kadlec et al., 2012). Hence, the detection of the Tn*559* in MRSA ST398 from horses is a novel observation, and reflects the antimicrobial resistance gene acquisition capacity of MRSA ST398 isolates of different origins. Primary target site for integration of Tn*559* is designated *att559*, and is located within the chromosomal *radC* gene (Kadlec et al., 2010). This evidences a preferable chromosomal location within the bacterial genome. Circular forms were also detected indicating a functional activity and therefore the possibility of exchange between bacteria.

The low occurrence of virulence genes in *S. aureus* isolates that belong to ST398 is in agreement with previous reports (Gómez-Sanz et al., 2010; Lozano et al., 2011a; Lozano et al., 2011b). By contrast, although very scarce data is available on the occurrence of virulence genes in *S. pseudintermedius* isolates, a few reports have shown ubiquity of the same set of virulence determinants; the leukocidin *lukS/F-I*, the exfoliatin *siet* and enterotoxin *se-int* genes among both, MSSP and MRSP of different lineages (Gómez-Sanz et al., 2011; van Duijkeren et al., 2011a; Ben Zakour et al., 2012; Gómez-Sanz et al., 2013).

Our MRSA ST398 strains showed closely related PFGE-patterns, in spite that they were isolated in three different years from unrelated horses. These results suggest clonal spread of MRSA ST398 in these animals and ensure further epidemiological investigations. Low discriminatory power of ApaI enzyme might be also speculated. Although contamination at the clinic should be negligible, this option cannot be discarded.

The first description of MRSA ST398 in horses in Spain and one of the possibly earliest MRSA ST398 isolates in this country is reported. This is the first report on *S. pseudintermedius* ST68 in horses and the first detection of this American associated lineage in Europe in a non-canine animal species. The detection of the Tn*559* in MRSA ST398 from horses is a novel observation, and reflects its high antimicrobial resistance gene acquisition ability. The finding of a *mec*A-positive *S. pseudintermedius* strain that shows susceptibility to oxacillin and cefoxitin enhances the need for testing the presence of the *mec*A gene in routine analysis to avoid treatment failures. Given that all *mec*A-positive strains were MDR, treatment options are compromised. Due to the close contact between horses and owners, potential public health implications on humans in contact to these animals are conceivable.

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Table 1. Characteristics of the *S. aureus* and *S. pseudintermedius* isolates obtained of animals tested

Characteristics of the isolates							Characteristics of the animals			
Strain	year	Type[a]	*spa-agr*-MLST-SCC*mec*	Resistance phenotype[b]	Resistance genes detected	Virulence genes detected	Animal ID	Age (years)	Breed	Clinical disease
C3865	2005	MRSA	t011-I-ST398-IVa	Pen-Oxa-Fox-Tet-Gen-Tob	*blaZ, mec*A, *tet*(K), *tet*(M), *aacA-aphD*	*cna, hla, hlb, hld, hlg*	Horse-1	5	Spanish Purebred	Mumps
C4667	2010	MRSA	t011-I-ST398-IVa	Pen-Oxa-Fox-Tet-Gen-Tob-Str-Tmp	*blaZ, mec*A, *tet*(K), *tet*(M), *aacA-aphD, str, dfr*(G), *dfrK*[c]	*cna, hla, hlb, hld, hlg*	Horse-2	4	Lusitano	Hardening of salivary gland
C4668	2011	MRSA	t011-I-ST398-IVa	Pen-Oxa-Fox-Tet-Gen-Tob-Tmp	*blaZ, mec*A, *tet*(K), *tet*(M), *a acA-aphD, dfr*(G), *dfrK*[c]	*cna, hla, hlb, hld, hlg*	Horse-3	9	KWPN[d]	Post-surgical injury (colic)
C4670	2011	MRSA	t011-I-ST398-IVa	Pen-Oxa-Fox-Tet-Gen-Tob-Tmp	*blaZ, mec*A, *tet*(K), *tet*(M), *aacA-aphD, dfrK*[c]	*cna, hla, hlb, hld, hlg*	Horse-4	12.5	Spanish Purebred	Post-surgical injury (colic)
C5343	2009	MSSA	t10576-II-ST1660	Pen	*blaZ*	*hla, hlb, hld, hlgv, cna, sem, sei*	Horse-5	8	Spanish Purebred	Arthritis
C5611	2010	MSSA	t1508-III-ST1	Pen	*blaZ*	*lukED, hlb, hld, hlgv, cna, seh*	Horse-6	7	Mixed Shire bred	Abscess axilla
C5337	2008	*mec*A-+ SP	t06-IV-ST68-V_T	Pen-Tet-Ery-Cli[e]-Kan-Str-Sxt-Cip[f]	*blaZ, mec*A[g], *tet*(K), *tet*(M), *aacA-aphD,* [*erm*(B)-*aadE-sat4-aphA3*][h], *dfr*(G)	*lukS/F-I*, *siet*, *se-int*	Horse-7	2.5	Spanish Purebred	Suppurative bone sequestration
C5348	2009	MSSP	NT[i]-II-ST184	Pen-Tet	*blaZ, tet*(K), *tet*(M)	*lukS/F-I*, *siet*, *se-int*	Donkey-1	6	Spanish Donkey	Emphysema of skin and injure

[a] MRSA, methicillin-resistant *Staphylococcus aureus*; *mec*A+ SP, *mec*A-positive *S. pseudintermedius*; MSSA, methicillin-susceptible *S. aureus*; MSSP, methicillin-susceptible *S. pseudintermedius*. [b] Pen, penicillin; Oxa, oxacillin; Fox, cefoxitin; Tet, tetracycline; Ery, erythromycin; Cli, clindamycin; Gen, gentamicin; Tob, tobramycin; Kan, kanamycin; Str, streptomycin; Sxt, trimethoprim/sulfamethoxazole; Cip, ciprofloxacin; Tmp, trimethoprim. [c] The *dfrK* gene was part of the transposon Tn*559*, which was inserted in the chromosomal *radC* gene. [d] Koninklijk Warmbloed Paardenstamboek Nederland. [e] The double-disk diffusion test (D-test) revealed a constitutive resistance phenotype to clindamycin. [f] Amino acid substitutions in Ser80Ile of GrlA protein and Ser84Leu in GyrA were detected. [g] The gene *mec*A is present but there is a susceptible phenotype to oxacillin and cefoxitin [h] Resistance genes physically linked. [i] Non typeable.

Table 2. Primers employed in this study to investigate the presence of the conserved *dfrK*-carrying transposon Tn*559* and PCR conditions used.

Gene or region amplified	Primer	Primer sequence (5'→3')	Amplicon (bp)	Reference (GenBank/EMBL accession no.)
dfrK	dfrK-fw	GAGAATCCCAGAGGATTGGG	423	Gómez-Sanz et al., 2010
	dfrK-rv	CAAGAAGCTTTTCGCTCATAAA		
tnpA	tnpA-fw	GCCACTTGGGACAATCAAAT	328	This study (FN677369)
	tnpA-rv	CAACATCCCATCCTTCCCTA		
tnpB	tnpB-fw	AAATCGTTGGGTTCGTTTTG	826	This study (FN677369)
	tnpB-rv	AAAGCGAGGTTTTGCTCTTG		
tnpC	tnpC-fw	GGCGAAGCAGAAATCACAA	235	This study (FN677369)
	tnpC-rv	AAGGATTTCCTCCGAACGAG		
radC	radC_SA-fw	CGGTGAGAGAAAATGCCAAT	114/4,403[a]	This study (FN677369)
	radC_SA-rv	TCAAACCACACTCCTTCAACC		
Tn*559*-circ	Tn559_circ-fw	TCCATGAACTCGTACAGCAA	778	Kadlec and Schwarz, 2010
	Tn559_circ-rv	TGGTTGTGAAATTGTCCATTC		

The PCR reactions were performed using BioTaq™ DNA Polymerase (Bioline) and the conditions were as follows: initial cycle of 3 min at 95°C followed by 30 cycles of 1 min at 95ºC, 1 min at 55°C, 1 min at 72°C, with a final step of 10 min at 72°C. An additional PCR reaction was performed for *radC* gene using LA Taq DNA polymerase (Takara) and following manufacturer recommendations.

[a] Amplicon size only obtained when Tn*559* is inserted within *radC* gene.

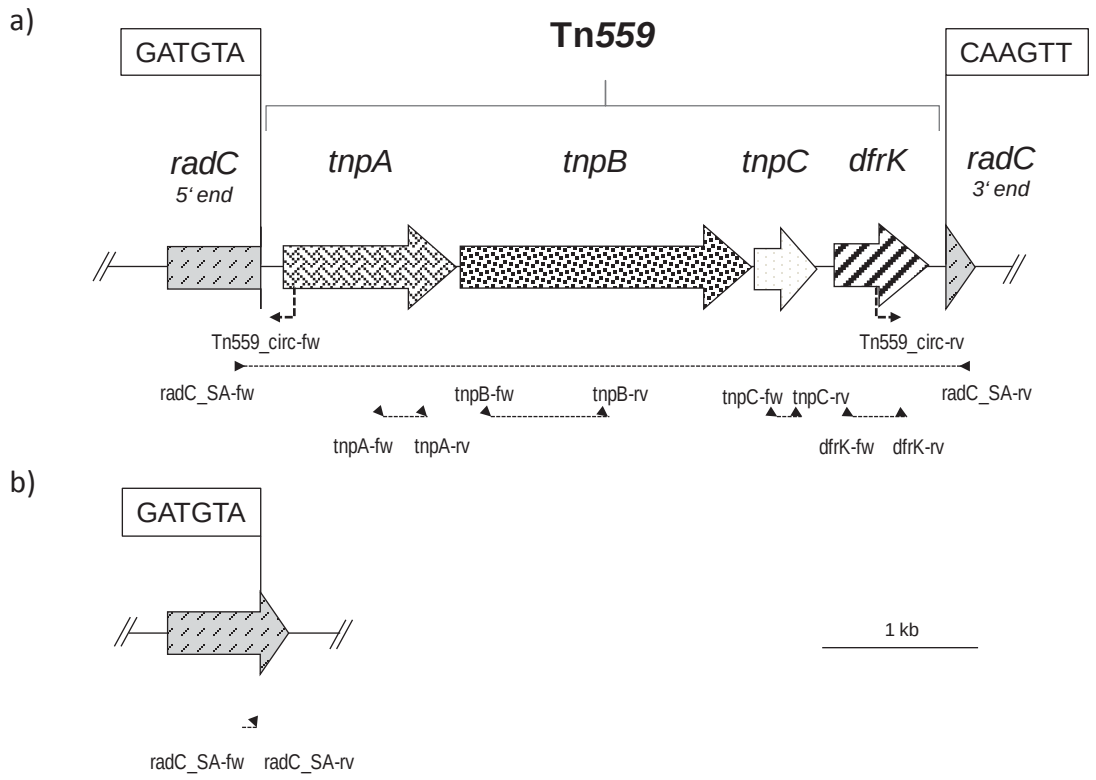

Figure 1. Schematic representation of Tn*559* and its chromosomal integration region within the chromosome of *S. aureus*. The arrows indicate the size and direction of transcription of the transposase (*tnpA*, *tnpB*, *tnpC*) and trimethoprim resistance (*dfrK*) genes as well as the gene coding a DNA repair protein (*radC*) that serves as integration site. Recognition site (5'-GATGTA-3') is boxed. A size in kb scale is given. a) Transposon Tn*559* integrated within the chromosomal *radC* of *S. aureus* and primers employed for its detection and potential functionality. b) Chromosomal segment of *S. aureus* carrying an integral copy of the *radC* gene.

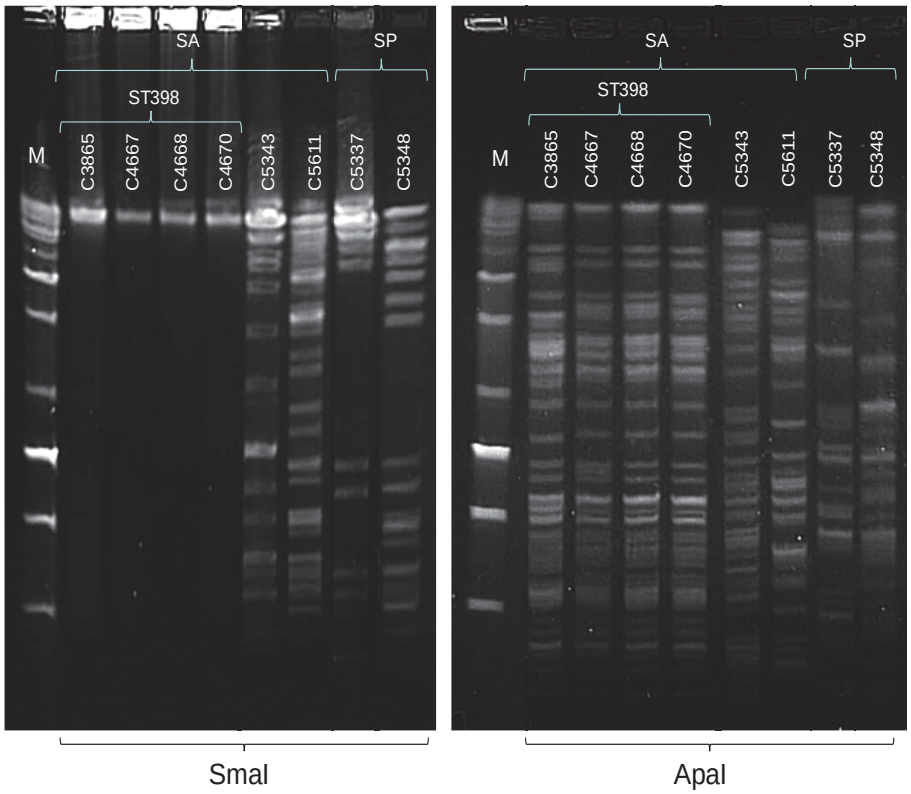

Figure 2. Pulsed-Field Gel Electrophoresis (PFGE) pattern of chromosomal DNA digested with SmaI and ApaI macrorestriction enzyme of all *S. aureus* (SA) and *S. pseudintermedius* (SP) strains investigated. Strains C3865, C4667, C4668 and C4670 (methicillin-resistant SA ST398); C5343 (methicillin-susceptible SA ST1660); C5611 (methicillin-susceptible SA ST1); C5337 (*mec*A-positive SP ST68); C5348 (methicillin-susceptible SP ST184). M, Low Range Pulse Marker (New England Biolabs).

Annex to paper 6

Table S8. **Period investigated, animals sampled, clinical condition of animals and CoPS isolates obtained.**

Year	Date or period of receiving samples (months)	No. of animals sampled	Clinical disease of animals	No. of CoPS
2005	4 (August-November)	6	2 intestinal infection 1 catheter insertion point 1 otic infection 1 vaginitis control **1 mumps**[a]	1
2006	10 (January-October)	7	2 arthritis 1 hip abscess 1 abscess retropharyngeal 1 vaginitis 1 colitis 1 lameness	-
2007	10 (January-October)	4	**1 postoperative wound infection** 1 abscess hindquarters 1 epistaxis 1 endometritis	(1)[b]
2008	11 (January-November)	10	3 metritis 1 umbilical cord 1 abortion **1 progressive thinning** 1 lameness 1 tracheotomy incision infection 1 bone cancer **1 Suppurative bone sequestration**	1 (1)[b]
2009	6 (January-June)	5	1 pneumonia 1 oral infection 1 respiratory infection **1 arthritis** **1 skin emphysema**	2
2010	9 (February-October)	4	1 respiratory infection 1 skin wound 1 placentitis **1 skin abscess** **1 postoperative wound infection**	2
2011	7 April; 25 May[c]	-[d]	**2 postoperative wound infection**	2

[a] In bold letters infection from which CoPS isolate was recovered.

[b] Within brackets the number of isolate/s that were recovered but not kept for further investigations.

[c] Not all samples swabbed were received at the Veterinary Laboratory in this period. Only two samples, both positive for MRSA ST398, were obtained.

[d] No information on the number of animals sampled.

Table S9. Phenotype of resistance to oxacillin and cefoxitin by both methodologies employed of the six *S. aureus* and both *S. pseudintermedius* investigated; responsible genetic determinants and its association to the genetic background of strains.

Strain	Bacterial species	*spa-agr*-MLST	Phenotype of resistance[a]				Genetic determinants and regulatory elements		
			Disk diffusion (mm)		MIC (µg/mL)		*mec*A	SCC*mec* (*mec*I/*mec*R1)	*bla*I[b]/*bla*R1
			Oxacillin	Cefoxitin	Oxacillin	Cefoxitin			
C3865	*S. aureus*	t011-I-ST398	10	16	16	16	+	IVa (-/Δ[c])	+[d]**/+**
C4667	*S. aureus*	t011-I-ST398	12	16	16	16	+	IVa (-/Δ)	+[d]**/+**
C4668	*S. aureus*	t011-I-ST398	6	15	32	16	+	IVa (-/Δ)	+[d]**/+**
C4670	*S. aureus*	t011-I-ST398	6	15	16	16	+	IVa (-/Δ)	+[d]**/+**
C5343	*S. aureus*	t10576-II-ST1660	19	30	0.25	2	-	- (-/-)	+[d]**/+**
C5611	*S. aureus*	t1508-III-ST1	19	29	0.5	2	-	- (-/-)	+/+
C5337	***S. pseudintermedius***	**t06-IV-ST68**	**22**	**35**	**0.25**	**0.5**	**+**	**V_T** (-/Δ)	**+/+**
C5348	*S. pseudintermedius*	NT[e]-II-ST184	21	40	0.25	0.25	-	- (-/-)	+/+

[a] Mean value obtained from three independent assays.

[b] *bla*I1 and *bla*I2 set of primers were employed (Black et al., 2011).

[c] truncated *mec*R1.

[d] Only *bla*I2 (internal region of *bla*I) pair of primers yielded positive results.

[e] Non-typeable.

Chapter 4

Characterization of novel genetic structures that carry antimicrobial resistance genes

CHAPTER 4. CHARACTERIZATION OF NOVEL GENETIC STRUCTURES THAT CARRY ANTIMICROBIAL RESISTANCE GENES.

MLS_B resistance in LA-MRSA ST398 is common (Kadlec et al., 2012). Recently, the MLS_B resistance gene *erm*(T) has been described for the first time in staphylococci in LA-MRSA ST398, with a plasmid location (Kadlec et al., 2010a). Further, its presence has been very recently reported on MSSA ST398 isolates of human origin without contact to livestock, with a chromosomal location (Uhlemann et al., 2012; Vanderdriese et al., 2011). The aim of this chapter was to investigate the *erm*(T) location in the genome of several *erm*(T)-positive LA-MRSA ST398 and MSSA ST398 strains and to characterized their genetic environments.

Three LA-MRSA ST398 of pig origin (**chapter 3**), two LA-MRSA ST398 from diseased humans (Lozano et al., 2012c), and one MSSA ST398 from healthy human (**chapter 1**) were subjected to plasmid extraction followed by transformation into *S. aureus* RN4220. Plasmids of interest were digested by EcoRI or BglII, cloned into a plasmid vector and transformed into *Escherichia coli* JM101. Plasmids were sequenced by primer walking using universal primers. Plasmid or chromosomal location of plasmids was investigated by hybridization experiments.

A novel 6,176-bp plasmid, named pUR3912, was identified in a MSSA ST398-t571 strain C3912 of human origin. This plasmid carried, in addition to the *erm*(T) gene, which was flanked by two identical copies of IS*431* in the same direction, a functionally active *cadDX* operon for cadmium resistance. This plasmid replicates via a RCR mechanism and the *ssoA* and *dso* regions were detected. pUR3912 represents the first *erm*(T) carrying plasmid that also harbours cadmium resistance determinants. Interestingly, pUR3912 was revealed to be co-located within the chromosomal DNA of the same strain C3912, with the *erm*(T)-*cadDX* region being flanked by both IS elements in direct orientation. This cointegrate was located within a transposase gene and 8 bp direct repeats at its integration site were observed. An integration model for pUR3912 was proposed in which both IS elements mediated such chromosomal integration.

Three novel MDR *erm*(T)-carrying plasmids, designated pUR1902 (porcine), pUR2940 and pUR2941 (human), were obtained from MRSA ST398 strains and characterized. In addition to the MLS_B resistance gene *erm*(T), all three plasmids carried the tetracycline resistance gene *tet*(L). In addition, plasmid pUR2940 harboured the

trimethoprim resistance gene *dfrK* and the MLS_B resistance gene *erm*(C) while plasmids pUR1902 and pUR2941 possessed the kanamycin/neomycin resistance gene *aadD*. Sequence analysis of approximately 18.1 kb of the *erm*(T)-flanking region from pUR1902, 20.0 kb from pUR2940 and 20.8 kb from pUR2941 revealed the presence of several copies of the recently described insertion sequence IS*Sau10*, probably involved in the evolution of the respective plasmids. All plasmids carried the functional cadmium resistance operon *cadDX*, in addition to the copper resistance/homeostasis genes *mco* and *copA.* This is the first description of MDR resistance plasmids carrying both cadmium and copper resistance determinants in MRSA ST398.

The physical linkage of antimicrobial resistance genes and genes that confer resistance to heavy metals may facilitate their persistence, co-selection and dissemination under the selective pressure imposed by any of the involved agents. The emergence of MDR plasmids that also carry heavy metal resistance genes is alarming and requires further surveillance.

The phenicol resistance gene *fexA*, enclosed within transposon Tn*558*, has been detected in *S. aureus* and several coagulase negative staphylococci from healthy and diseased bovine, swine, equine and human samples (Argudín et al., 2011; Fessler et al., 2010; Lozano et al., 2012d; Kadlec et al., 2009a; Kehrenberg et al., 2006; Shore et al., 2010; Wang et al., 2012), but never in *S. pseudintermedius* or in canine isolates. MSSP ST20 strain C2719, recovered from one household dog, was revealed to carry a *fexA* variant (*fexAv*) that only confers resistance to chloramphenicol but not to florfenicol. PCR mapping and sequencing revealed *fexAv* to be part of the transposon Tn*558* and was located within the chromosomal *radC* gene. Cloning experiments and SDM revealed that the combined presence of two nucleotide substitutions that differed from the prototype sequence *fexA* of *S. lentus* (Kehrenberg et al., 2004) were implicated in the absence of resistance to florfenicol.

This is the first detection of the *fexA* gene and transposon Tn*558* in *S. pseudintermedius*. The first report of a *fexA* variant that only confers resistance to chloramphenicol is reported. Continued surveillance on antimicrobial resistance in *S. pseudintermedius* is required.

These studies have resulted in two already published papers, one paper in advanced in stage of revision and another submitted in international peer-reviewed journals.

4.1. PAPER 7.

Gómez-Sanz E, Kadlec K, Fessler AT, Billerbeck C, Zarazaga M, Schwarz S, Torres C. Analysis of a novel *erm*(T)- and *cadDX*-carrying plasmid from methicillin-susceptible *Staphylococcus aureus* ST398-t571 of human origin. J Antimicrob Chemother. 2013;68(2):471-3.

4.2. PAPER 8.

Gómez-Sanz E, Zarazaga M, Kadlec K, Schwarz S, Torres C. Chromosomal integration of the novel plasmid pUR3912 from methicillin-susceptible *Staphylococcus aureus* ST398 of human origin. Clinical Microbiology and Infection (advanced stage of revision).

4.3. PAPER 9.

Gómez-Sanz E, Kadlec K, Feßler AT, Zarazaga M, Torres M, Schwarz S. Novel *erm*(T)-carrying multiresistance plasmids from porcine and human methicillin-resistant *Staphylococcus aureus*ST398 that also harbor cadmium and copper resistance determinants. Antimicrobial Agents and Chemoteraphy (*in press*).

4.4. PAPER 10.

Gómez-Sanz E, Kadlec K, Fessler AT, Zarazaga M, Torres C, Schwarz S. Detection and analysis of transposon Tn*558* carrying a *fexA* variant that only confers resistance to chloramphenicol in canine *Staphylococcus pseudintermedius*. Antimicrobial Agents and Chemoteraphy (submitted).

Paper 7

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Analysis of a novel *erm*(T)- and *cadDX*-carrying plasmid from methicillin-susceptible *Staphylococcus aureus* ST398-t571 of human origin

Elena Gómez-Sanz[1], Kristina Kadlec[2], Andrea T. Feßler[2], Carmen Billerbeck[2], Myriam Zarazaga[1], Stefan Schwarz[2] and Carmen Torres[1]*

[1]*Biochemistry and Molecular Biology, University of La Rioja, Logroño, Spain;* [2]*Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany*

*Corresponding author. Tel: +34-941-299750; Fax: +34-941-299721; E-mail: carmen.torres@unirioja.es

Keywords: antimicrobial resistance, macrolide–lincosamide resistance, horizontal gene transfer, insertion sequences

Sir,

The major mechanism of resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS_B) is the methylation of the adenine at position A2058 in domain V of 23S rRNA. Of the rRNA methylase genes so far detected in staphylococci, *erm*(T) has rarely been identified.[1] It was described for the first time in staphylococci on plasmid pKKS25 in a porcine livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) ST398 isolate.[2] Recent studies also revealed its presence in the chromosomal DNA of methicillin-susceptible *S. aureus* (MSSA) ST398 isolates in humans.[3,4] In this study, we investigated a previously identified *erm*(T)-positive MSSA ST398-t571 isolate from a healthy human to gain insight into the genetic environment of *erm*(T), its possible association with other resistance genes and its plasmid location.

The MSSA ST398-t571 isolate C3912 was obtained in 2011 from the nasal swab of a healthy human.[5] This isolate showed only inducible MLS_B resistance. Plasmids were prepared and transformed into *S. aureus* RN4220 with subsequent selection on medium containing erythromycin (15 mg/L). A single plasmid, designated pUR3912, was identified and shown to confer the aforementioned resistance phenotype. Plasmid pUR3912 was linearized by EcoRI, cloned into the plasmid vector pBlueScript II SK+ (Stratagene) and the recombinant plasmid was transformed into *Escherichia coli* JM101. The complete plasmid pUR3912 was sequenced by primer walking on both strands starting with M13 universal and reverse primers. A schematic map of the 6182 bp plasmid pUR3912 is shown in Figure 1. The nucleotide sequence of plasmid pUR3912 determined in this study has been deposited in the EMBL database under accession number HE805623.

The *erm*(T) gene encoded a 244 amino acid rRNA methylase which was indistinguishable from the recently described chromosomal Erm(T) of MSSA ST398 strain ST398NM01,[3] and shared 98.9% identity (99.6% similarity) with Erm(T) of plasmid pKKS25 from LA-MRSA ST398.[2] A comparison between the sequenced part of pKKS25 and pUR3912 revealed that the *erm*(T) gene was the only common feature between both plasmids (Figure 1a). A complete translational attenuator, which consisted of two pairs of inverted repeat sequences of 12 bp each and a reading frame for a regulatory peptide of 19 amino acids, was identified immediately upstream of the *erm*(T) gene (Figure 1b). Detailed analysis of the *erm*(T) region of pUR3912 showed that the *erm*(T) gene was flanked by two identical copies of the insertion sequence IS*431* [named $IS431_L$ and $IS431_R$ based on their positions with respect to *erm*(T)], both located in the same orientation. A 709 bp region immediately downstream of $IS431_R$ showed 92.1% identity to the corresponding region of plasmid pSSP1 of *Staphylococcus saprophyticus* ATCC 15305 and included 120 bp of a truncated *rep* gene.[6] A complete *rep* gene encoding a 204 amino acid replication initiation protein and a cadmium resistance operon were detected adjacent to this region. The *rep* gene showed 96.6% identity to that of pSSP1. The *cadD* gene encodes a 206 amino acid P-type metal efflux ATPase protein involved in cadmium resistance and *cadX* for a protein of 116 amino acids that serves as a transcriptional regulator of the cadmium resistance operon.[7,8] Broth microdilution assays revealed that the *S. aureus* RN4220 transformant carrying pUR3912 exhibited a 128-fold increase in the MIC of $CdSO_4$ (128 mg/L) as compared with *S. aureus* RN4220 (MIC $CdSO_4$ 1 mg/L), and thus confirmed the functionality of the *cadDX* operon.

The comparison shown in Figure 1 suggested that a pUR3912-like plasmid has been incorporated into the chromosomal DNA of MSSA ST398NM01 and that insertion elements of the type IS*431* were most likely involved in this process.[3,9] Three structural differences were noted between pUR3912 and the pUR3912-like plasmid in the chromosomal DNA of MSSA ST398NM01 (Figure 1): (i) two direct repeats of 64 bp are present between *cadX* and *rep* in MSSA ST398NM01, while only one of these sequences is present in pUR3912; (ii) an additional IS*431* copy, named $IS431_L$, was present in pUR3912—the detection of the 8 bp direct repeats immediately up- and downstream of the $IS431_L$ in pUR3912 strongly suggested that the integration of this insertion sequence into a pUR3912 precursor was an independent process; and (iii) the 709 bp pSSP1-like segment is missing in the chromosomally integrated plasmid, although the 9 bp (5′-AAAAATTTG-3′) corresponding to the 5′ terminus of the truncated *rep* gene are present. It is unknown when and by which way this 709 bp segment became part of pUR3912, but

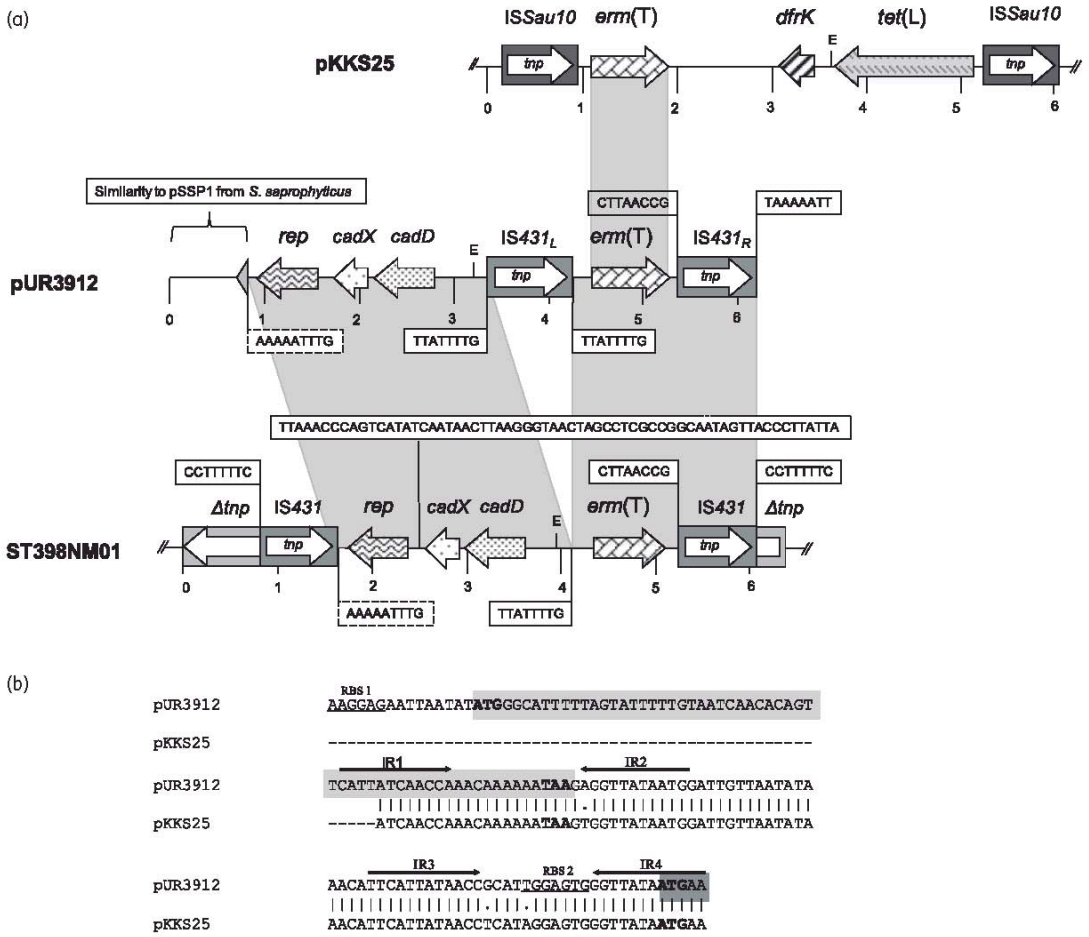

Figure 1. (a) Comparison of the complete plasmid pUR3912 (accession number HE805623) identified in this study with the *erm*(T)-*cadDX* chromosomal segment of MSSA strain ST398NM01 (accession number CP003045) and the sequenced part of plasmid pKKS25 of LA-MRSA ST398 (accession number FN390947). The arrows indicate the extents and directions of transcription of the genes *erm*(T) (combined resistance to MLS_B), *rep* (plasmid replication), *cadX* (transcriptional regulator), *cadD* (P-ATPase metal efflux), *dfrK* (trimethoprim resistance) and *tet*(L) (tetracycline resistance). IS*431* (including $IS431_L$ and $IS431_R$), IS*Sau10* and the truncated IS element (Δ*tnp*) are shown as dark grey, black and light grey boxes, respectively, with the white arrow indicating the transposase gene *tnp*. The EcoRI cleavage site (E) is indicated. The 8 bp direct repeats at the IS*431* integration sites within a transposase gene—related to the transposase gene of IS*712G*—of the chromosomal DNA of ST398NM01 as well as the 8 bp direct repeats upstream and downstream of the $IS431_L$ and $IS431_R$ within pUR3912 are shown in boxes. The 9 bp (5′-AAAAATTTG-3′) of ST398NM01 identical to the last 9 bp of the 709 bp region of plasmid pSSP1 from *S. saprophyticus* ATCC 15305 in pUR3912 are indicated in a dashed box. The regions of similarity between pUR3912 and the segments of ST398NM01 and pKKS25 are indicated by grey shading. An additional copy of the 64 bp sequence present in ST398NM01 is shown in the stippled box. A size scale in kilobase pairs (kb) is given below each map. (b) Comparison of the complete *erm*(T) regulatory region of pUR3912 with the truncated pKKS25. The 55 bp deletion in pKKS25 is indicated by dashes. Vertical bars indicate bases that are identical in the two sequences while dots reflect the presence of different bases. The two pairs of inverted repeat sequences, IR1–IR2 and IR3–IR4, are marked by arrows, the ribosomal binding sites RBS 1 and RBS 2 are underlined and the reading frames for the 19 amino acid regulatory peptide and the 5′-terminal part of the *erm*(T) gene are indicated by grey shading.

the presence of several copies of IS*431* in pSSP1 may suggest a potential involvement.

To determine whether pUR3912 is present in other *S. aureus* ST398 strains, eight recently described *erm*(T)-positive strains from our strain collection (two MSSA ST398 and two LA-MRSA ST398 of human origin and four LA-MRSA ST398 of porcine origin) were investigated.[10–12] Plasmid pUR3912 appeared to be present in the two unrelated MSSA ST398 strains of human origin. In these strains, a plasmid of the expected size was detected that carried the *erm*(T) and *cadX* genes at the expected distance—as confirmed by PCR analysis [erm(T)-fw 5′-ATTGGTTCAGGGAAAGGTCA-3′ + cadX-fw 5′-TTAGTCGTTGGATTATGGCT-3′]. However, the two unrelated LA-MRSA ST398 of human origin and the four LA-MRSA ST398 of porcine origin harboured the *erm*(T) gene on larger plasmids. In these six plasmids, *erm*(T) was associated with the *tet*(L) gene, as confirmed by PCR analysis [erm(T)-fw + tet(L)-fw CATTTGGTCTTATTGGATCG]. These observations suggest that plasmid pUR3912 may occur more

often in the animal-independent MSSA ST398, whereas plasmid pKKS25 or other larger plasmids seem to occur preferentially in LA-MRSA ST398. However, the small number of *erm*(T)-positive *S. aureus* strains does not allow reliable conclusions to be drawn.

This is the first description of an *erm*(T)-harbouring plasmid that also carried a cadmium resistance operon. The observation that *erm*(T) is present either on a small plasmid, such as pUR3912, or on larger plasmids that also carry *tet*(L), such as pKKS25, or that it is located in the chromosomal DNA of MSSA ST398NM01 suggests that the *erm*(T) gene has been acquired at independent occasions by MSSA and MRSA ST398 strains that are adapted to different hosts.[13] The physical linkage of antimicrobial resistance genes and genes that confer resistance to heavy metals may facilitate their persistence and dissemination under the selective pressure imposed by any of the involved agents.

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Transparency declarations

None to declare.

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The enterococcal ABC transporter gene *lsa*(E) confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*

Sarah Wendlandt[1]†, Carmen Lozano[2]†, Kristina Kadlec[1], Elena Gómez-Sanz[2], Myriam Zarazaga[2], Carmen Torres[2] and Stefan Schwarz[1]*

[1]*Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany;* [2]*Biochemistry and Molecular Biology, University of La Rioja, Logroño, Spain*

*Corresponding author. Tel: +49-5034-871-241; Fax: +49-5034-871-143; E-mail: stefan.schwarz@fli.bund.de
†These authors contributed equally to this study.

Keywords: antimicrobial multiresistance, inter-genus transfer, staphylococci, enterococci

Sir,

In recent years, combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in staphylococci has been attributed to ABC transporters of the Vga type. Besides variants of the *vga*(A) gene,[1] novel genes such as *vga*(C) and *vga*(E) have

Paper 8

Chromosomal integration of the novel plasmid pUR3912 from methicillin-susceptible *Staphylococcus aureus* ST398 of human origin

Elena Gómez-Sanz[1], Myriam Zarazaga[1], Kristina Kadlec[2], Stefan Schwarz[2], Carmen Torres[1#]

[1]Biochemistry and Molecular Biology, University of La Rioja, Logroño, Spain

[2]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany

The novel *erm*(T)-*cadDX*-carrying plasmid pUR3912 has recently been described in the methicillin-susceptible *Staphylococcus aureus* ST398-t571 strain C3912 from a healthy human in Spain. Structural analysis revealed that pUR3912 belongs to the pC194 replicon family, replicates via a rolling-circle mechanism and harbors putative double-strand (*dso*) and single-strand (*sso*) origins of replication. Besides its plasmid location, a copy of pUR3912 was also found in the chromosomal DNA of strain C3912. Two IS*431* copies, which flank the plasmid, most probably mediated its chromosomal integration. Its ability to exist extrachromosomally, but also to integrate into the chromosomal DNA ensures persistence and dissemination of pUR3912.

Keywords: *erm*(T)-*cadDX*, rolling-circle replication, pC194, IS*431*, co-integrate

Recently, plasmid pUR3912, which carries the macrolide-lincosamide-streptogramin B resistance gene *erm*(T), the cadmium resistance operon *cadDX* and two IS*431* copies, has been identified and completely sequenced (Fig. S1) [1]. This plasmid originated from the methicillin-susceptible *Staphylococcus aureus* (MSSA) strain C3912, which has the–sequence-type (ST) 398 and *spa*-type t571 and was obtained from a healthy human [1]. The genetic content and part of the gene organization of pUR3912 resembled a chromosomal segment of MSSA ST398-t571 strain ST398NM01, the whole genome sequence of which has been recently published [2]. In the present study, we investigated plasmid pUR3912 for its ability to integrate into the chromosomal DNA of strain C3912 and analyzed its reorganized chromosomal structure.

Structural analysis of pUR3912 identified this plasmid as a rolling circle replication (RCR) plasmid of the pC194 family [3,4]. Many RCR plasmids carry antimicrobial and heavy metal resistance genes and/or insertion sequences and transposons [3]. Upstream of the *rep* gene in pUR3912, a putative *dso* with homology to that of pC194 was observed (Fig. S1). An additional sequence involved in initiation of the synthesis of the lagging-strand, the single-strand origin of replication (*sso*) [3-5] was detected 80 bp upstream of the cadmium resistance gene *cadD* in pUR3912 (Fig. S1). Due to the great similarity of pUR3912 and the corresponding chromosomal segment of strain ST398NM01, strains C3912, *S. aureus* RN4220, and a *S. aureus* RN4220 protoplast transformant carrying pUR3912 (RN4220/pUR3912) [1] were subjected to Southern blot analysis to evaluate the possible location of an additional pUR3912 copy in the chromosomal DNA. After digestion of genomic DNA with I-CeuI (New England Biolabs), pulsed-field-gel-electrophoresis was conducted [6]. I-CeuI digests were transferred to a nylon-membrane and hybridization and detection with specific probes comprising the complete pUR3912, and with a 16S rDNA probe, were conducted according to the manufacturer's recommendations (Roche). Southern blot analysis confirmed the presence of pUR3912 also in the chromosomal DNA of strain C3912, but not in RN4220 and RN4220/pUR3912.

Assuming that pUR3912 is located in the chromosomal DNA of C3912 at the same integration site as in strain ST398NM01, i.e. within the *tnp* transposase gene of an IS*712G*-like insertion sequence (IS) [2], long-range PCR with primers tnp-fw (5'-CCAAATTATGCTGAGCTTGGTC-3') and tnp-rv (5'-ACCGGGATTAGTTTCTACGC-3'),

located in the upstream and downstream region of this potential integration site, and LA Taq DNA polymerase (Takara) were employed. Two amplicons of 6,551 and 312 bp were obtained (Fig. 1). This observation indicated that (i) strain C3912 harbors more than one copy of this IS element, and (ii) the integration of a 6,239-bp fragment has occurred at least within one of the present copies of this IS element. Complete sequence analysis of the 6,551 bp chromosomal fragment of C3912 after amplicon purification using QIAquick Gel extraction kit (Qiagen) revealed two identical IS*431* copies located in the same orientation that flank an *erm*(T)-*cadDX* segment closely related to that of pUR3912 (Fig. 2).

It is known that IS*431* and similar ISs, such as IS*257*, are able to integrate small plasmids into larger plasmids, but also small plasmids into the chromosomal DNA [7,8]. This process requires the presence of one copy of the IS element on the small plasmid and another copy in the structure in which the small plasmid will be integrated, followed by a recombination between the two IS elements. To explain the findings obtained from strain C3912, the following model has been generated based on the sequence of plasmid pUR3912 [1] and the knowledge of IS*431*/IS*257*-mediated co-integrate formation [7] (Fig. 2). In a first step, either the IS431_L of pUR3912, which – based on its 8-bp direct repeats up- and downstream of its integration site – seems to have independently integrated into a pUR3912 precursor, or a free IS*431* copy has integrated into the *tnp* gene of a chromosomal IS*712G*-like element and thereby produced the 8-bp direct repeats (5'-CCTTTTTC-3') (Fig. 2). In a second step, this chromosomal IS*431*, has undergone homologous recombination with the IS431_R of a pUR3912-like plasmid resulting in the integration of pUR3912 into the chromosomal DNA flanked by both recombined IS*431* copies. This chromosomal structure is highly similar to the corresponding chromosomal region of strain ST398NM01. However, a 700 bp segment (with identity to plasmid pSSP1 of *S. saprophyticus* ATCC15305) was present in both, pUR3912 and the chromosomal co-integrate in strain C3912, but absent in ST398NM01 [1]. Two very similar sequences, 5'-TAAAAATT-3' and 5'-TAAAAAATT-3', that flank this 0.7 kb segment were detected and may have played a role in its acquisition. The only difference between the free pUR3912 and its co-integrate was the presence of a 64-bp duplication within the *dso* of the integrated pUR3912.

Two unrelated *erm*(T)-positive MSSA ST398 strains of human origin from our collection (strains C2549 with *spa* type t571 and C2679 with t1451) [9], which, based on the expected size and gene content [*erm*(T) and *cadDX*], carried plasmid pUR3912 [1], also harbored an integrated copy of pUR3912 within the same genetic context (Fig. 1). On the other hand, this segment was not present in three additional recently described multiresistant *erm*(T)- and *cadDX*- carrying plasmids from animal associated MRSA ST398 or porcine and human origin (Fig. 1) [10]. Chromosomal integration within this IS element in unrelated ST398-t571 and -t1451 MSSA strains points towards a preferable integration site. Moreover, a recent study that analyzed a collection of different *S. aureus* ST398 isolates revealed that 95% of human-associated MSSA ST398 carried the *cadDX* operon, in contrast to only 25% of pig-associated MRSA ST398 isolates investigated [11]. Unfortunately, the location of *cadDX* in these isolates was not investigated. The same study reported φ3 bacteriophage, which carries the human-specific immune evasion cluster (IEC) genes *scn* and *chp*, as the best genetic marker of the human-specific ST398 clade. PCR analysis of the IEC genes [12] revealed the presence of both *scn* and *chp* in the three isolates. As plasmid pUR3912 is neither conjugative nor carries mobilization genes, co-resident plasmids that carry the respective genes for horizontal transfer are needed. Such plasmids have so far not yet been detected in the strains that carry pUR3912-like plasmids. This may point towards limited options for horizontal transfer of pUR3912 to other MRSA/MSSA lineages, including the animal-associated ones, under the current conditions. Further epidemiological studies are warranted to elucidate whether pUR3912 and/or its chromosomal co-integrate are preferentially present in the animal-independent ST398 MSSA clade.

The ability of pUR3912 to integrate into the chromosomal DNA of different MSSA

ST398 strains via ISs, as well as its co-localization as extrachromosomal element within the same cell, ensure its persistence and dissemination. The observations made in this study underline the role of insertion sequences in the acquisition, maintenance and dissemination of antimicrobial and/or metal resistance genes.

Nucleotide sequence accession number. The 6551-bp nucleotide sequence of the chromosomal integrated plasmid pUR3912 of C3912 has been deposited in the EMBL database (accession number HF677199).

Acknowledgements

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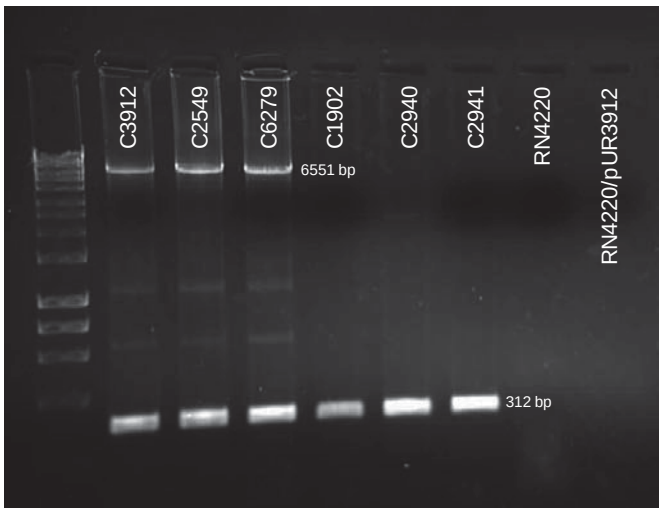

Figure. 1. Gel electrophoresis profile (running conditions, 1 hour at 96V) of the PCR products obtained from the three pUR3912-carrying ST398 methicillin-susceptible *S. aureus* (MSSA) strains C3912, C2549 and C2679, three ST398 methicillin-resistant *S. aureus* (MRSA) strains (C1902, C2940 and C2941), the pUR3912-recipient *S. aureus* RN4220 strain and its protoplast transformant (RN4220/pUR3912) using primers tnp-fw and tnp-rv. M, HyperLadder™ I (Bioline); band sizes: 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 kb. The faint bands detected in C3912, C2549 and C2679 are unspecific PCR products.

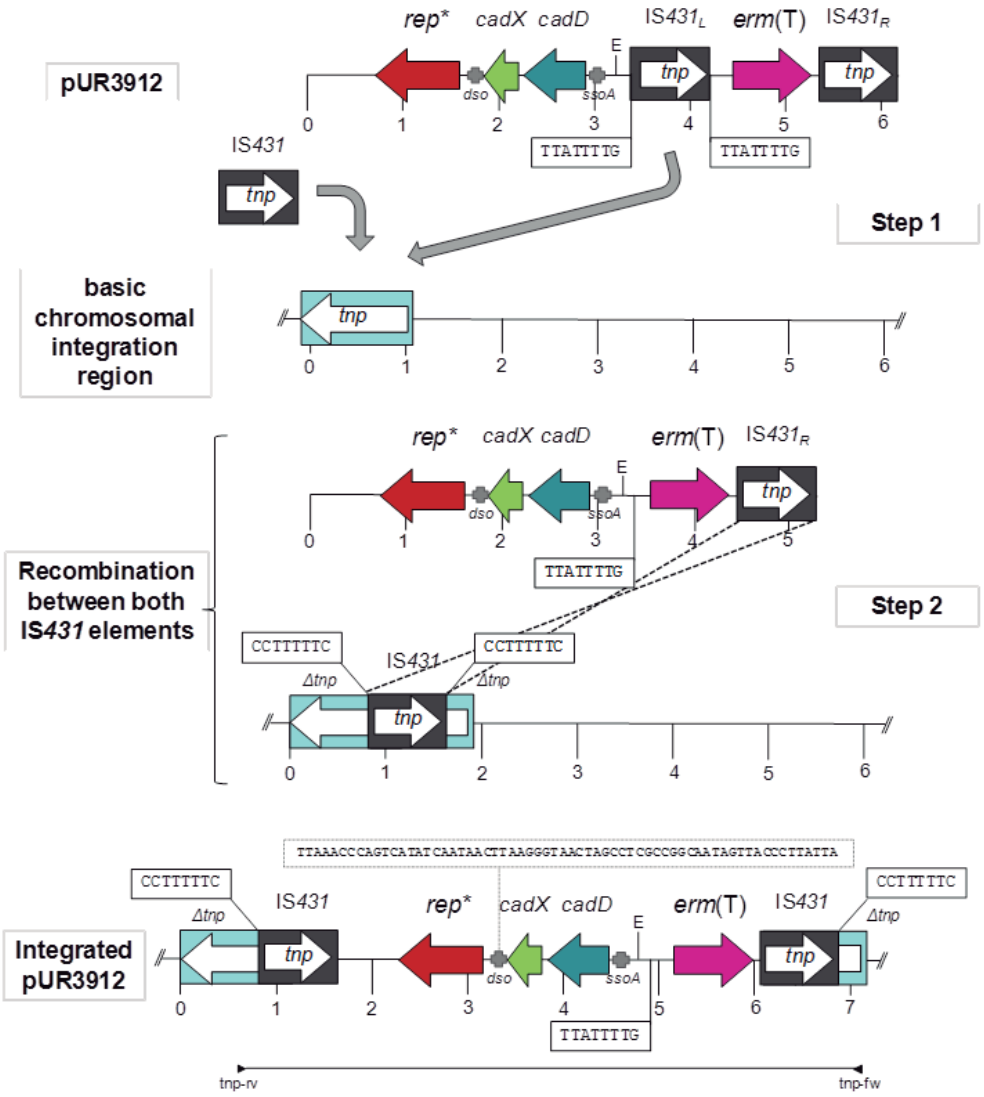

Figure. 2. Schematic representation of the IS*431*-mediated integration of plasmid pUR3912 (accession no. HE805623; the plasmid was adjusted for this figure, the database entry starts with the EcoRI (E) cleavage site) into the chromosomal DNA of strain C3912. The different steps include the integration of IS431_L or a free IS*431* copy into the chromosomal *tnp* followed by reciprocal homologous recombination of the integrated IS*431* with the IS431_R of a pUR3912-like plasmid. This latter process led to the integration of pUR3912 into the chromosome. The positions of primers used for the detection of the putative chromosomal location of pUR3912 are labeled tnp-fw and tnp-rv and indicated by arrow heads with the joining line representing the extension of the amplified fragment. In-depth analysis of the pUR3912 and its co-integrate DNA and protein sequences revealed that real size of the *rep* gene and Rep protein corresponded to 846 bp and 281 aa, respectively, instead of the original size estimated for the *rep* of pUR3912 (617 bp). The updated *rep* gene was indicated as *rep**. The arrows indicate the extents and directions of transcription of the genes *erm*(T) (resistance to macrolides-lincosamides-streptogramin B), *rep** (plasmid replication), *cadX* (translational regulator) and *cadD* (P-ATPase metal efflux). The *dso* and the *ssoA*, involved in plasmid replication, are indicated. The IS*431* and IS*712G*-like elements are shown as dark and faint grey boxes, respectively, with the white arrow indicating the transposase gene (*tnp*). The 8-bp direct repeats at the IS*431* integration sites within the chromosomal DNA (in *tnp*) as well as the 8-bp direct repeats of IS431_L at its integration sites within pUR3912 are shown in boxes. The 64-bp duplication within the *dso* in the integrated pUR3912 is shown within a dashed box. A size scale in kb is given below each map.

Annex to paper 8

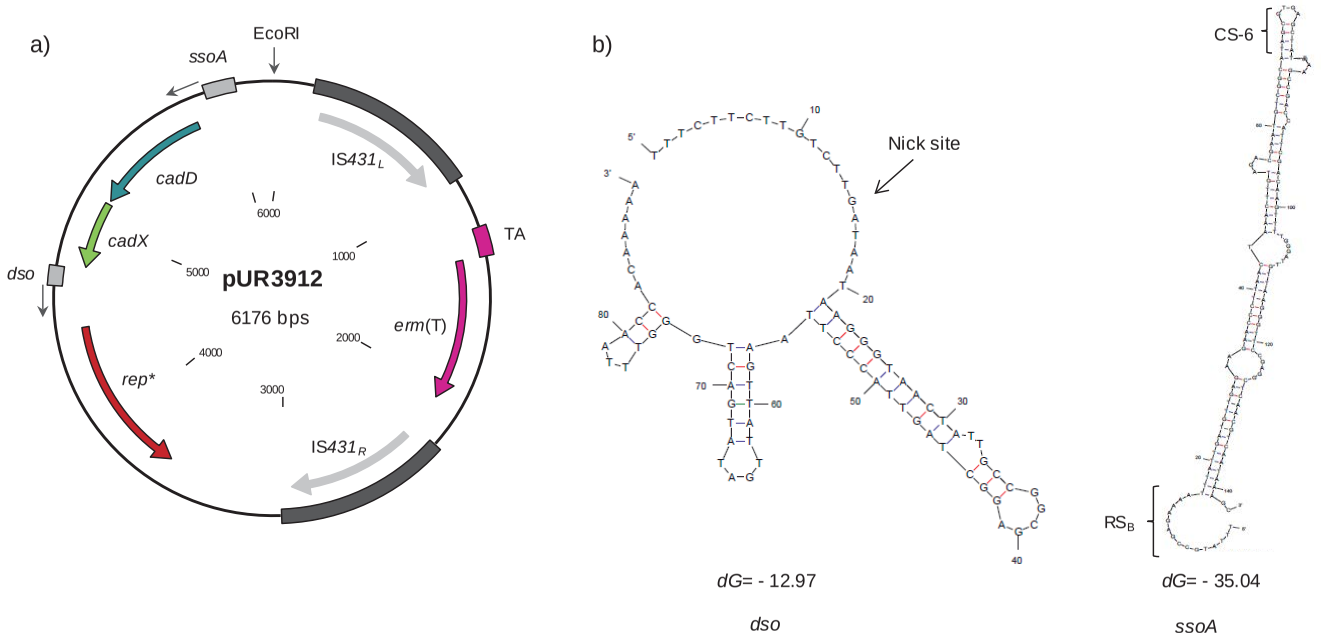

Figure S3 (Fig. S1 paper 8). Schematic presentation of the circular free copy of pUR3912 and the *dso* and *ssoA* detected. a) Circular map of pUR3912 showing the location and direction of transcription (indicated by arrows) of the 6 open reading frames detected using Clone Manager Suite9 program. Location of the *dso* and *ssoA* is shown within grey boxes with thin grey arrows indicating the direction of replication, while the *rep** gene (with its updated length, 846 bp) is displayed as a red arrow. The complete IS elements are shown as grey boxes with the transposase gene (*tnp*) as a faint grey arrow. Antimicrobial [*erm*(T), resistance to macrolides-lincosamides-streptogramin B] and metal [*cadX*, translational regulator and *cadD*, P-ATPase metal efflux] resistance genes are represented as pink, green or blue arrows, respectively. The complete translation attenuator (TA) region of the *erm*(T) gene is shown as a pink box. The single EcoRI site, which was used for cloning and sequencing pUR3912 is also indicated. A size scale in kb is given inside the map. b) Nucleotide sequence prediction of the secondary structures of the *dso* (left) and *ssoA* (right) regions of pUR3912 using Mfold version 3.5 program [1]. The free energy of the DNA hairpin formation (*dG*) of both structures is displayed below the DNA sequence. The nick site for the Rep protein of the *dso* as well as the 6-bp sequence (5'-TAGCGT-3'), termed CS-6, and the recombination site B (RS_B) regions of *ssoA* are indicated. The number of base pairs included within the predicted structures is indicated at several points.

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FUNCTIONAL ANALYSIS OF pUR3912

Sequence comparison analysis of the novel pUR3912 revealed a great percentage of similarity to the basic pC194 replicon, the prototype of a family of RCR plasmids (del Solar et al., 1998). DNA alignment analysis of the *rep* genes of pC194 and pUR3912 evidenced their homology. Plasmid pUR3912 presented an open reading frame -corresponding to the *rep* gene- of 846 bp, coding a 281-aa RepA-type replication initiation protein with endonuclease activity. This type of Rep proteins, are characteristics of plasmids that present a RCR mechanism. Upstream of the *rep* gene of pUR3912, a putative double-strand origin (*dso*) of replication with homology to that of pC194 was observed and its possible secondary structure was predicted using the Mfold 3.5 program (**Fig.S3**). Homology between the *dso* of pC194 and pUR3912 started 3 nucleotides upstream of the 5' end of the nick sequence (5'-CTTCTTGTCTTG/ATAATA-3', where / represents the putative nick site), a conserved region in replicons of the pC194 family on which the cognate Rep protein introduces the specific replication initiator nick (del Solar et al., 1998) (**Fig. S3**). Homology with pC194 was lost 431 bp downstream of the *rep* gene of pUR3912. Sequence comparison analysis revealed the presence of a *sso* region (spanning 142 bp) of the type A, 80 bp upstream of the cadmium resistance gene *cadD*. This region was predicted to form a long and complex secondary structure, which was highly similar to that of pC194. Both *ssoA*-conserved regions, the RS_B and the CS-6, were detected in the *ssoA* of pUR3912 (**Fig. S3**). However, an RS_B complementary sequence (RS_B-C) was not found further downstream of the *ssoA* 3' end region in pUR3912.

An interesting aspect of the *ssoA* origins is that they have a very narrow host range and only function efficiently in their natural hosts (del Solar et al., 1998). Although replication persists even when the lagging monocatenary structure is not replicated, the accumulation of single-stranded plasmid DNA may implies an increment of the fitness cost, and may compromise its maintenance in the non natural bacterial genus, what may suggest that pUR3912 is a *S. aureus* adapted plasmid.

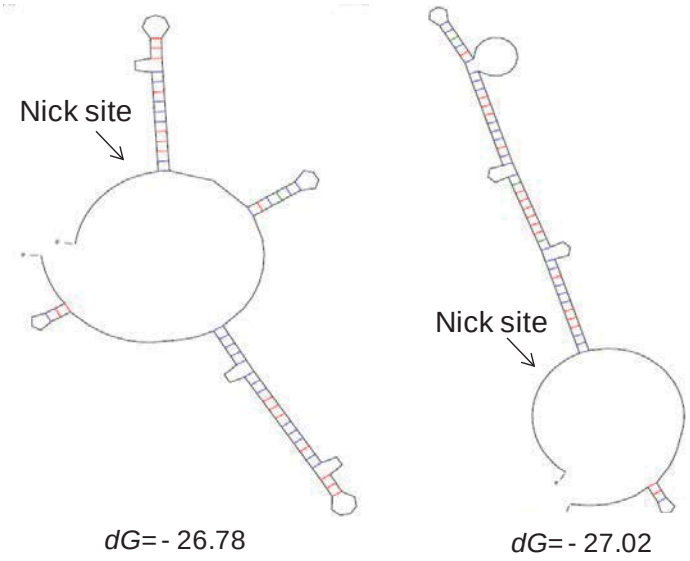

Figure S4. Outline prediction of two possible secondary structures formed in the enlarged *dso* of the integrated pUR3912 using Mfold version 3.5 program. The free energy of the DNA hairpin formation (*dG*) of both structures is displayed below the DNA sequence. The nick site of the extended *dso* is specified.

Paper 9

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Editor: Rossolini | Section: Mechanisms of Resistance | Designation: T

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Novel *erm*(T)-Carrying Multiresistance Plasmids from Porcine and Human Methicillin-Resistant *Staphylococcus aureus* ST398 Harbor Cadmium and Copper Resistance Determinants

AQ: au Elena Gómez-Sanz,[a] Kristina Kadlec,[b] Andrea T. Fessler,[b] Myriam Zarazaga,[a] Carmen Torres,[a] Stefan Schwarz[b]

Biochemistry and Molecular Biology, University of La Rioja, Logroño, Spain[a]; Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany[b]

This study describes three novel *erm*(T)-carrying multiresistance plasmids that also harbor cadmium and copper resistance determinants. The plasmids, designated pUR1902, pUR2940, and pUR2941, were obtained from porcine and human methicillin-resistant *Staphylococcus aureus* (MRSA) of the clonal lineage ST398. In addition to the macrolide-lincosamide-streptogramin B (MLS_B) resistance gene *erm*(T), all three plasmids also carry the tetracycline resistance gene *tet*(L). In addition, plasmid pUR2940 harbors the trimethoprim resistance gene *dfrK* and the MLS_B resistance gene *erm*(C), while plasmids pUR1902 and pUR2941 possess the kanamycin/neomycin resistance gene *aadD*. Sequence analysis of approximately 18.1 kb of the *erm*(T)-flanking region from pUR1902, 20.0 kb from pUR2940, and 20.8 kb from pUR2941 revealed the presence of several copies of the recently described insertion sequence IS*Sau10*, which is probably involved in the evolution of the respective plasmids. All plasmids carried a functional cadmium resistance operon with the genes *cadD* and *cadX*, in addition to the multicopper oxidase gene *mco* and the ATPase copper transport gene *copA*, which are involved in copper resistance. The comparative analysis of *S. aureus* RN4220 and the three *S. aureus* RN4220 transformants carrying plasmid pUR1902, pUR2940, or pUR2941 revealed an 8-fold increase in $CdSO_4$ and a 2-fold increase in $CuSO_4$ MICs. The emergence of multidrug resistance plasmids that also carry heavy metal resistance genes is alarming and requires further surveillance. The colocalization of antimicrobial resistance genes and genes that confer resistance to heavy metals may facilitate their persistence, coselection, and dissemination.

Studies of the resistance genes present in livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) of multilocus sequence type (ST) 398 identified a certain heterogeneity of genes encoding the same resistance phenotype (1). This was particularly evident for genes conferring combined resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS_B) (2–8). The major mechanism of resistance to MLS_B is the methylation of the adenine at position A2058 in domain V of 23S rRNA. So far, at least four rRNA methylase genes, *erm*(A), *erm*(B), *erm*(C), and *erm*(T), have been described in LA-MRSA ST398 (1). Among them, the *erm*(T) gene, although initially reported in other Gram-positive bacteria, such as lactobacilli, streptococci, and enterococci (9–11), has been described for the first time in staphylococci on plasmid pKKS25 in a porcine LA-MRSA ST398 strain (12). This gene has also been detected in LA-MRSA ST398 strains from cattle (3) and food/food products of poultry origin (4). Its presence among MRSA ST398 of human origin has also been described (8). A study of *erm* genes in livestock manure and manure management systems found *erm*(T) at a high frequency in bovine and swine manure (13). Whenever *erm*(T) was detected in staphylococci, the corresponding strains showed constitutive resistance to clindamycin, implying the presence of structural alterations in the *erm*(T)-associated translational attenuator (5, 8, 12).

Recent studies have also revealed the presence of the *erm*(T) gene among methicillin-susceptible *S. aureus* (MSSA) ST398 strains (usually of *spa* type t571) in humans who had no contact to livestock (14–18), and these strains may be involved in serious infections in humans (19–21). The *erm*(T) gene has been shown to confer an inducible clindamycin resistance phenotype and to be located in the chromosomal DNA (15, 16). Very recently, the first *erm*(T)-carrying resistance plasmid, namely, pUR3912, from an MSSA isolate of human origin was described (22). This plasmid carried a functionally active cadmium resistance operon (*cadDX*) and represented the first *erm*(T)-carrying plasmid that also harbored heavy metal resistance determinants. Despite the fact that cadmium is a highly toxic metal which is neither used in agriculture nor found in the community or in the hospital sector, the presence of cadmium resistance determinants on staphylococcal plasmids has been described before (23, 24). In contrast, copper and zinc compounds are commonly used as feed supplements in livestock (25), and zinc resistance has been recently identified as part of type V SCC*mec* cassettes, which are commonly found in LA-MRSA ST398 (26). Thus, it has been assumed that zinc resistance plays a role in the coselection and emergence of methicillin resistance in LA-MRSA ST398 of animal origin.

This study described novel staphylococcal multiresistance plasmids that also carry cadmium and copper resistance determinants. The aim of the present study was to determine the location and the genetic environment of the resistance genes on *erm*(T)-carrying multiresistance plasmids obtained from five LA-MRSA ST398 strains of porcine and human origin recently detected in Spain (5, 8).

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Address correspondence to Carmen Torres, carmen.torres@unirioja.es.

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TABLE 1 Comparative analysis of the MICs of the original *erm*(T)-carrying MRSA strains, *S. aureus* RN4220, and *S. aureus* RN4220 transformants carrying the three novel *erm*(T)-positive plasmids

Bacterial strain	Resistance genes detected for:		MIC (μg/ml) of antimicrobial agent[b]											MIC (mM) of metal		
	Antimicrobial agents[a]	Metal compounds	PEN	OXA	ERY	CLI	TIA	TET	GEN	KAN	NEO	SPE	TMP	$CdSO_4$	$CuSO_4$	$ZnCl_2$
RN4220			0.03	0.12	0.5	0.12	0.5	0.5	0.25	4	0.5	64	1	0.015	8	1
C1902[c]	*blaZ*, *mecA*, *erm*(T), *tet*(L), *tet*(M), *aadD*, *aacA-aphD*	*cadD*, *cadX*, *mco*, *copA*	16	≥16	≥64	≥128	1	64	8	≥512	128	128	2	0.25	8	2
RN4220/pUR1902	*erm*(T), *tet*(L), *aadD*	*cadD*, *cadX*, *mco*, *copA*	≤0.015	0.12	≥64	≥128	0.25	64	0.25	32	16	64	0.5	0.125	16	1
C2940	*blaZ*, *mecA*, *erm*(T), *erm*(C), *vga*(A), *tet*(L), *tet*(M), *dfrK*, *aacA-aphD*	*cadD*, *cadX*, Δ*mco*, *copA*, *czrC*	8	8	≥64	≥128	≥128	64	128	≥512	2	128	≥256	0.5	16	8
RN4220/pUR2940	*erm*(T), *erm*(C), *tet*(L), *dfrK*	*cadD*, *cadX*, Δ*mco*, *copA*	≤0.015	0.06	≥64	≥128	0.5	64	0.25	4	0.5	64	≥256	0.125	8	1
C2941	*blaZ*, *mecA*, *erm*(T), *erm*(C), *tet*(L), *tet*(M), *aadD*	*cadD*, *cadX*, *mco*, *copA*, *czrC*	16	≥16	≥64	≥128	0.5	64	0.5	64	64	≥512	0.25	0.5	16	8
RN4220/pUR2941	*erm*(T), *tet*(L), *aadD*	*cadD*, *cadX*, *mco*, *copA*	≤0.015	0.12	≥64	≥128	0.25	64	0.25	32	32	64	0.5	0.125	16	1

[a] The antimicrobial resistance genes present in the original strains C1902, C2940, and C2941 were described in previous studies (5, 8).
[b] PEN, penicillin; OXA, oxacillin; ERY, erythromycin; CLI, clindamycin; TIA, tiamulin; TET, tetracycline; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; SPE, spectinomycin; TMP, trimethoprim.
[c] Strains C1905 and C1906 showed the same profiles to the antimicrobials and metal compounds tested.

MATERIALS AND METHODS

Bacterial strains investigated and molecular typing. Five *erm*(T)-positive MRSA ST398 strains identified in previous studies were included (5, 8). Strains C1902, C1905, and C1906 were isolated from healthy pigs and presented the *agr* allotype I, *spa* type t011, and SCC*mec* IVa, while strains C2940 and C2941 came from humans with different diseases, showed *agr* allotype I, *spa* type t011, and harbored the SCC*mec* type V (5, 8). All strains were multiresistant (Table 1). To estimate the clonal relatedness of the strains, pulsed-field gel electrophoresis (PFGE) of total DNA after digestion with ApaI (Roche Pharma, Madrid, Spain) was performed by following the HARMONY protocol (27). ApaI fragments were separated for 20 h at 6 V/cm using pulse time ramping from 2 to 5 s (6).

Isolation and transfer of *erm*(T)-carrying plasmids. Plasmids were extracted and purified using a modified alkaline lysis method (12). Obtained plasmids were transformed by protoplast transformation into *S. aureus* RN4220 with subsequent selection on regeneration plates containing erythromycin (15 μg/ml). The presence of the *erm*(T) gene in the transformants was confirmed by a specific PCR (Table 2). The approximate sizes of the transformed plasmids pUR1902, pUR2940, and pUR2941 were calculated as the sum of the fragment sizes obtained after digestion of the plasmids with the restriction endonucleases EcoRI and BglII (Roche) in independent experiments.

Cloning of *erm*(T) and flanking regions from plasmids pUR1902, pUR2940, and pUR2941. Plasmids were digested with EcoRI (for pUR1902 and pUR2941) and by EcoRI and BglII (for pUR2940). The corresponding fragments were cloned into the plasmid vector pBluescript II SK(+) (Stratagene, Amsterdam, The Netherlands), and recombinant plasmids were transformed into *Escherichia coli* JM101. The cloned fragments of interest were sequenced by primer walking on both strands, starting with M13 universal and reverse primers (22). Linkage between sequenced fragments, as well as determination of DNA regions that could not be cloned in repeated experiments, was performed by PCR mapping. For this, primers were designed from the sequences of already known segments and the amplicons were sequenced.

Antimicrobial susceptibility testing. MICs for the antimicrobial agents listed in Table 1 were determined for the original strains and their *S. aureus* RN4220 transformants by broth microdilution (28) using custom-made microtiter plates (MCS Diagnostics, Swalmen, The Netherlands). *S. aureus* ATCC 29213 served as a quality control strain.

MIC determinations for cadmium, copper, and zinc compounds and testing of the resistance genes involved. The MIC for cadmium sulfate ($CdSO_4$; PanreacQuímica S.L.U., Barcelona, Spain) was determined by agar dilution in three independent assays on Mueller-Hinton (MH; Becton, Dickinson, Madrid, Spain) agar plates, while the MICs for copper sulfate and zinc chloride ($CuSO_4$ and $ZnCl_2$; Scharlau, Barcelona, Spain) were likewise determined on cation-adjusted Mueller-Hinton II (MH-II; Becton, Dickinson, Madrid, Spain) agar with the pH of the medium adjusted to 5.5 for $ZnCl_2$ or to 7.4 for $CuSO_4$ (25, 26). For this, the original strains C1902, C1905, C1906, C2940, and C2941, the recipient strain *S. aureus* RN4220, and also the isogenic *S. aureus* RN4220 transformants were used. Concentration ranges for $CdSO_4$ were 0.001 to 2 mM, while those for $CuSO_4$ and $ZnCl_2$ were 0.125 to 128 mM (26). Plates were incubated for 20 h at 37°C under aerobic conditions. The presence of genes responsible for heavy metal resistance (cadmium, copper, zinc) was investigated by PCR and subsequent sequencing of the respective amplicons (Table 2).

Southern blotting for the possible chromosomal localization of *erm*(T) and associated genes. Southern blot analysis of genomic DNA after previous digestion with endonuclease I-CeuI (New England BioLabs, Barcelona, Spain) was performed. Agarose plugs with genomic DNA of strains C1902, C2940, and C2941 were digested with I-CeuI (10 U) for 4 h at 37°C. Fragments were separated in a 1% (wt/vol) PFGE agarose gel (18 h, 6 V/cm, and 5–30 s at 14°C) and I-CeuI PFGE digests were transferred to a nylon membrane. Hybridization with probes for *erm*(T), *cadDX*, *copA*, *mco*, and the 16S rRNA gene and detection were conducted according to the manufacturer's recommendations (Roche, Madrid, Spain).

Nucleotide sequence accession numbers. The nucleotide sequences of the sequenced parts of plasmids pUR2941 (20,776 bp), pUR1902 (18,126 bp), and pUR2940 (19,957 bp) have been deposited in the EMBL database under accession numbers HF583290, HF583291, and HF583292, respectively.

RESULTS AND DISCUSSION

PFGE profile, antimicrobial resistance patterns, and plasmid profiles. All five strains shared closely related ApaI PFGE profiles, with the porcine strains C1902, C1905, and C1906 exhibiting even indistinguishable fragment patterns (data not shown). MIC values

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TABLE 2 Primers and PCR conditions employed[a]

Gene or region amplified	Primer designation	Primer sequence (5′→3′)	Nucleotide position in published sequence	Amplicon size (bp)	Reference	GenBank accession nos.[b]
erm(T)[c]	ermT_fw	ATTGGTTCAGGGAAAGGTCA	109–128 in *erm*(T)	536	3	HF583290, HF583291, HF583292
	ermT_rv	GCTTGATAAAATTGGTTTTTGGA	622–644 in *erm*(T)			
cadD	cadD-fw2	TGCTAGAGCAAAGACTAGGAAAGA	93–116 in *cadD* (81–104 in *cadD* of pUR3912)	460	This study	HF583290, HF583291, HF583292, HE805623
	cadD-rv	AGCCATAATCCAACGACCAA[d]	533–552 in *cadD* (521–540 in *cadD* of pUR3912)			
cadX	cadX-fw	TGCTTGTGATGTGATCTGTGT	15–35 in *cadX*	213	This study	HF583290, HF583291, HF583292, HE805623
	cadX-rv	TGATGTGAAGTTGAAGCAACAC	206–227 in *cadX*			
Promoter *cadDX*	Pr_cadDX-fw	CTGACGATGCCAGGAAACTT	438–457 upstream of *cadD*	579, 419[e]	This study	HF583290, HF583291, HF583292, HE805623
	Pr_cadDX(pUR3912)-fw	AGTAAGGGTGCAGTGCCAAT	290–309 upstream of *cadD* of pUR3912			
	Pr_cadDX-rv2	CGATATTCTTTCCTAGTCTTTGCTC	98–122 in *cadD* (86–110 in *cadD* of pUR3912)			
copA	copA-fw	CATGCTTTAGGCTTGGCAAT	931–950 in *copA*	662	This study	HF583290, HF583291, HF583292
	copA-rv	TCTTCTGGCATGAGTTGTGC	1573–1592 in *copA*			
mco	mco-fw	TCCCTCCCCAAATACAGCTA	537–556 in *mco*	699	This study	HF583290, HF583291, HF583292
	mco-rv	GTTCCGTGGATATGGAATGG	1216–1235 in *mco*			
czrC	czrC-fw	TAGCCACGATCATAGTCATG	48–67 in *czrC*	655	20	JCSC6944
	czrC-rv	ATCCTTGTTTTCCTTAGTGACTT	680–702 in *crzC*			

[a] Primers and PCR conditions employed in this study to detect genetic determinants for resistance to the different metal compounds detected in the three novel plasmids described in the study and those detected in the recently described pUR3912, as well as those for the MLS_B resistance gene *erm*(T). The PCRs were performed using BioTaq DNA polymerase (Bioline; Cultek, Madrid, Spain) and the following conditions: initial cycle of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C [45°C for the *erm*(T) gene], and 1 min at 72°C, and with a final step of 5 min at 72°C.

[b] Published sequences from which primers were designed and/or coordinates have been established.

[c] It should be noted that this pair of primers could not properly amplify the *erm*(T) gene of a set of *erm*(T)-positive MSSA isolates of the sublineage ST398, which seems to be associated with humans.

[d] The underlined nucleotide indicates ●●●.

[e] Amplicon size obtained using the combination of Pr_cadDX(pUR3912)-fw and Pr_cadDX-rv2.

determined for the original strains and their *S. aureus* RN4220 transformants are shown in Table 1. In addition to the previously described data (5, 8), strain C2940 also revealed resistance to tiamulin (MIC, ≥128 μg/ml), due to the presence of the *vga*(A) gene, and strain C2941 showed elevated MIC values for spectinomycin (≥512 μg/ml), although it was negative for the presence of the spectinomycin resistance gene *spc*.

On the basis of plasmid sizes, EcoRI and BglII restriction patterns, and antimicrobial resistance profiles, three different types of *erm*(T)-carrying plasmids were distinguished. Strains C1902, C1905, and C1906 of porcine origin carried plasmids of ~22 kb that showed the same restriction patterns. As a representative, plasmid pUR1902 from strain C1902 was included in a further analysis; strain C2940 harbored the ~25-kb plasmid pUR2940 and strain C2941 harbored the ~33-kb plasmid pUR2941. MIC testing and PCR analysis of the transformants revealed that, in addition to MLS_B resistance due to the *erm*(T) gene, transformants carrying plasmid pUR1902, pUR2940, or pUR2941 were tetracycline resistant and carried the *tet*(L) gene. In addition, plasmids pUR1902 and pUR2941 conferred resistance to kanamycin/neomycin and had an *aadD* gene, while plasmid pUR2940 conferred trimethoprim resistance via the gene *dfrK*. Moreover, plasmid pUR2940 harbored the additional MLS_B resistance gene *erm*(C) (Table 1).

Analysis of the novel pUR1902, pUR2940, and pUR2941 plasmids. Figure 1 shows maps of the sequenced parts of the three novel multiresistance plasmids in comparison to previously known staphylococcal plasmids that share significant structural similarities. All three plasmids, pUR1902, pUR2940, and pUR2941, had in common the resistance genes *erm*(T) and *tet*(L), a plasmid mobilization gene, *mob*, a 3′-truncated replication gene, *rep49*, the cadmium resistance *cadDX* operon, a *repA*-like gene (70.9% identity to *repA* of pUB101), and two copies of the insertion sequence IS*Sau10* (Fig. 1).

Plasmid pUR2940 exhibited a 5,436-bp resistance region that was 99.9% identical to that of plasmid pKKS25 (12) (Fig. 1). This region comprised the resistance genes *erm*(T), *tet*(L), and the *dfrK*, in addition to one IS*Sau10* copy. A pair of 21-bp imperfect direct duplications (5′-TTCCATTAAC/AGGGCGCGATTG-3′ [●●●]) was found that flanked the 1,590-bp region that comprised the *dfrK* gene (Fig. 1). As previously suggested (29), this sequence might have served for the integration of the *dfrK* region into pUR2940. Immediately downstream of the aforementioned IS*Sau10*, a region delimited by another IS*Sau10* copy was observed. The region between both IS elements was 98.9% identical to the small *erm*(C)-carrying plasmid pNE131 (Fig. 1). The *repL* gene of this small plasmid was interrupted by two IS*Sau10* elements that were located in the same orientation. An 8-bp target site duplication, 5′-GACTGTTC-3′, was detected upstream of the left-hand IS*Sau10* and downstream of the right-hand IS*Sau10* (Fig. 1). The *erm*(C) gene was located between the two *repL* segments. This region exhibited the typical structural characteristics for the integration of a small plasmid into a larger one via ISs (24). Immediately downstream of this segment, the copper resistance genes *copA* and Δ*mco*, whose 3′ ends were truncated by IS*Sau10*, were detected (Fig. 1).

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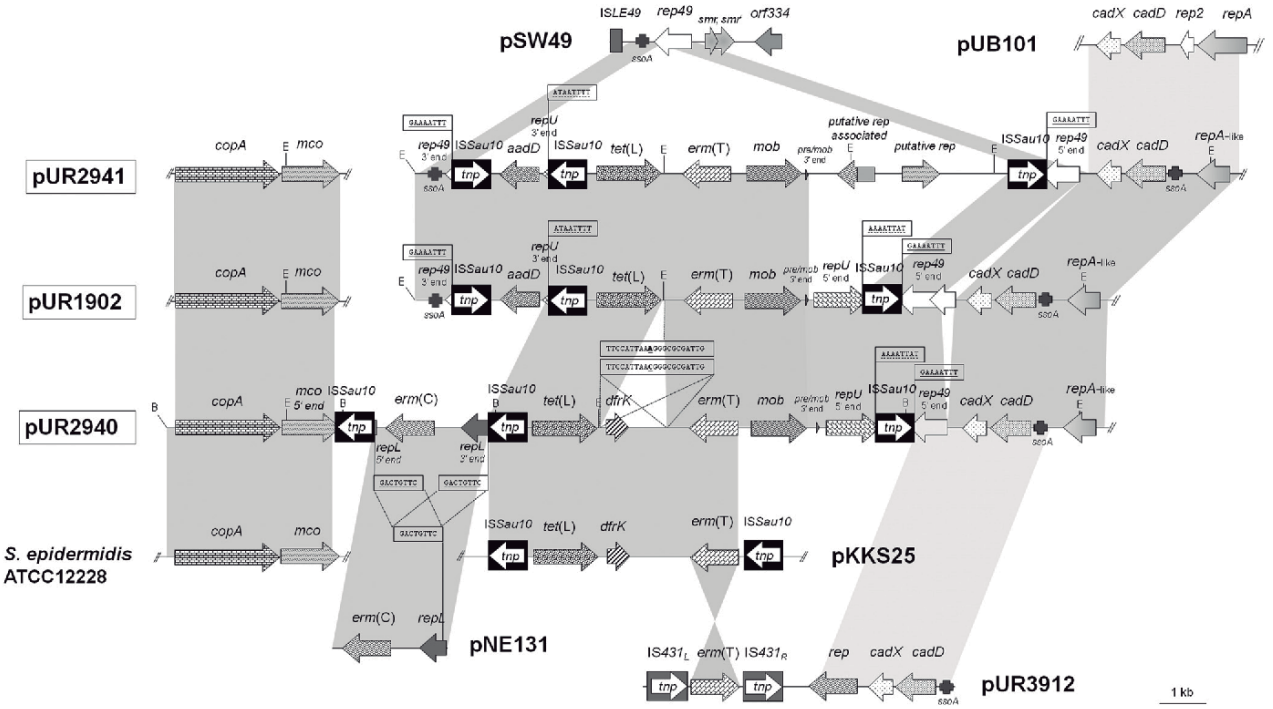

FIG 1 Comparative schematic presentation of the sequenced segments of the three novel *erm*(T)-carrying plasmids (pUR2941 [accession number HF583290], pUR1902 [HF583291], and pUR2940 [HF583292]) described in this study and the previously reported plasmids pSW49 (AM040730), pUB101 (AY373761), pKKS25 (FN390947), pNE131 (M12730), and pUR3912 (HE805623), as well as a chromosomal fragment of *S. epidermidis* ATCC 12228 (AE015929). The arrows indicate the extents and directions of transcription of antimicrobial [*erm*(T), *tet*(L), *aadD*, *dfrK*] and metal (*cadD*, *cadX*, *copA*, *mco*) resistance genes as well as genes involved in replication (*rep49*, *repU*, *repL* *repA-like*, *rep-associated*, putative *rep*, *repA*, and *rep2*), mobilization (*mob*), and others (ISLE49, *smr*, *smr'*, *orf334*). The 5′ and 3′ ends of the truncated *rep49*, *repU*, and *repL* genes and the 5′ end of the truncated *mco* gene in pUR2940 are likewise displayed. The different SSO regions detected are indicated as *ssoA*. The IS*Sau10* and IS*431* copies are shown as black or gray boxes with a white arrow indicating the transposase gene *tnp*. The 8-bp direct target site duplications at the extremes of IS*Sau10* are shown in boxes and underlined. The 21-bp sequences of the integration site of the *dfrK*-carrying region are also boxed. The regions of >90% homology are shown in dark gray, while those between 80 and 90% similarity are displayed in light gray. The EcoRI (E) and BglII (B) cleavage sites are indicated. A size scale in kilobases is displayed in the lower right corner.

Two discontinuous regions were sequenced in plasmid pUR1902 and pUR2941: (i) *erm*(T)-carrying segments of 14,626 bp and 17,276 bp, respectively, obtained from several contiguous EcoRI fragments, and (ii) a *copA*- and *mco*-carrying segment of 3,500 bp obtained by PCR mapping (Fig. 1). Despite extensive attempts, these two regions could not be joined by PCR mapping. Except for a single mismatch, the *copA-mco* segments were identical to that of pUR2940 but harbored the complete *mco* gene. At the left terminus of the *erm*(T)-flanking region of both plasmids, a region of 2,702 bp was detected that was absent in pUR2940. This small fragment carried another IS*Sau10* copy, whose upstream region showed the highest identity to a segment of the *Staphylococcus warneri* small plasmid pSW49, which includes a single-strand origin of replication, *ssoA*, and the 3′ end of *rep49* (Fig. 1). The region downstream of the IS*Sau10* contained the *aadD* gene and the 3′ end of *repU*, which were identical to the corresponding regions of plasmid pKKS825 from MRSA ST398 (30) (Fig. 1). In addition, plasmid pUR2941 exhibited a unique segment of 4,140 bp in which two putative *rep* genes of 744 bp and 726 bp were detected.

Neither the *erm*(T) gene nor the cadmium nor copper resistance determinants (*cadD*, *cadX*, *copA*, or *mco*) detected were present on any of the chromosomal DNA fragments obtained after I-CeuI digestion of C1902, C2940, and C2941 genomic DNA. In contrast, the 16S rRNA gene probe yielded the expected positive hybridization results for all chromosomal DNA bands visualized in all three strains (data not shown).

Role of IS elements in the development of novel multiresistance plasmids. IS*Sau10*-related IS*257* and IS*431* have been shown to be responsible for the cointegration of small plasmids within larger plasmids or within the chromosomal DNA (24, 31, 32). The different copies of IS*Sau10* disrupted diverse *rep* genes of small plasmids as well as the *mco* gene of pUR2940. It has been suggested that the truncation of the *rep* genes in the cointegration process of different plasmids is important for the maintenance of the replication system of the original replicon (24). In addition, the presence of two IS*Sau10* copies in the same orientation with the typical 8-bp direct target site duplications at both ends and bracketing a sequence that closely resembles a small *erm*(C)-carrying plasmid in pUR2940 strongly suggested that its integration was IS*Sau10* mediated. IS elements play an important role in the evolution of multidrug resistance plasmids because of their capacity to integrate, split, and undergo homologous recombination with related IS elements. The presence of several copies of the recently described IS*Sau10* element in the sequenced regions of all three plasmids suggests that these IS*Sau10* copies play a role in the mosaic structure of plasmids pUR1902, pUR2940, and pUR2941.

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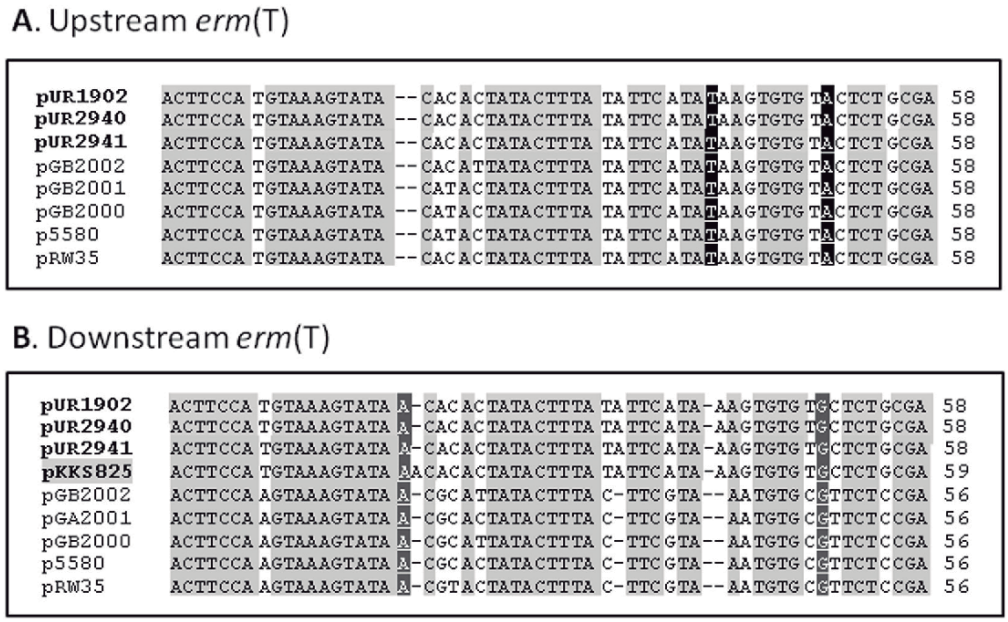

FIG 2 Sequence alignment of the homologous regions (56 to 59 bp) located upstream (A) and downstream (B) of the *erm*(T) gene in the three novel MRSA ST398 plasmids (pUR1902, pUR2940, and pUR2941), plasmid pGB2002 (*Streptococcus agalactiae*), pGB2001 (*Streptococcus agalactiae*), pGA2000 (*Streptococcus pyogenes*), p5580 (*Streptococcus dysgalactiae*), pRW35 (*Streptococcus pyogenes*), and a possible related precursor, pKKS825 (MRSA ST398) potentially used for the integration of an *erm*(T)-containing segment into the hypothetical pUR2940 *erm*(T)-free precursor. Displayed are nucleotides (nt) at the following up- and downstream positions of the *erm*(T) gene, respectively: nt 339 to 396 and 142 to 199 in pUR2940 (accession number HF583292); nt 524 to 581 and 142 to 197 in pRW35 (EU192194); nt 394 to 451 and 142 to 197 in pGB2002 (JF308629); nt 393 to 450 and 142 to 197 in pGB2001 (JF308630), pGA2000 (JF308631), and p5580 (HE862394); nt 1282 to 1340 downstream of the *dfrK* gene in pKKS825 (FN377602). Nucleotides in faint gray are those identical in all sequences, those in dark gray are those identical in all downstream regions, and nucleotides in black show the identical bases specific for the upstream segments.

Comparative analysis of the *erm*(T) gene and its immediate upstream and downstream regions. Comparative analysis of the complete 735-bp *erm*(T) genes with those deposited in the GenBank/EMBL databases revealed that the *erm*(T) genes of plasmids pUR2940 and pUR2941 were identical to those of streptococcal plasmids and that of plasmid pKKS25 of was identical to that of MRSA ST398. In contrast, the *erm*(T) gene present in plasmid pUR1902 showed a unique sequence that differed from the aforementioned *erm*(T) genes by a single nucleotide (A416G), resulting in the amino acid change Asn139Ser. A truncated translational attenuator region of *erm*(T) in the three novel plasmids was observed and showed the same 57-bp deletion as found on plasmid pKKS25 (12). Consequently, the three novel plasmids expressed *erm*(T) constitutively and were clindamycin resistant.

Comparison of the up- and downstream sequences of the *erm*(T) genes identified a common region of ~1.4 kb, including the *erm*(T) gene, that was embedded in largely nonhomologous segments in the *erm*(T)-carrying streptococcal plasmids and in the plasmids described in this study. Exactly at the junctions between these homologous and nonhomologous segments, imperfect direct repeats of 56 to 58 bp were detected. These imperfect direct repeats were also present up- (396 bp) and downstream (199 bp) of the *erm*(T) gene in the three novel plasmids (Fig. 2). It is likely that these imperfect direct repeats have played a role in the acquisition of *erm*(T) via recombination by *erm*(T)-free precursors of these plasmids. Interestingly, these regions were not present in the corresponding parts of pUR3912 or strain ST398 NM01, what may suggest an alternative *erm*(T) acquisition pathway or subsequent divergence of such regions. Analysis of the *erm*(T)-lacking MRSA ST398 plasmid pKKS825, which also carries the *aadD*, *tet*(L), and *dfrK* genes, identified one such short region of 59 bp (30). This region in pKKS825 differed by only 1 bp from the 58-bp sequence found downstream of *erm*(T) in pUR1902, pUR2940, and pUR2941 (Fig. 2) and may represent a suitable acceptor site for an *erm*(T)-carrying segment.

Susceptibility to cadmium, copper, and zinc compounds and the presence of resistance determinants. Table 1 shows the MIC data for $CdSO_4$, $CuSO_4$, and $ZnCl_2$ of the aforementioned original strains and transformants. An 8-fold increase in the MIC for $CdSO_4$ in *S. aureus* RN4220 transformants carrying pUR1902, pUR2940, or pUR2941 (0.125 mM) was observed compared with the *S. aureus* RN4220 strain (0.015 mM). Original strains and transformants carried the cadmium resistance operon *cadDX*, which consisted of the *cadD* gene, encoding a 209-amino-acid (aa) P-type metal efflux pump involved in cadmium resistance, and *cadX*, which codes for a 115-aa protein that serves as a transcriptional regulator.

S. aureus RN4220 transformants carrying pUR1902 or pUR2941 exhibited a 2-fold increase in the MIC values for $CuSO_4$, from 8 mM to 16 mM in comparison to the recipient strain. *S. aureus* RN4220 carrying pUR2940 did not show an increase in the MIC values for $CuSO_4$ in comparison to the recipient strain. The three original strains and their transformants—but not *S. aureus* RN4220—harbored the copper transport gene *copA*, which codes for a 660-aa P1-type ATPase protein involved in copper resistance, in addition to the multicopper oxidase gene *mco* involved in cop-

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per homeostasis. However, accordingly, *S. aureus* RN4220/pUR2940 harbored a truncated Δ*mco* gene, which seemed responsible for the absence of variation in the $CuSO_4$ MIC values, regardless of the presence of the *copA* gene. Although the *copA-mco* genes of pUR1902 and pUR2941 increased the MIC values for copper by only one dilution, it should be noted that this MIC difference is in the high concentration range (16 mM corresponds to >2,000 μg/ml) and as such represents a substantial change in the copper MIC.

Strains C2940 and C2941 revealed 4- to 8-fold-higher MIC values for $ZnCl_2$ (8 mM) in comparison with strain C1902, *S. aureus* RN4220, and the transformants carrying pUR2940, pUR2941, or pUR1902 (1 mM) (Table 1). Accordingly, the presence of the *czrC* gene, which codes for a 644-aa putative cadmium and zinc transporter, was evidenced by PCR and sequencing in the original *S. aureus* strains C2940 and C2941 but neither in C1902 nor in the three *S. aureus* RN4220 transformants.

Simultaneous presence of antimicrobial and metal resistance genes on staphylococcal plasmids. The presence of cadmium resistance determinants in plasmids of *S. aureus* of different lineages is relatively common (23, 24, 33). Likewise, genes involved in copper resistance, especially *copA* and *mco* or variants (*copB* and *copC*), have been detected in different staphylococcal species and other Gram-positive bacteria, such as *Listeria monocytogenes* and *Macrococcus caseolyticus* (33–41). In LA-MRSA ST398, cadmium, copper, and other heavy metal resistance genes had been only detected in the chromosomal DNA, either within the SCC*mec*V(5C2&5)$_C$, which carries the cadmium/zinc resistance gene *czrC*, or in the novel SCC*mec*IX and -X, which carry cadmium (*cadDX*), copper (*copB-mco*), and arsenic (*arsRBC* and *arsDARBC*) resistance elements (26, 42). The gene cluster *copA-mco* detected in the three plasmids showed the highest percentage of identity to that identified in the chromosomal DNA of *Staphylococcus epidermidis* ATCC 12228 (92.8%), *Staphylococcus haemolyticus* JCSC1435 (92.6%) and the novel SCC*mec*X and SCC*mec*IX elements of MRSA ST398 strains JCSC6945 (91.9%) and JCSC6943 (91.3%), respectively. The current study presents the first report of LA-MRSA ST398 harboring a plasmid that carries cadmium and copper resistance genes.

Elevated concentrations of cadmium in feedstuffs may also occur due to the application of sewage sludge or phosphate fertilizers with high levels of cadmium into agricultural soils (43). Even though the accepted maximum levels of cadmium in feedstuffs for livestock are regulated (43), the possibility that such intake might be in part responsible for the selection of cadmium resistance determinants in the present bacterial population of pigs cannot be excluded. In humans, smoking cigarettes is an additional important source of cadmium (44). However, the major route of cadmium intake for the nonsmoking and non-occupationally exposed population is through ingestion of contaminated food (including food of animal origin) and water (43, 45). This exposure might also select for cadmium resistance in transferable elements in the *S. aureus* human population. In contrast, copper and zinc are essential trace elements for animals and humans (46, 47). They are added as feed supplements for livestock, particularly pigs, in great quantities to increase the daily growth rate of piglets (up to 8 to 10 weeks of age), to prevent gastrointestinal infections, and to limit and control cases of postweaning wanting and wasting (26, 28, 46, 47). The presence and maintenance of genetic elements that carry genes for copper and zinc resistance/tolerance in LA-MRSA ST398 are most probably favored by the extensive use of copper and zinc in pig production.

Staphylococcal plasmids that contain antimicrobial and metal resistance genes have been known for a long time. Plasmids of the pI258 type (GenBank accession no. NC_013319) have been disseminated in *S. aureus* ST30 (48) and contain, in addition to the beta-lactamase *blaZ* and the MLS_B resistance gene *erm*(B), also genes conferring resistance to cadmium (*cadA* and *cadC*), arsenic (*arsRBC*), and mercury (*mer* operon), as well as copies of the IS*431* element. In the current study, all three novel plasmids conferred a multidrug resistance phenotype, carried tetracycline and MLS_B resistance genes, as well as kanamycin/neomycin and/or trimethoprim resistance genes. Due to the elevated use of antimicrobial agents, in particular tetracyclines, macrolides, lincosamides, and aminoglycosides (1, 49) in veterinary medicine and food animal production, the colocation of antimicrobial resistance and heavy metal resistance genes on the same plasmids may favor their maintenance and dissemination under the selective pressure imposed by the use of either antimicrobial agents or heavy metals.

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Annex to paper 9

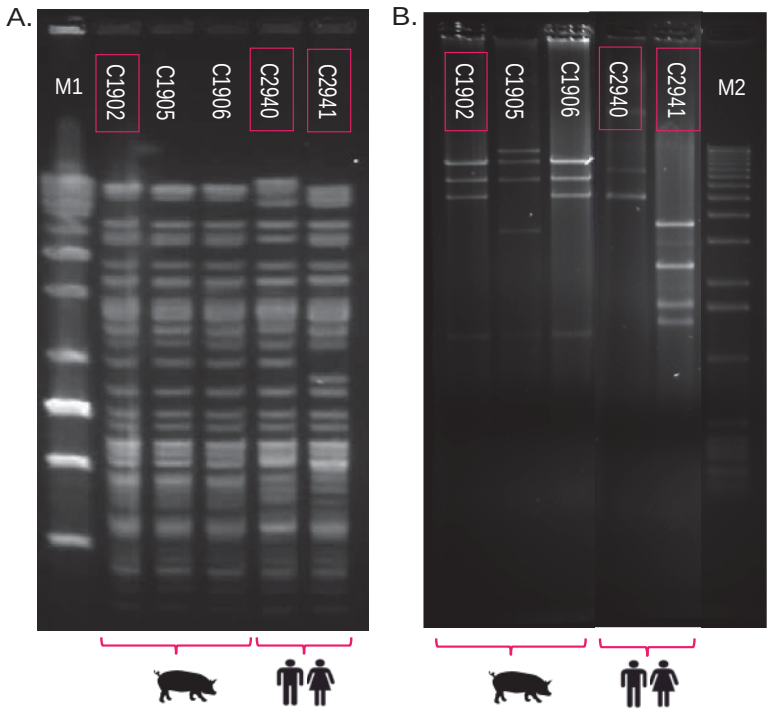

Figure S5. A. ApaI-PFGE band profile of the five LA-MRSA ST398 strains investigated in this study (C1902, C1905, C1906, C2940 and C2941). **B.** EcoR1-restriction profile of the *erm*(T)-carrying plasmid of strains investigated in RN4220 protoplast transformants. The strains further analysed are those squared. M1, Low Ranger Marker (New England Biolabs); M2, 1 Kb DNA Ladder (Invitrogen).

Table S10. Characteristics of the reading frames detected in the sequenced fragments of the novel plasmids pUR1902, pUR2940 and pUR2941 from methicillin-resistant *S. aureus* ST398 strains of porcine and human origin in this study.

	Novel plasmid	Gene or element	Size (bp)	Size (aa)	Function of integral protein	Compound affected
Antimicrobial resistance	pUR1902, pUR2940, pUR2941	*erm*(T)	735	244	23s rRNA methyltransferase	MLS_B
	pUR1902, pUR2940, pUR2941	*tet*(L)	1377	458	Energy-dependent efflux pump	Tetracycline
	pUR1902, pUR2941	*aadD*	771	256	Aminoglycoside adenyltransferase	Neomycin/kanamycin
	pUR2940	*erm*(C)	735	244	23s rRNA methyltransferase	MLS_B
	pUR2940	*dfrK*	492	163	Dihydrofolate reductase	Trimethoprim
Metal resistance/homeostasis	pUR1902, pUR2940, pUR2941	*cadD*	630	209	P-Type metal efflux pump	Cadmium
	pUR1902, pUR2940, pUR2941	*cadX*	348	115	Transcriptional regulator of *cadD*	Cadmium
	pUR1902, pUR2940, pUR2941	*copA*	1983	660	P1-Type ATPase efflux pump	Copper
	pUR1902, pUR2941	*mco*	1434	477	Multicopper oxidase	Copper
	pUR2940	Δ*mco* (5'end)	1378	-	Multicopper oxidase	Copper
Mobile Genetic Elements	pUR1902, pUR2940, pUR2941	IS*Sau10* [n=3]	793	224	Transposase	-
Replication/mobilization	pUR1902, pUR2940, pUR2941	*mob*	1212	403	Plasmid mobilization (relaxase)	-
	pUR1902, pUR2940, pUR2941	Δ*pre/mob* (3'end)	50	-	Plasmid mobilization (relaxase)	-
	pUR1902, pUR2940, pUR2941	*repA*-like	444	147	Replication initiation	-
	pUR1902, pUR2940, pUR2941	Δ*rep49* (5'end)	813	-	Replication initiation (nuclease)	-
	pUR1902, pUR2941	Δ*rep49* (3'end)	114	-	Replication initiation (nuclease)	-
	pUR1902, pUR2940	Δ*repU* (5'end)	990	-	Replication initiation	-
	pUR1902, pUR2941	Δ*repU* (3'end)	70	-	Replication initiation	-
	pUR2940	Δ*repL* (5'end)	24	-	Replication initiation (nuclease)	-
	pUR2940	Δ*repL* (3'end)	461	-	Replication initiation (nuclease)	-
	pUR2941	Putative *rep* associated	744	247	Replication initiation	-
	pUR2941	Putatitve *rep*	726	241	Replication initiation	-

Table S11. Analysis of representative MGEs (plasmids and SCC*mec*) analyzed in this study that share identity to the novel plasmids pUR1902, pUR2940 and pUR2941 and regions of homology identified.

Mobile genetic element	Origin	Size sequenced (bp)	Resistance gene/s or operon/s	Plasmid or SCC*mec* replication / mobilization	MGEs carried	Accesion no.	Comparative common region/s in representative novel plasmid			
							Size (bp)	Plasmid	*orf* enclosed	nt identity (%)
SCC*mec* X	*S. aureus*	51,483	*arsR/B/C, mecA, cadD/X, copB, mco*	*ccrB6, ccrA1*	IS*431*, IS*ShaI*	AB505630	3,353	pUR2940	*copA*, Δ*mco* (5'end)	93.9
SCC*mec* IX	*S. aureus*	44,355	*mecA, cadD/X, arsR/B/C, copB, mco, arsDARBC*	*ccrB1, ccrA1*	IS*431*	AB505628	3,353	pUR2940	*copA*, Δ*mco* (5'end)	93.5
ATCC 12600 plasmid	*S. aureus*	28,000	*copA, mco*	-	-	HQ663882	1,378	pUR2940	Δ*mco* (5'end)	95.8
pNE131	*S. epidermidis*	2,355	*erm*(C)	*repL*	-	M12730	2,373	pUR2940	*erm*(C), Δ*repL* (5', 3' end)	98.9
pKKS25	*S. aureus*	6,229	*tet*(L), *dfrK*, *erm*(T)	-	IS*Sau10*	FN390947	5,436	pUR2940	IS*Sau10*, *tet*(L), *dfrK*, *erm*(T)	99.9
pK214	*Lactococcus lactis*	29,871	*mdt*(A), *str*, *cat*, *tet*(S)	*repB*, *repD*, Δ*rep*, *mob*	*tnpR*, IS*904*, *tnpA*, IS*215*, IS*216*, IS*214*	X92946	1,574	pUR2940	*mob*	85.6
pKKS627	*S. aureus*	6,242	*tet*(L), *dfrK*	*repU*, *pre/mob*	-	FN390948	1,263	pUR2940	Δ*pre/mob* (3'end), Δ*repU* (5'end)	99.9
pUB110	*S. aureus*	4,548	*aadD*, *ble*	*repU*, *pre/mob*	-	M19465	1,263	pUR2940	Δ*pre/mob* (3'end), Δ*repU* (5'end)	99.9
pUR3912	*S. aureus*	6,176	*erm*(T), *cadD/X*	*rep*, *ssoA*	IS*431*	HE805623	735 / 2,275	pUR2940	*erm*(T) / Δ*rep49* (5'end), *cadD/X, ssoA*	98.9/85.5
pSW49	*S. warneri*	3,552	*smr/smr'*	*rep49*, *ssoA*	ISLE49	AM040730	1,061	pUR2940	Δ*rep49* (5' end)	75.5
							428	pUR1902	*ssoA*, Δ*rep49* (3' end)	87.6
pUB101	*S. aureus*	21,845	*cadD/X*, *blaI/R/Z*, *far1*	Δ*rep1*, Δ*rep2*, *repA*, *repB*	IS*431/257*, *tnp2*, *tnp1*	AY373761	2,838	pUR2940	*cadD/X*, *ssoA*, *repA*-like	80.1
							260	pUR2941	5' end of putative *rep*	72
pKKS825	*S. aureus*	14,362	*aadD*, *tet*(L), *dfrK*, *vga*(C)	*pre/mob*, *repU*-like, *mob*, *rep*	-	FN377602	1,112	pUR1902	*aadD*, Δ*repU* (3'end)	100
pERGB	*S. aureus*	15,259	*cfr*, *aadD*, *tet*(L), *dfrK*	*repU*, *pre*	IS*21-558*, *tnp*, *tnpA*	JN970906	1,112	pUR1902	*aadD*, Δ*repU* (3'end)	100
SAP024A	*S. epidermidis*	47,352	*cadD/X*	*mob*, *rep* associated, *repA*, *rep*	IS*431*	GQ900469	495	pUR2941	5' end of putative *rep* associated	86.9
SAP047A	*S. aureus*	28,974	*cadD/X*, *blaI/R/Z*	*repA*, *rep*	Δ*pre*, IS*431mec*	GQ900405	348	pUR2941	3' end of putative *rep* associated	78.5

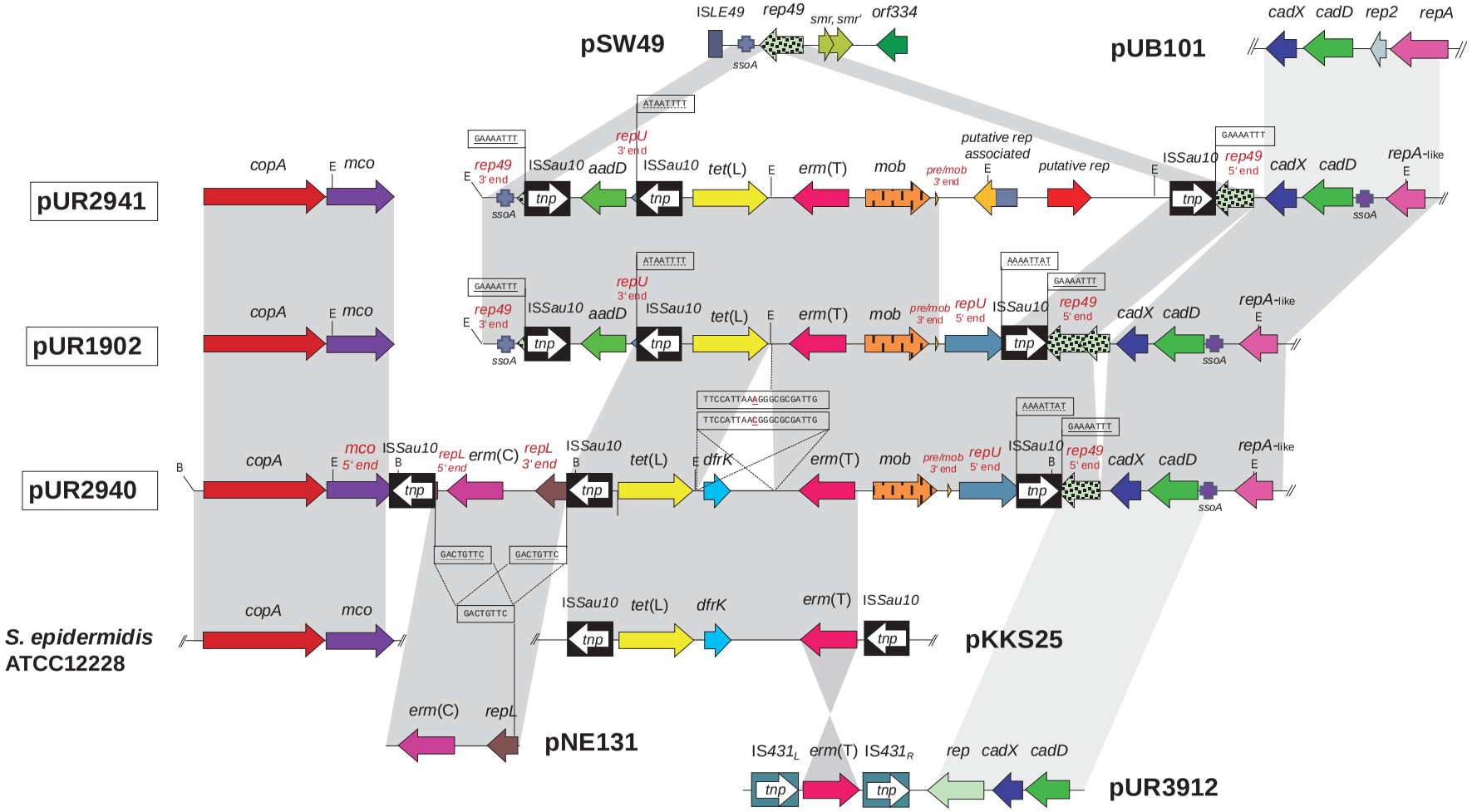

Figure S6. Colour version of figure 1 (**paper 9**).

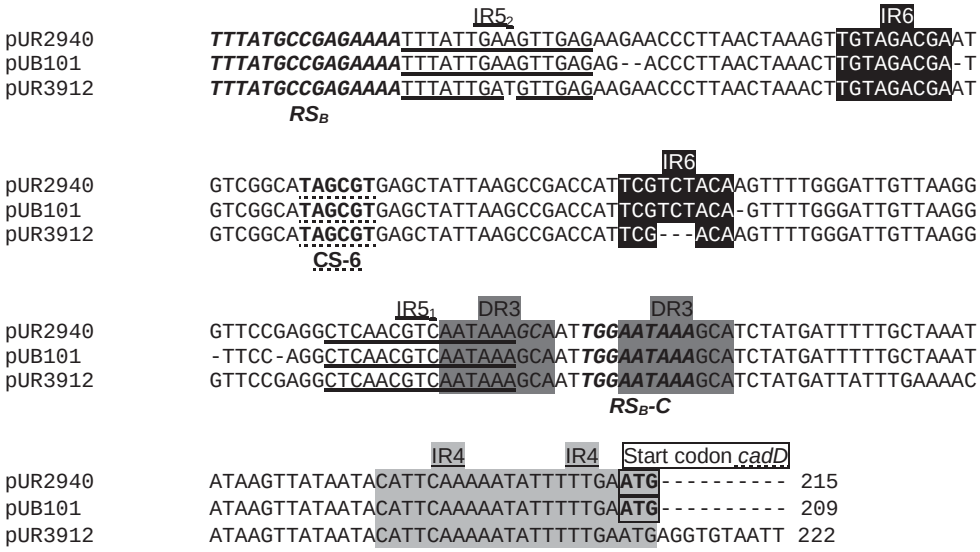

Figure S7. Comparative analysis of the 215-bp upstream region of representative pUR2940 [including the *cadD* start codon and covering the *ssoA* region (Kramer et al., 1998, O'Brien et al., 2002)] and the corresponding segment of pUR3912 (HE805623) and pUB101 (AY373761). The start codon of *cadD* is boxed and the direct (DR3) and indirect repeats (IR4 and IR6) are indicated by different gray shaded. Inverted repeat 5 ($IR5_1$ and $IR5_2$) are underlined. The recombination site B and complementary (RS_B and RS_B-C) of *ssoA* are indicated in italics and in bold letters. The conserved 6-nucleotide sequence (CS-6) of *ssoA* is dashed and in bold letters. Nomenclature of the direct and indirect repeats is based on that described by O'Brien et al., (2002).

pUR1902 PLASMID CURING IN C1902

Curing of plasmid pUR1902 in C1902. Plasmid curing was performed on selected strain C1902 [MLS_B resistance due to the presence of *erm*(T) gene alone] by culturing it in Brain Heart Infusion (BHI; Oxoid, Madrid, Spain) broth at 43ºC for 24h at 215 rpm followed by subculture into fresh BHI broth and incubation as before for ten consecutive rounds. Individual colonies obtained were plated on BHI agar and BHI agar supplemented with erythromycin (15 µg/ml) by replica plating. Colonies only grown on medium without erythromycin were subjected to plasmid extraction for evaluation of its plasmid content. MICs to the different antimicrobials and heavy metals investigated were determined on plasmid-free colonies as indicated above (Table 1). In addition, they were tested by PCR for the presence of the *erm*(T) gene (Table 2) as well as the rest of antimicrobial and metal resistance genes present in the original strain C1902.

Pheno- and genotypic characteristics of strain C1902/pUR1902$_{cured}$. Strain C1902/pUR1902$_{cured}$ revealed the maintenance of tetracycline, gentamicin and kanamycin resistance, encoded by the genes *tet*(M) and *aacA-aphD*, in addition to β-lactams resistance by *blaZ* and *mecA* genes. All resistance genes carried in C1902 [*erm*(T), *tet*(L), *aadD, cadD, cadX, mco, copA*] were absent in its isogenic pUR1902$_{cured}$ strain. Resistance values to $CdSO_4$ and $CuSO_4$ showed 4-fold [0.25 mM (64 µg/ml) to 0.06 mM (16 µg/ml)] and 2-fold [8 mM (1024 µg/ml) to 4 mM (512 µg/ml)] decreases, respectively, with respect to original C1902, confirming the functional activity of the pairs *cadD-cadX* and *copA-mco*, while resistance levels to $ZnCl_2$ did not vary.

Paper 10

Detection and analysis of transposon Tn*558* carrying a *fexA* variant that only confers resistance to chloramphenicol in canine *Staphylococcus pseudintermedius*

Elena Gómez-Sanz[1], Kristina Kadlec[2], Andrea T. Feßler[2], Myriam Zarazaga[1], Carmen Torres[1#], Stefan Schwarz[2]

[1] Biochemistry and Molecular Biology, University of La Rioja, Logroño, Spain

[2] Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany

Transposon Tn*558* integrated in the chromosomal *radC* gene was detected for the first time in *Staphylococus pseudintermedius*. It carried a novel *fexA* variant (*fexAv*) that only confers chloramphenicol resistance. The exporter FexAv exhibited two amino acid substitutions, Gly33Ala and Ala37Val, both of which seem to be important for substrate recognition. Site-directed mutagenesis that reverted the mutated basepairs to those present in the original *fexA* gene, restored the florfenicol resistance phenotype.

Keywords: florfenicol, efflux, substrate recognition, antimicrobial

In staphylococci, phenicol resistance is mediated either by the chloramphenicol acetyltransferase encoding *cat* genes, which confer resistance to non-fluorinated phenicols (e.g. chloramphenicol) or any of the two genes *fexA* (coding for a phenicol-specific efflux pump) or *cfr* (coding for a rRNA methyltransferase), both of which mediate combined resistance to fluorinated (e.g. florfenicol) and non-fluorinated phenicols (1). Since the first description of the *fexA* gene in a bovine *Staphylococcus lentus* isolate (2), this gene has been detected - either as part of the small non-conjugative transposon Tn*558* or in combination with the *cfr* gene in transposition-deficient Tn*558* variants - in *S. aureus* and several coagulase-negative staphylococci from healthy and diseased cattle, swine, horses or humans (3-9). The gene *fexA* has also been detected in a *Bacillus* isolate from swine faeces and in environmental pollutants from swine feedlots in China (10, 11).

In a previous study that focused on the occurrence of methicillin-resistant coagulase-positive staphylococci in dogs in La Rioja, Spain (12), one methicillin-susceptible *S. pseudintermedius* isolate, named C2719, was identified. Susceptibility testing by agar disk diffusion and/or broth microdilution (13) showed that this isolate was resistant to penicillin (due to the *blaZ* gene) and to chloramphenicol (MIC 64 µg/ml), but exhibited a low MIC of 2 µg/ml for florfenicol. None of the three *cat* genes known to occur in staphylococci - cat_{pC194}, cat_{pC221} and cat_{pC223} (1) - was detected by PCR (14). The objective of this study was to investigate the genetic basis for chloramphenicol resistance in this strain and to characterize its genetic environment.

PCR analysis for the chloramphenicol/florfenicol resistance genes *fexA* and *cfr* (5) revealed that, despite its low florfenicol MIC, strain C2719 harbored the *fexA* gene. PCR mapping and sequencing revealed that this *fexA* variant, named *fexAv*, showed 99.7% nucleotide sequence identity (99.2% identity and 99.6% similarity at protein level) to the prototype *fexA* gene of *S. lentus* (GenBank accession no. AJ549214) (2). Moreover, the *fexAv* gene was found to be part of the transposon Tn*558* (3, 15) (Fig. 1a). The chromosomal/plasmid location of Tn*558,* as well as its specific integration site, was determined by plasmid preparation, specific PCRs, and sequencing (16, 17). As no plasmids were detected in *S. pseudintermedius* C2719, a chromosomal location of the *fexAv*-carrying transposon appeared to be likely. Primers radC_SP-fw and radC_SP-rv (Table 1) were used to determine whether Tn*558* was integrated within the chromosomal *radC* gene (coding for a DNA repair protein), as it has been observed for this

transposon and others of this family, such as Tn*559* and Tn*554*, in other staphylococci and enterococci (17-19). An amplicon of 7,082 bp that comprised the complete Tn*558* (6,645 bp) and part of the *radC* gene (437 bp) was obtained (Fig. 1a). The attachment site (*att558*) of Tn*558* in the *radC* gene of isolate C2719 and its comparison with that observed in the prototype Tn*558* of *S. lentus* are shown in Fig. 1b. Complete sequence analysis of this transposon revealed 99.7% nucleotide identity to that of *S. lentus* (GenBank accession no. AJ715531). It presented three semi-conserved amino acid (aa) substitutions in two reading frames: (i) Gly321Ala in the transposase protein TnpA (nucleotide G962C in the transposase gene *tnpA*), and (ii) Gly33Ala and Ala37Val in the FexAv protein (nucleotides G98C and C110T, respectively, within the *fexAv* gene) (Fig. 1a). Four additional conserved aa substitutions were observed in Tn*558*: (i) Lys515Gln and Val526Leu in the transposase TnpB (nucleotides A1543C and G1576C, respectively) and (ii) Leu67Ile and Ile131Val within the FexAv protein (nucleotides C199A and A391G, respectively). No circular intermediates of Tn*558*, which are indicative for the mobility of the transposon, were detected neither under normal growth conditions nor after exposure to ultra-violet light, anaerobiosis or heat shock conditions.

To confirm that the *fexAv* gene is in fact responsible for chloramphenicol but not for florfenicol resistance, a PCR assay using primers entire_fexA-1 and entire_fexA-2 (Table 1) that amplified the complete *fexAv* gene (1,428 bp) including 201 bp and 452 bp of its upstream and downstream region, respectively, was conducted. This 2,081-bp amplicon was first cloned into the pCR® 2.1-TOPO® vector and transformed into the recipient strain *Escherichia coli* TOP10 using the TOPO TA Cloning® Kit (Invitrogen, Groningen, The Netherlands). Subsequent transformation into *E. coli* HB101 was conducted to test the functionality of the *fexAv* gene in an appropriately phenicol-susceptible *E. coli* recipient strain (chloramphenicol MIC 2 µg/ml and florfenicol MIC 4 µg/ml). *E. coli* HB101 carrying the recombinant vector exhibited a 16-fold increase in the chloramphenicol MIC (MIC 32 µg/ml) while the florfenicol MIC value remained unchanged.

In a study on the chloramphenicol/florfenicol efflux protein FloR, Braibant et al. (20) identified by site-directed mutagenesis (SDM) that the residue Asp23 seems to participate directly in the affinity pocket involved in phenicol derivative recognition. When Asp23 was mutated to Glu23, the respective FloR protein still conferred chloramphenicol resistance, but lost its ability to export florfenicol. A model (http://www.ch.embnet.org/software/TMPRED_form.html) of the transmembrane segments (TMS) of the 12-TMS-protein FloR and the 14-TMS-protein FexA predicted that the residues Asp23 in FloR and the Gly33/Ala37 in the prototype FexA are all located in the transmembrane segment 1 and hence may have similar functions. Based on these data, SDM was conducted on the *fexAv* nucleotide substitutions G98C and C110T to revert them back to those of the prototype *fexA* from *S. lentus.* For this, an inverse PCR with specific "mutagenesis primers" (fexA_33+37-inv1 + fexA_33+37-inv2) was performed (Table 1). A plasmid preparation (Qiagen Plasmid Midi Kit, Qiagen, Hilden, Germany) of the aforementioned recombinant pCR® 2.1-TOPO® vector served as template for SDM. After SDM, digestion of the obtained SDM products with the restriction enzyme *Dpn*I (Fermentas, St. Leon-Rot, Germany) was performed to eliminate the original methylated template plasmid DNA. An aliquot of the SDM approach was then transferred by electrotransformation into *E. coli* HB101. Correctly mutated recombinant vectors (TOPO/$fexA_v^{C98G+T110C}$) were confirmed by PCR of the complete *fexA* gene and sequence analysis of the amplicon (Table 1). Macrodilution assays for chloramphenicol and florfenicol (13) showed a 4-fold higher florfenicol MIC of 16 µg/ml for the *E. coli* HB101 transformants carrying TOPO/$fexA_v^{C98G+T110C}$ compared to the recipient strain alone (MIC 4 µg/ml), while the MICs to chloramphenicol remained unchanged (MIC 32 µg/ml). This increase in the florfenicol MIC was similar to that described for *E. coli* JM109 carrying the cloned *fexA* gene from *S. lentus* (2) and confirms that reversion of the naturally mutated positions restores the florfenicol resistance phenotype.

Nucleotide sequence accession number. The 7,698-bp nucleotide sequence enclosing the complete Tn*558* and the *radC* gene of *S. pseudintermedius* strain C2719 has been deposited on the EMBL under accession number HF679552.

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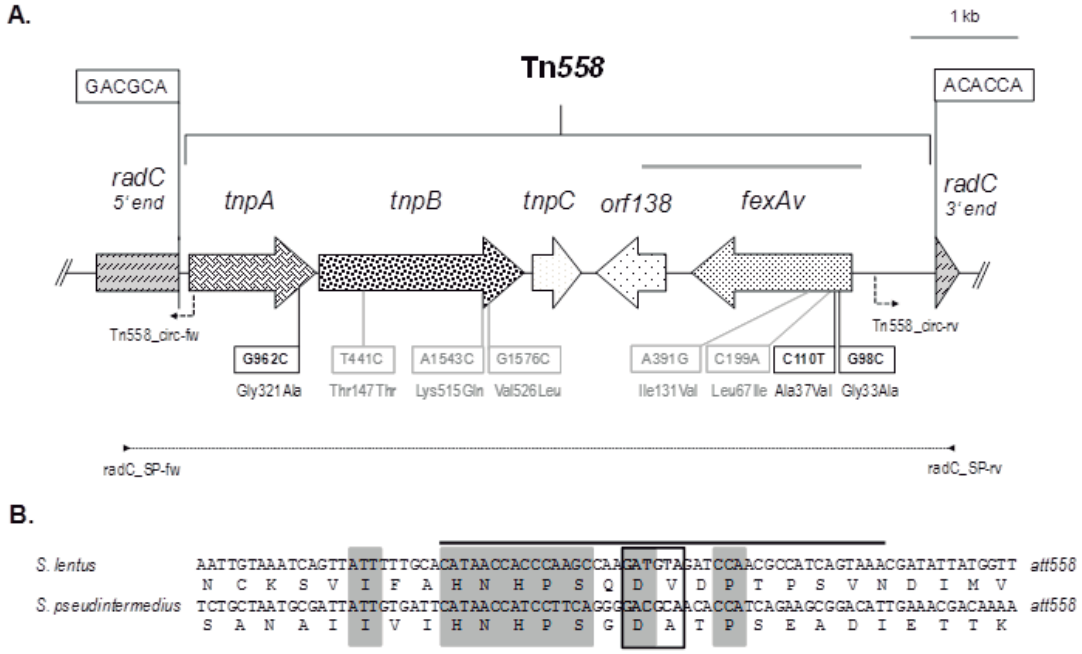

Figure 1. A) Schematic presentation of the Tn*558* structure carrying the gene *fexA*$_V$ detected in this study as well as its integration region (within the *radC* gene) into the chromosomal DNA of *S. pseudintermedius* C2719 (EMBL accession no. HF679552). The arrows indicate the extents and directions of transcription of chloramphenicol resistance (*fexAv*) and transposase (*tnpA, tnpB, tnpC*) genes, as well as the reading frame *orf138*. The 6-bp core nucleotide sequences at the transposon junctions are shown within boxes. The positions of primers used to amplify the region between both *radC*-gene extremes is indicated as an arrow head with a dashed-line showing the extension length. The primers employed to detect circular intermediates, as well as the direction of amplification, are also shown. The gray bar over the *fexAv* gene indicates the region that has been used for cloning and mutagenesis experiments. A size scale in kilobases (kb) is displayed in the upper right-hand corner of the figure. B) Nucleotide and amino acid sequence alignment of the attachment sites *att558* in *S. lentus* (GenBank accession no. AJ715531) and *S. pseudintermedius* C2719 (EMBL accession no. HF679552). Gray boxes indicate identical amino acids found in both sequences. The hexanucleotide core sequences of the integration sites are framed. The black bar above the *att558* sequences indicate the minimum sequence required for transposition into this site as determined by deletion analysis.

Table 1 Primers used to detect the chromosomal integration site of the *fexA*-carrying Tn*558* of *S. pseudintermedius* isolate C2719, those to amplify the complete *fexA* gene and those to perform the site-directed mutagenesis (SDM).

Name	Primer (5'→3')	Amplicon (bp)	PCR conditions	Polymerase
radC_SP-fw	GTTTGTCGGAATAGGGCGTA	437[a]/7,082	1 min 94ºC; 30 cycles of 10 sec at 98ºC, 15 min at	La Taq (Takara)
radC_SP-rv	ACGATTCTTCCCCAATCACA		68ºC; 10 min at 72ºC	
entire_fexA-1	GATCCGTAAGCCCATCCATA	2,081	3 min at 94°C; 30 cycles of 30 sec at 94ºC, 45 sec at	BioTaq (Bioline)
entire_fexA-2	AGGCACCGGTTGTTAAACTG		55°C, 2 min at 72°C; 5 min at 72°C.	
fexA_33+37-inv1	CTATCTGTACTTGTAG**G**TGCAATTACGG**C**TGATTTAGTCAATCCC	6,012[b]	3 min at 98°C; 20 cycles of 1 min at 98ºC, 1 min at	Phusion (Finnzymes)
fexA_33+37-inv2	GGGATTGACTAAATCA**G**CCGTAATTGCA**C**CTACAAGTACAGATAG		55°C, 2.5 min at 72°C; 10 min at 72°C.	

Nucleotides in bold type and underlined are those modified by the SDM PCR.

[a] Amplicon obtained if an intact copy of the *radC* gene was present.

[b] Size of the complete recombinant and mutated plasmid, composed of the pCR® 2.1-TOPO® vector (3,931 bp) and the insert obtained with the entire_fexA-1 and entire_fexA-2 primers (2,081 bp).

Annex to paper 10

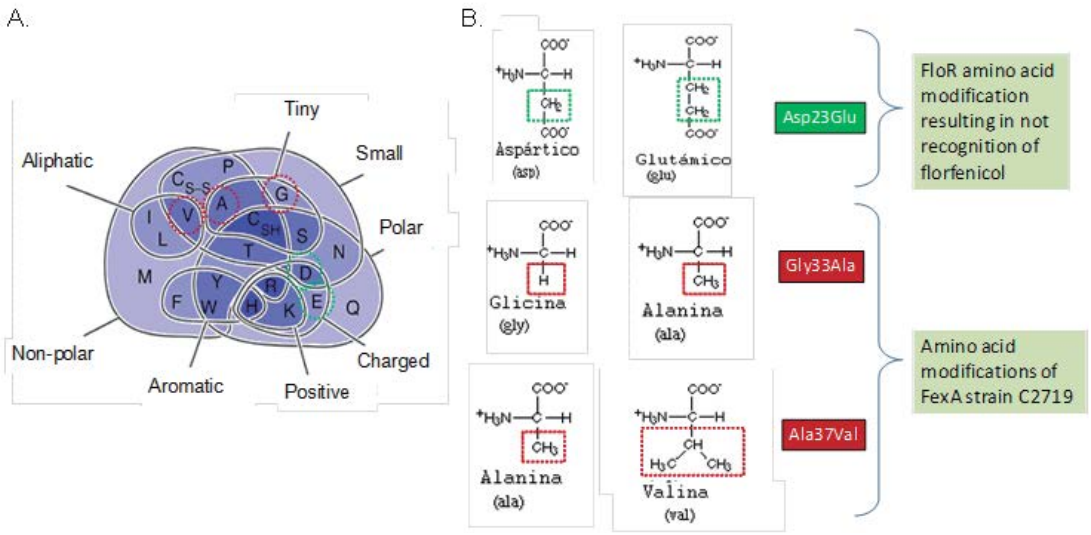

Figure S8. **A.** A Venn diagram showing the relationship of the 20 naturally occurring amino acids to a selection of physio-chemical properties thought to be important in the determination of protein structure. The amino acids of interest for the FexAv are circled in red while those relevant for FloR are circled in green. **B.** Amino acid substitution in FloR protein of *Salmonella* that led to non-recognition of florfenicol (Braibant et al., 2005) and amino acid substitutions found in FexA of *S. pseudintermedius* that were revealed to be involved in absence of florfenicol resistance.

Discussion

CHAPTERS 1 and 2. *S. aureus* AND *S. pseudintermedius* IN DOGS OF DIFFERENT ORIGIN, OWNERS AND COLONIZATION DYNAMICS OF CASES OF INTERSPECIES TRANSMISSION.

S. aureus and *S. pseudintermedius* in dogs and their owners at the household and clonal dynamics of cases of interspecies transmission.

A high *S. aureus* prevalence of 41.8% (40.3% MSSA and 1.5% MRSA) was observed among tested owners. *S. aureus* is a natural colonizer of the nasal mucosae of humans, and approximately 27% of healthy persons carry *S. aureus* (Wertheim et al., 2005). This elevated occurrence observed here could be, in part, attributed to the coexistence with pet animals, although other causes such as the role of contaminated surfaces or fomites were not investigated in these settings. None of the tested owners presented occupational exposure to the healthcare system, what could also have explained the high *S. aureus* values detected. In contrast, a recent study reported an unexpectedly low *S. aureus* nasal occurrence among dog owners (18.5%) (Walther et al. 2012). However, in that study, pre-enrichment of the samples was not performed, what may be responsible for the low rates detected. Values of 24 (Boost et al., 2008) and 28% (Hanselman et al., 2009) were found among owners in former studies at the household setting. Therefore, surveillance is needed to unveil the role of pets in the propagation of *S. aureus* among in-contact persons. On the other hand, nasal MRSA colonization among healthy persons is very low, normally less than 1% (Lozano et al., 2011a). In this study, a single *S. aureus* isolate from one owner corresponded to a MRSA strain, it belonged to the lineage ST8, was PVL positive and MDR. ST8 is enclosed within the traditional CC8 and is a worldwide distributed MRSA lineage, especially in the US (known as USA300). MRSA PVL+ occurrence in healthy carriers is low, normally corresponding to isolates of such lineage. MRSA PVL + strains have been already detected among companion animals (Rankin et al., 2005). Moreover, human MRSA PVL+ infections caused by a strain originally detected in a pet animal has been already detected (van Duijkeren et al., 2005), and dog infections caused by CC8-MRSA PVL+ have been recently observed (Haenni et al., 2012), evidencing host transmissibility of toxigenic MRSA strains.

Three of tested humans (4.5%) carried *S. pseudintermedius* while MRSP was not recovered in this group. *S. pseudintermedius* in humans without dog contact is expected to be highly uncommon (<1%) (Mahoudeau et al., 1997; Talan et al., 1989). Despite the narrow host spectrum believed for this bacterium, sporadic cases of both MSSP colonization and infection in humans coexisting with pet animals have been recently reported (Boost et al., 2008; Hanselman

et al., 2009; Walther et al., 2012). A recent study revealed 3.9% MRSP nasal occurrence among small animal practise veterinarians (Paul et al., 2011), whereas MSSP was not detected. The authors suggested that MRSP might have a particular ability to colonize humans compared to MSSP. However, in the present study, only MSSP was recovered from owners. Hanselman et al., (2009) also reported that 4.5% of tested pet owners carried *S. pseudintermedius*; although 42% of tested owners were veterinarians. In the present study, all owners were healthy and did not exhibited occupational exposure to animals. These results suggest that dog contact do increment the possibility to carry *S. pseudintermedius*.

A total of 9.3% of dogs and 25% of cats (12% of pets) carried *S. aureus*, whereas no MRSA was detected. The presence of *S. aureus* in pets is relatively common. Hanselman et al., (2009) reported rates of 14% among healthy dogs, Boost et al., (2008) observed 8.8% nasal occurrence among the dogs sampled whereas Walther et al, (2012) revealed a remarkably low value of 1.8%. *S. aureus* in dogs and cats has traditionally been associated with the presence in their pet owners (Loeffler et al., 2010a). The high *S. aureus* occurrence detected in cats in the present study should be taken carefully, due to the low number of cats investigated. Similarly to humans, MRSA prevalence among pets is very low (0-1%) (Loeffler et al., 2010a; Weese et al., 2010b) only with elevated values when coexisting with people with higher risk of colonization, such as MRSA colonized or infected owners, or healthcare workers, among others (Faires et al., 2009; Morris et al., 2012).

S. pseudintermedius was detected in 26% of dogs and 8.3% of cats. Dogs are the natural host for *S. pseudintermedius*, with different rates depending on the body-site. Highly variable nasal values have been detected in dogs (16-64%), with mouth (42-74%) and perineum (28-72%) as the sites with higher *S. pseudintermedius* recovery rates (Bannoehr et al., 2012). According to published data, MRSP rates among healthy dogs are lower than 4.5%, what matches with the values detected in this study, with 3.7% of dogs positive for MRSP. This bacterial species is an opportunistic pathogen whose vigilance is necessary, in special MRSP, for several reasons: (i) it is an emerging canine pathogen, (ii) MRSP occurrence is increasing in the canine population and virtually all strains are MDR and (iii) it has zoonotic potential and human infections caused by this bacterium have been reported.

One of both MRSP isolates corresponded to the ST71, the well-known clonally spread European MDR MRSP clone, which has been for the first time described in Spain in this thesis.

(see **paper 3**). The other strain belonged to ST92, an uncommon lineage that was detected for the first time in a canine MSSP isolate in Switzerland (unpublished data).

In 28 of the 43 sampled households (65%) there was at least one individual (either owner or pet) that carried CoPS. In 10 of these 28 positive households, there was at least one owner and one pet positive for CoPS. These data show a real flow of these bacterial species within household settings, and subsequently, the risk of bacterial transference among the different in-contact individuals.

Molecular characterization of isolates allowed the identification of five cases of direct interspecies transmission in 5 households (11.6% of sampled residences) (**Fig. 22** and **Fig. 23**). In 4 of them, an identical MSSA clone was isolated from at least one owner and one pet, suggesting a human origin (anthropozoonotic transmission). In two cases, strains recovered belonged to CCs classically associated with hospital acquired *S. aureus* (CC45 and CC121), what supports the hypothesis of a human origin. A novel lineage (ST1654, singleton) was involved in a third household, whose plausible origin was at the moment unknown. A longitudinal carriage study was performed in members (humans and pets) of these households, which aimed at unveiling the real origin of the isolates (see below). In the remaining household, the human and canine recovered *S. aureus* clone belonged to ST109, which is included in CC9. *S. aureus* ST9, the predicted founder of this traditional clonal group, was first detected among MSSA isolates from pigs in France (Armand-Lefevre et al., 2005) and it is nowadays a well-known LA-MRSA lineage, being detected in pigs and pig handlers in China and in Southeast Asia (Crombé et al., 2013; Wagenaar et al., 2009 Monecke et al., 2011;). The positive individuals neither had contact to livestock nor to rural areas. Unfortunately, due to the owner's refusal to participate in the longitudinal study, the plausible source and direction of transmission of this clone remains unknown.

The last case of direct suspected interspecies transmission corresponded to a MDR MSSP that was recovered from both owner and dog, with a presumable animal origin (zoonotic transmission). This represents the first description in literature of a case of interspecies transmission due to a MDR MSSP, and enhances the interest in MSSP when addressing the *S. pseudintermedius* potential zoonotic transmission. Fortunately, these individuals could be followed up in the longitudinal study.

In five additional households*, S. aureus* or *S. pseudintermedius* indirect interspecies transmission was suspected (**Fig. 22** and **Fig. 23**). Interestingly, two unrelated owners from also

carried *S. pseudintermedius*, while their dogs were negative. A suspected canine origin is expected, and both cases could be included in the longitudinal study.

In addition, pets from three households (2 cats, 1 dog) were *S. aureus* carriers whereas their tested owners were negative. A presumable human origin was suggested. The dog carried a *S. aureus* CC121 strain, which is a common hospital-associated *S. aureus* lineage. The longitudinal study was also conducted in this dog and its owner. The positive cats carried either *S. aureus* CC5 or CC8, well-known community-associated human lineages. Nevertheless, the source of *S. aureus* cannot be always attributed to the human as *S. aureus* of CC5 and CC8 in pets are not exceptional. In fact, CC5 appears to be the predominant lineage of *S. aureus* that causes infection of small animal patients and that colonize small animal veterinary practitioners (Morris et al., 2012; Pinto-Ferreira et al., 2012; Weese et al., 2006;). Furthermore, *S. aureus* CC8 has been also detected in several occasions in pet animals, including cases of infections (Haenni et al., 2012). Unfortunately, none of these households could be followed up in the longitudinal study and so, the evolution of *S. aureus* in cats and its possible interspecies transmissibility could not be established.

Seven of the 10 interspecies transmission cases could be followed up in a longitudinal approach to investigate the evolution of the reported cases as well as the population structure of both *S. aureus* (**Fig. 22**) and *S. pseudintermedius* (**Fig. 23**) along time.

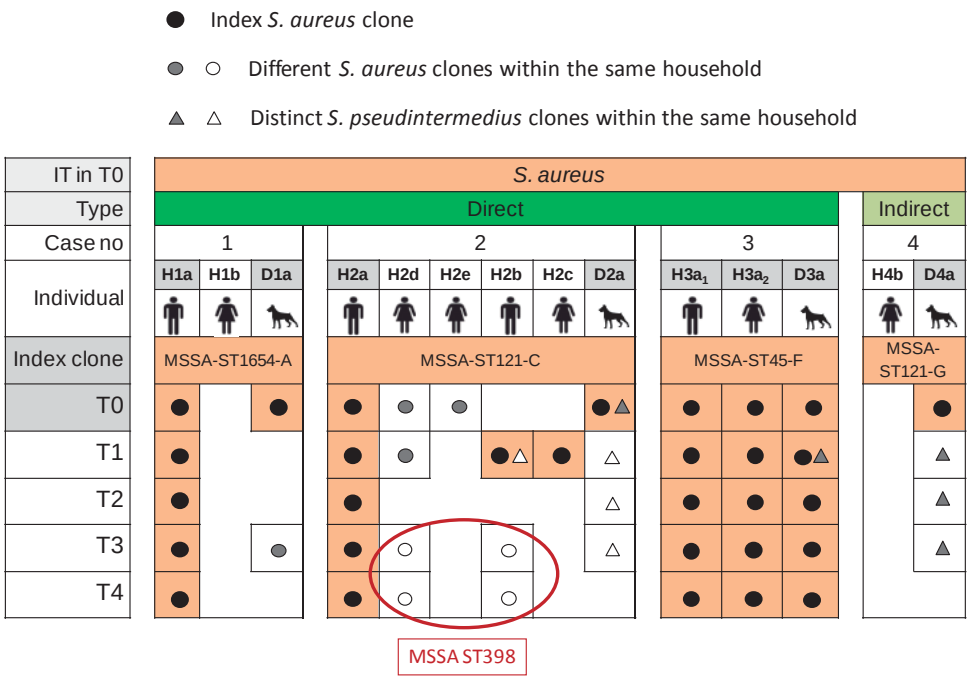

Figure 22. Direct and indirect cases of suspected *S. aureus* interspecies transmission (IT) between owner and pet. Index clone, clone detected in both owner and pet at the same sampling in T0 (cross-sectional study). Orange background represents the presence of the index clone.

All cases of suspected *S. aureus* direct interspecies transmission (cases 1 to 3) evidenced that the owner was the real sources of the MSSA pet acquisition, since all index persons were persistent carriers of the clone involved, while only one dog resulted a persistent carrier (**Fig. 22**).

Results obtained in case 3 deserve special attention because an identical *S. aureus* CC45 was detected in all members the household (**Fig. 22**). The longitudinal study unveiled that MSSA CC45 was persistently present in both owners and dog (the cat stopped living in the household soon after the first sampling), what evidenced either (i) a recurrent contamination of the dog from its positive owners, (ii) recurrent acquisition from contaminated surfaces of the household or (iii) a real colonization status of this animal. All in all, this pet represents a source of *S. aureus* for human re-acquisition and maintenance, what emphasizes the role of pets in the global epidemiology of *S. aureus*; data that should be taken into account when addressing infection control and prevention strategies.

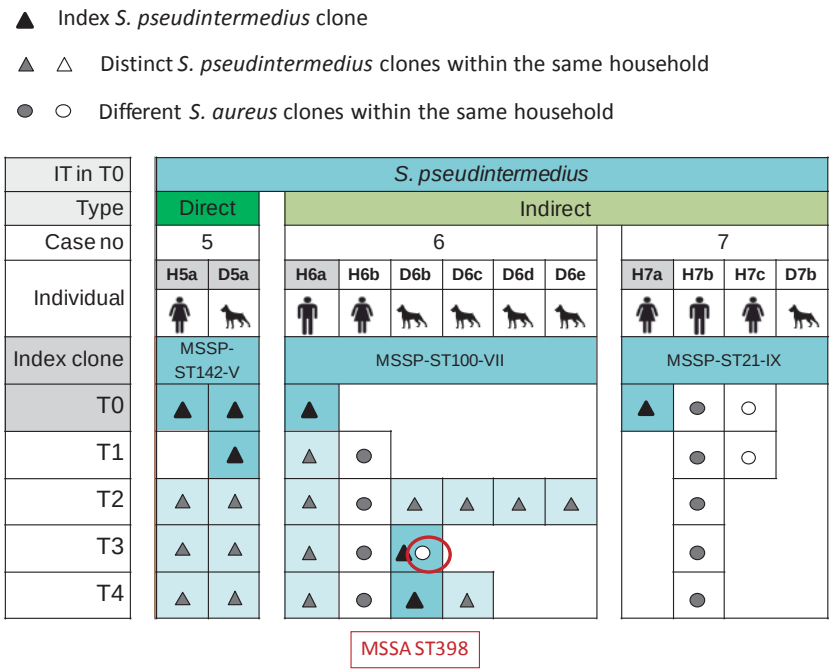

Figure 23. Direct and indirect cases of suspected *S. pseudintermdius* interspecies transmission (IT) between owner and pet. Index clone, clone detected in both owner and pet at the same sampling in T0 (cross-sectional study). Intense blue background represents the presence of the index clone; faint blue background represents the detection of a different *S. pseudintermedius* clone in the index individuals or their dogs, where index individual refers to those individuals involved in the different cases of interspecies transmission.

The longitudinal study on the single case of direct zoonotic transmission by a MDR MSSP strain (ST142) (case 5) revealed that both, owner and dog were persistent carriers of *S. pseudintermedius*. However, they were colonized by a different MSSP clone in the subsequent samplings, what evidences the clonal diversity and strain replacement of *S. pseudintermedius* within the same host. A recent study that focused on the dynamics of *S. pseudintermedius* in healthy dogs with multiple sampling sites, revealed that between two and five clones were present in the same animal (Paul et al., 2012). Further, replacement of *S. pseudintermedius* clones in an individual's microbiota over time has been previously reported as common in a former longitudinal study on *S. pseudintermedius* in dogs (Paul et al., 2012). Both owner and dog lived separately for several months between the first and the second sampling, which *S. pseudintermedius* absence in owner in T1 matches with the idea that this strain was transmitted from the dog. As aforementioned for *S. aureus* CC45 in the dog, possible recurrent contamination of *S. pseudintermedius* from the dog or common surfaces is also plausible.

Addressing the indirect cases reported in the cross-sectional study, the longitudinal approach on *S. aureus* CC121 (case 4) positive dog (**Fig. 22**) revealed that this animal was only a

sporadic *S. aureus* carrier, and a *S. pseudintemedius* was only recovered in subsequent samplings. Further, continued surveillance of *S. pseudintermedius*-carrying owners from the remaining two households (cases 6 and 7) (**Fig. 23**) revealed that one index owner was a persistent *S. pseudintermedius* carrier, while his dogs were intermittent or sporadic carriers. Again, strain replacement was observed in the isolates recovered from the owner and their dogs. Remarkably, both MSSP clones exhibited related MLST profiles and carried the exfoliatin *expA* gene, what might point to a divergent evolution of these isolates from a common ancestor.

The fact that *S. pseudintermedius* was maintained along the whole sample year in two owners reveals that humans can either be colonized for prolonged periods of time with this bacterial species or present easiness for recurrent acquisition. In these terms, the transmission capacities of these bacteria, as well as their survival ability on non-natural hosts, may have been overlooked.

Focussing on the overall carriage rates in the longitudinal study of the different individuals, a great rate of owners (81.3%) and dogs (90%) were positive for *S. aureus* or *S. pseudintermedius* at least at one sampling. In addition, the high percentage of *S. aureus* or *S. pseudintermedius* persistent carriers in investigated owners (56.3%: 43.8% by *S. aureus* and 12.5% by *S. pseudintermedius*) by the same or different clone is relevant. Although these data should be taken carefully due to the limited number of individuals investigated, the obtained values for *S. aureus* persistence are slightly higher than those reported in previous longitudinal studies on *S. aureus* in humans (≤36%) (Lebon et al., 2008; Lombholt et al., 2005; Sakwinska et al., 2010; van Belkum et al., 1997; Vanderbergh et al., 1999; VasanthaKumari et al., 2009).

The presence of *S. pseudintermedius* persistent owners, together with the absence of intermittent *S. pseudintermedius* human carriers might point to favourable conditions in specific individuals to be colonized by *S. pseudintermedius*. In contrast, both bacterial species were present in all carriage types (persistent, intermittent, sporadic) in dogs, with *S. aureus* being in most cases sporadically detected. These data suggest that *S. aureus* may be more frequently detected in the nares of dogs than *S. pseudintermedius* in the nasal cavity of humans but that both bacterial species can persistently colonize non natural hosts, at least in those cases with reiterate direct contact.

S. aureus and *S. pseudintermedius* carriage evolution showed different trends depending on the host from which they were recovered: (i) a single owner was positive for both bacterial species, what contrasts with the elevated number of positive dogs from which *S. aureus* and *S.*

pseudintermedius was recovered (4/9), and (ii) *S. pseudintermedius* was not detected in the *S. aureus*-persistent owners, and *S. aureus* was not present in the *S. pseudintermedius*-persistent, while two of the three persistently colonized dogs were sporadic nasal carriers of the other CoPS. These observations reflect the ability of dogs to temporary carry different staphylococci and suggest that these bacteria might behave differently depending on the host.

This work gains knowledge in the ecology of *S. aureus* and *S. pseudintermedius* in humans and in-contact dogs and represents the first approach to understand the role that dogs may play in the epidemiology of *S. aureus* and *S. pseudintermedius* in the human host. Results from both, the cross-sectional and the longitudinal study give evidence of a real flow of both bacterial species within pet-owning household settings. The detection of interspecies transmission cases for both bacterial species, their ability to persistently colonize the human host, as well as the apparent predisposition of dogs to be sporadic carriers of *S. aureus* should be taken into account in subsequent developments of infection control measurements. Further longitudinal studies on *S. aureus* and *S. pseudintermedius* with a larger cohort in humans and in-contact dogs are essential for confident assertions.

Although none of the animals tested carried MRSA and dogs are not the major host of *S. aureus*, its vigilance is important for their potential role in the transmission of *S. aureus* at the household. Close contact between pets and their owners creates favourable conditions for MRSA transmission (Bramble et al., 2011), but it should not be forgotten that they may also represent secondary reservoirs of MSSA.

Concurrent carriage of *S. aureus* or *S. pseudintermedius* and MRCoNS

Concurrence of *S. aureus* or *S. pseudintermedius* and MRCoNS was common among our tested population: high rates of owners (53.9%) and dogs (33.3%) presented both types of staphylococci in at least one sampling, what reflects a plausible punctual coexistence of such bacteria. Concurrence of *S. aureus* and MRCoNS has been previously reported to have a negative association due to competition for the same ecological niche (Johannessen et al., 2012; Weidenmaier et al., 2012). Moreover, *S. epidermidis* is considered to “protect” the host from *S. aureus* colonization (Johannessen et al., 2012).

Persistent concurrence of *S. aureus* or *S. pseudintermedius* and (MDR) methicillin-resistant S*. epidermidis* was observed in two owners (12.5% of investigated humans), one of them carrying the identical *S. pseudintermedius* and methicillin-resistant *S. epidermidis* clones thorough the sample year. Although the possible negative/positive influence that methicillin-resistant S*. epidermidis* may exert on the *S. pseudintermedius* colonization has not yet been investigated, these data show that co-colonization of both bacterial species is possible.

In addition, although the acceptance of exogenous genetic material between staphylococci does seem to require special circumstances not yet properly understood (Bloemendaal et al., 2010), transmission of the *mec*A gene between MRCoNS and *S. aureus* has been previously assumed (Berglund et al., 2008; Bloemendaal et al., 2010; Hanssen 2004). With this regard, it is important to remark that half of MRCoNS clones were MDR with a high diversity of AMR profiles. Macrolides resistance was commonly observed in among MRCoNS recovered isolates.

Our data give evidence on the possible coexistence of MDR staphylococci and *S. aureus* or *S. pseudintermedius* within the same ecological niche, and suggest that such concomitance may increment the possibility for horizontal gene transference.

S. aureus and *S. pseudintermedius* in pound dogs.

Pound dogs sampled in this thesis corresponded to the 3.9% of dogs rescued in La Rioja in 2009 (98/2,492). All animals tested were euthanized and nasal samples were taken once the animal was deceased. A total of 82 dogs were rescued in rural areas (<25,000 inhabitants) and 16 dogs originated from urban areas (>150,000 inhabitants).

Twenty-two dogs were positive for *S. pseudintermedius* (22.5%) (only four animals came from urban areas and the rest from rural). These values are approximate to the overall mean rate recovered from the nasal passages of these animals (31%) (Bannoehr et al., 2012). Interestingly, 8% of animals carried MRSP (half from urban areas and half from rural). Since MRSP rates in household healthy dogs in the community are relatively low (≤4.5%) (Bannoehr et al., 2012; EMAa, 2010; Walther et al., 2012; van Duijkeren et al., 2011; Weese et al., 2010), the values obtained in this work are remarkably high. On the other hand, there is very scarce data on the MRSP occurrence in shelter animals. One report found 3% of shelter dogs positive for MRSP

(Gingrich et al., 2011), while another publication detected a MRSP occurrence of 16.7% among individuals from a dog rescue shelter in Hong Kong (Epstein et al., 2009). However, no information was facilitated on the living conditions or potential animal contact of tested dogs in the later study. In the present study, positive pound dogs were captured in different locations, at different periods, and were hosted in different cages prior sacrifice. Therefore, it can be estimated that they were not directly related animals. However, possible transmission within the shelter house cannot be discarded.

The data reported here indicate that MRSP seem to be more prevalent in shelter than in household animals (household, 3.7%; kennel, 8%). The reason for this difference could be due to several factors: (i) the dog-to-dog contact, (ii) the bacteria present in the environment of the shelter house, (iii) poor hygienic conditions of the kennels and (iv) the in-contact environment of the animals prior rescue. Whether the origin of the animal capture (rural, urban) is relevant for the MRSP occurrence remains unknown. Based on this finding, occupational exposure to these animals (shelter workers, volunteers, veterinarians, etc), and persons adopting dogs, might be at higher risk to present MRSP.

The detection of MRSP ST71 (in 7 of the 8 MRSP isolates) represented the first description in Spain of this clonally spread MDR lineage at that time. As reported previously, this clone is the predominant MRSP lineage detected in Europe, and MSSP strains of this lineage have not yet been reported (Bannoehr et al., 2012). This clone has been already detected as causative agent of human infections in people in contact to positive pets (Stegmann et al., 2010), what evidences its zoonotic pathogenic properties. The remaining MRSP was typed as ST26. This lineage in MRSP had been previously detected in Japan and in the USA (Bannoehr et al., 2007; Black et al., 2011), but, to our knowledge, this study reports its first description in Europe.

MRSP has been shown to be relatively common in healthy dogs, especially dogs from shelter houses, with MRSP ST71 as predominant clone. These data give additional evidence of the success and emerging spread of this MDR clone. The presence of multiple AMR genes and the plausible coinheritance of some of them lead us to consider this MRSP as an important reservoir of antimicrobial resistance determinants, with the corresponding difficulties implied in the treatment of infections. Given the close contact of pet animals and humans and its description as causative agent of human infections, monitoring studies are warranted to properly assess possible human health implications.

An unexpectedly high *S. aureus* (24.5%) nasal occurrence was detected. These values are notoriously higher than previous reports among dogs with direct human contact (1.8-14%) (Boost et al., 2008; Hanselman et al., 2009; Walther et al., 2012). However, as abovementioned, very scarce data is available on the occurrence of *S. aureus* in shelter or pound dogs, and most studies are biased in favour of MRSA (Gingrich et al., 2011; Kasprowicz et al., 2011; Loeffler et al., 2010b). There was a surprisingly high *S. aureus* occurrence among dogs rescued in urban areas (43.8%) in comparison to those from rural areas (20.7%). However, also animals from rural areas presented high *S. aureus* nasal carriage rates, which double the values detected in the former cross-sectional study at the hosuehold (9.3%). These results are intriguing, since it is assumed that *S. aureus* in dogs originates from in-contact humans. The possibility that dogs that are exposed to different and variable external conditions (absence of animal cleansing as well as the surfaces in contact, especially kennels) favour their chances to carry *S. aureus* cannot be discarded. The high rate observed among dogs from urban areas might also respond to increase likelihood of exposure to human bacteria. Since *S. aureus* has been shown to be able to persist for prolong periods on inanimate surfaces, this possibility cannot be discarded. However, due to the limited number of animals tested, this suggestion is pure speculative.

No MRSA was detected in this study, what is in accordance to other reports with very low rates of MRSA among healthy dogs and also among those available from shelter animals (<1%) (Gingrich et al., 2011; Kasprowicz et al., 2011; Loeffler et al., 2010b).

According to the last report from Affinity Foundation on abandonment of dogs and cats, the number of adoptions from shelter homes is steadily increasing in the last years in Spain (www.fundacion-affinity.org). From the animals adopted in 2010, 78.5% of animals were adopted in Spain and 21.5% in other countries. These data raise interest in the presence of *S. aureus* and *S. pseudintermedius* in kennel animals, due to their possible role in the global epidemiology of these bacteria. Therefore, caution should be taken when facing staphylococcal human infections in persons with close contact to these animals.

This is the first report on *S. aureus* ST398 in dogs in Spain. Moreover, the presence of MSSA of the lineage ST398 in the majority of MSSA isolates obtained (29.2% of *S. aureus* recovered) is outstanding, and represents the first detection of MSSA ST398 in dogs in an epidemiological study. Very few data is available on *S. aureus* ST398 in dogs, all reporting MRSA, either as commensal or as causative agent of infection (Fessler et al., 2012; Floras et al., 2010; Haenni et al., 2012; Nienhoff et al., 2009; Reischl et al., 2009; Witte et al., 2007; Zhang et al.,

2011;). Among these, a respiratory disease MRSA ST398 outbreak in a litter of dogs at a breeding kennel in Ontario, Canada, has been reported (Floras et al., 2010). In that study, *S. aureus* was present in almost half of animals tested. These data shows the ability of this lineage to be present in "kennel environments" and to cause canine infections. To our knowledge, a single MSSA ST398 canine strain was included in a recent comparative genetic analysis of a convenience sample collection of human and canine isolates (Vinzce et al., 2012). Remarkably, that study concluded that high degree of similarity (by PFGE and DNA-microarray) was observed between the human and canine *S. aureus* strains investigated, pointing to the EHSGs of the investigated lineages (CC5, CC8, CC15, CC22, CC30, CC45, CC398), all of which were also detected among tested individuals in this thesis.

Few common lineages were detected in animals captures in rural and urban areas. ST133/CC133 [small ruminant associated lineage (Eriksson et al., 2012; Guinane et al., 2010)] and ST1/CC1 [community-associated lineage but also frequent in different animal species (horses, pigs, cattle, dog) (Aspiroz et al., 2010; Cuny et al., 2010; Pantosti, 2012)] were only detected in animals coming from small villages. Although the possible contact with farm animals could not be determined, the absence of the IEC genes in these isolates supports the idea of an animal origin.

Typical human associated lineages were also detected among tested animals, such as ST78/CC88 and ST45/CC45. It is interesting to remark that all isolates that belonged to traditionally host-narrowed human-associated lineages (all but ST398, ST133, ST1, ST2329) presented the *scn*, *chp* and/or the *sak* genes. Our IEC-carrying values (54.2%) are higher than those described by Verkaik et al., (2011) in cattle (33.8%) but remarkably lower than those recently detected by Vinzce et al., (2012) in canine isolates (79%). Further surveillance on the presence of the IEC genes in different animal species is needed to unveil its role in the estimation of the possible animal or human adaptation of a given *S. aureus* isolate.

Our results give evidence that dogs can be *S. aureus* carriers of not only typical human associated lineages but also of common lineages detected among farm animals. These data suggest that dogs may act as secondary reservoirs of the predominant *S. aureus* lineages of a given environment. However, the real origin of the isolates remains unknown and the cohort investigated was small for conclusive assertions. Continue surveillance on MSSA but also MSSP strains in animals is required to track the evolution of current successful lineages.

Comparative analysis of *S. aureus* and *S. pseudintermedius* isolates investigated from household individuals and pound dogs.

A comparative analysis of the *S. aureus* and *S. pseudintermedius* circulating lineages, concomitant carriage, antimicrobial resistance profile and virulence genes content of the isolates recovered from both cross-sectional studies was conducted to evaluate possible differences that might be related to the origin of the samples.

CIRCULATING GENETIC LINEAGES

High lineage diversity was detected in both bacterial species and in all individuals (household/kennel pets, owners), what contrasts with the high clonality of MRSP.

S. aureus. Seventeen different STs and 21 *spa* types were observed among the 36 *S. aureus* isolates from household individuals. It is important to remark that all *S. aureus* present in household pets exhibited lineages also detected among the human population under study. These data supports the idea that *S. aureus* lineages from companion animals usually reflect the epidemiology of human clones within the same geographic region (Loeffler et al., 2010a). Clonal lineages ST30, ST121 and ST45 were predominant (**Table S3**). MRSA of these lineages are well-known hospital and/or community acquired clones (Monecke et al., 2011). The presence of MSSA of these lineages in healthy individuals has been also documented, although few data are available on the epidemiology of MSSA lineages (Lozano et al., 2011a). Similar lineage diversity rates were detected among *S. aureus* from pound dogs, with 11 different STs and 13 *spa* types. Both farm animal (ST398, ST133) and human associated lineages (ST78, ST45, ST5) were observed (**Table S3**). Lineages present in both types of individuals (household, pound) and hosts (animal, human) were ST398, ST45, ST5 and ST146 (SLV of ST5) reflecting the ability of *S. aureus* from this genetic background to be present in both canine and human hosts. These lineages have been already considered EHSGs, being capable of infecting a broad range of hosts (Vinzce et al., 2012).

In addition, three distinct singletons were revealed, what highlights the capacity of MSSA isolates to evolve and diversify. Singletons reported here could represent novel lineages which future evolution and host tropism should definitely be tracked. As observed in the longitudinal study, ST1654 (present in both owner and his dog) seem to present a host tropism towards humans, since it was persistently present in owner and only sporadically detected in his dog.

***S. pseudintermedius*.** High diversity of lineages was detected in both populations investigated, with 14 different STs among the 18 *S. pseudintermedius* from household individuals and 16 from the 24 kennel dogs (**Table S4**). High clonal diversity of *S. pseudintermedius* is a common characteristic *mec*A-negative isolates, and evidence the great level of genetic diversity of this bacterial species. On the other hand, as stated above, methicillin-resistant isolates are represented by a limited number of highly successful clonal types. This is the case of our methicillin-resistant strains, with 80% of isolates belonging to ST71 (European clone), while ST68 (American associated clone) was not detected. MRSP ST71 was the only lineage detected in both populations sampled.

MSSP ST142 was present in a human, a dog, and a cat suggesting an EHSG. However, due to the recent development of an MLST scheme for this bacterial species (Solyman et al., 2013), and given that epidemiological data on the different MSSP lineages is not well documented, preferential host spectrum and ecology of *S. pseudintermedius* of different lineages cannot be estimated. In addition, the recent description of a novel and extended MLST scheme will challenge the few epidemiological data available on the former STs.

A total of 28.6% of *S. pseudintermedius* STs from household individuals were new, what contrast with the 58.8% observed among kennel animals. These later values are higher than recent reports on *S. pseudintermedius* from household dogs (<43%) (Walther et al., 2012). Since very scarce data is available on staphylococci from kennel dogs, it could be expected that this population of animals exhibited higher lineage diversity. However, this was not the case of *S. aureus*. Possible *S. aureus* contamination and spread at the pound house may be suggested, and would point to a higher promiscuity of *S. aureus* to be present in non natural environments than *S. pseudintermedius*.

Finally, our results on *spa* typing are in agreement with previous reports that showed non-typeability of the *spa* region among MSSP (Moodley et al., 2009), since 86.7% of MSSP isolates were *spa*-non typeable.

CONCURRENT CoPS CARRIAGE

Of the 152 dogs investigated, eight carried more than one CoPS isolate. Of these, seven animals corresponded to pound dogs (7.1% of positive pound animals) and only one from household settings (1.9% of the positive pets) (**Table 5**). Curiously, MSSA ST398 (4 of 8) and MRSP ST71 (3 of 8) were overrepresented in concomitance with other strain. The fact that 4 of

these animals presented MSSA ST398 isolates warrants further investigations on their potential favoured status to, at least sporadically, share the nasal ecological niche with other MSCoPS species. Given that all but one animal that carried two different staphylococci were pound dogs, a higher risk of acquisition of these bacteria in kennel environments is suggested.

Table 5. Characteristics of the CoPS detected in dogs that concurrently present more than one CoPS.

Description	**Dog origin**	**CoPS concomitant isolates**			
		Isolate	Type and ST	Isolate	Type/ST
Different CoPS species	Pound	C3473	**MSSA ST398**	C2382	**MRSP ST71**
	Pound	C3491	**MSSA ST398**	C2720	MSS ST181
	Pound	C3509	**MSSA ST398**	C2726	MSSP ST190
	Pound	C3487	**MSSA ST398**	C3510	MSSSc -
	Pound	C3485	MSSA ST5	C2724	MSSP ST184
	Household	C3929	MSSA ST121	C3930	**MRSP ST71**
Distinct *S. pseudintermedius*	Pound	C3464	MSSP ST20	C2604	**MRSP ST71**
	Pound	C2725	MSSP ST185	C3467	MSSP ST190

ANTIMICROBIAL RESISTANCE PROFILE

S. aureus. In general, isolates from household individuals showed higher rates of resistance to different antimicrobials than those from pound dogs (**Table S5**). A single *S. aureus* isolate was MRSA, which showed additional resistance erythromycin, kanamycin and ciprofloxacin. Most of the MSSA isolates from household individuals were only penicillin resistant. A total of 13.9% of *S. aureus* from household individuals were also resistant to erythromycin and showed inducible clindamycin resistance, mediated by *erm*(A), *erm*(C) or *erm*(T) (**Table S5**). Resistance to MLS_B among healthy human isolates is not rare and it may be attributed to the extended use of macrolides and lincosamides in the treatment of Gram-positive infections (Lozano et al., 2011a). Interestingly, MLS_B resistance was higher among kennel dogs (20.8%) (**Table S5**), what could respond to a clonal spread of a single strain since 4 of the 5 resistant isolates exhibited an identical PFGE profile and belonged to the same lineage (ST78/CC88). The low rate of penicillin resistance (20.8%) detected among pound dogs is outstanding, since over 90% of human *S. aureus* isolates are resistant to penicillins and it is also a common trait among *S. aureus* from pet animals (Guardabassi et al., 2004; Schwarz et al., 2001). Absence of human exposure might indirectly justify the lack of antimicrobial resistance among these isolates.

High correlation was observed between the absence of the IEC genes and absence of antimicrobial resistance (100%), and also between the presence of the IEC genes and resistance to at least one antimicrobial (92.3%) in pound dogs. This observation points towards the

possibility that the absence of human-related contact (kennel animals, IEC-negative) may render to lower antimicrobial resistance levels.

If only MSSA isolates are taken into account, isolates from household individuals were still more resistant than those from pound dogs (with the exception of MLS_B). Remarkably, half of MSSA from pound dogs were susceptible to all antimicrobials tested (**Fig. 24**).

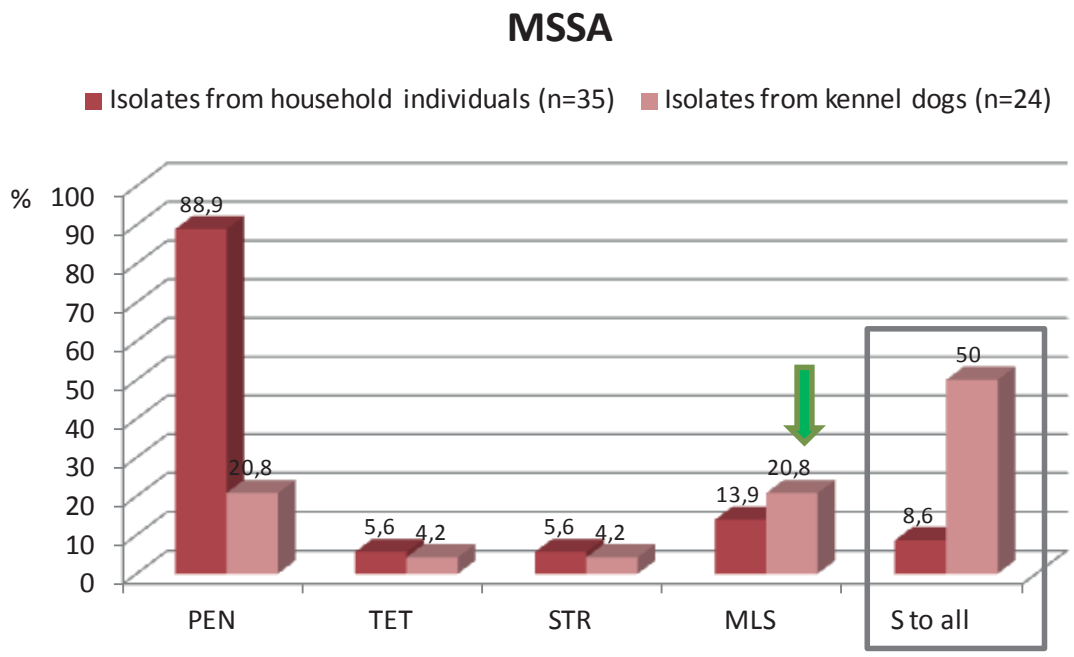

Figure 24. Percentages of resistance to the different antimicrobials tested in the MSSA isolates recovered from household individuals and pound dogs. PEN, penicillin; TET, tetracycline; STR, streptomycin; MLS, macrolides/lincosamides/streptogramines B; S to all, susceptible to all antimicrobials.

***S. pseudintermedius*.** In general, *S. pseudintermedius* isolates were more resistant to the antimicrobials tested than the investigated *S. aureus* population (**Table S6**). High rates of MDR were observed among *S. pseudintermedius* of both populations sampled, with a total of 39% (7/18) of *S. pseudintermedius* from household individuals and 41.7% (10/24) from pound animals being MDR. All the MRSP recovered were MDR and predominantly presented the same antimicrobial resistance genes, with few exceptions. The homology of the pheno- and genotype of MRSP (ST71 and non-ST71) described to date is noteworthy, and suggest predisposition for acquisition and maintenance of specific resistance genes or MGEs. The presence of *tet*(K) among all investigated MRSP ST71, which are normally located on small plasmids is remarkable and ,together with the common presence of the chloramphenicol resistance gene cat_{pC221} (also normally located in small plasmids), evidence its ability to acquire plasmid-mediated

antimicrobial resistance determinants. However, it has been observed that *S. pseudintermedius* appears to prefer transposon-borne resistance genes such as the tetracycline resistance gene *tet*(M) (Tn*916*) and the MLS_B resistance gene *erm*(B) (Tn*917*), which are normally integrated in the chromosomal DNA (Kadlec et al., 2012; Guardabassi et al., 2004). This may be also the case of the Tn*5405* (*aadE*, *aphA3*, *sat4*) and its common physical linkage with the *erm*(B) gene. In fact, MDR in the investigated *S. pseudintermedius* (both, MRSP and MSSP) was always associated to the presence of the Tn*5405* elements and the *erm*(B) gene.

Counting all *S. pseudintermedius* isolates, those from pound animals were in general more resistant to the tested agents (**Table S6**). However, this observation was associated to the presence of nine MDR MRSP among pound dogs. If only MSSP were considered for evaluation, this trend presented a prominent inverse pattern (**Fig. 25**). Again, these relatively low resistance values among MSSP from pound dogs suggests reduced antimicrobials exposure and/or limited contact with acquired antimicrobial resistant bacteria. In total, 31.3% (5/16) of all MSSP isolates from households and 12.5% (2/16) of those from pound dogs were MDR.

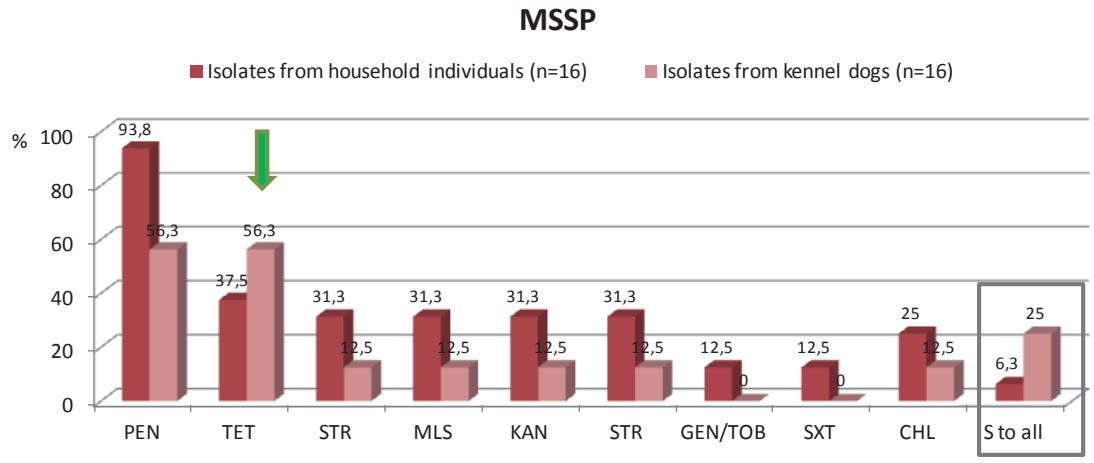

Figure 25. Percentages of resistance to the different antimicrobials tested in the MSSP isolates recovered from household individuals and pound dogs. PEN, penicillin; TET, tetracycline; STR, streptomycin; MLS, macrolides/lincosamides/streptogramines B; KAN, kanamycin; GEN/TOB, gentamycin/tobramycin; SXT, thrimetoprim/sulphametoxazol; CHL, chloramphenicol; S to all, susceptible to all antimicrobials.

Resistance to penicillin, tetracycline, MLS_B, streptomycin and ciprofloxacin was observed in both bacterial species (*S. aureus* and *S. pseudintermedius*). Interestingly, β-lactams resistance and resistance to fluoroquinolones were correlated in both bacterial species since ciprofloxacin

resistance was only present in methicillin-resistant isolates (the single MRSA and 9 of the 10 MRSP isolates).

The role of dogs as reservoirs of antimicrobial resistant bacteria needs further investigations. Transmission of antimicrobial resistance between bacteria may be facilitated by the close physical contact between positive household pets and humans as well as by the selective pressure imposed by the used of antimicrobials in both human medicine and small animal practice.

VIRULENCE GENE CONTENT

S. aureus. High number and variety of virulence factors was detected among the investigated *S. aureus* population. Higher virulence gene content and diversity was detected among household individuals (owner and pets) than among pound dogs (**Fig. 26**).

The presence of the PVL in a MRSA ST8 strain of human origin is notable, while none of the animals tested were positive. In addition, the toxin shock syndrome toxin gene *tst* was only detected among owners, suggesting a host tropism towards human isolates (**Fig. 26**). The exfoliatin genes *eta* and *etb* were only detected among household individuals, and positive animals corresponded to direct cases of interspecies transmission. One of these cases could be followed up and revealed that *S. aureus* was persistent in the owner and only sporadic in the dog. Based on these results, and due to absence of these toxin genes among the pound dog population, it can be estimated that they are preferentially present in human isolates, and that their detection in pet animals may respond to a human-to-pet *S. aureus* transmission. Some of the positive isolates belonged to CC121, an *S. aureus* lineage, usually methicillin-susceptible, which commonly causes SSTIs worldwide (Monecke et al. 2011). This lineage was predominant carrying the leukocidin gene *lukE/D*. Curiously, this toxin gene was the only one more prevalent among pound animals (**Fig. 26**). Its presence was also more common among household animals than among their owners, what suggest, a possible animal host tropism.

Enterotoxins, which are responsible for staphylococcal food poisoning outbreaks, were unexpectedly highly prevalent in household individuals, with remarkably lower rates among pound dogs (**Fig. 26**). In total, 93% and 75% of *S. aureus* from human and household pets, respectively, carried at least one enterotoxin gene. Of these, all but 2 human isolates (83.3%) harboured the complete *egc* operon, egc_{like}, or *egc* variants. These rates are notoriously higher than previous reports on both healthy humans and animals (Abdel-Moein et al., 2011). It is

important to remark that all *S. aureus* involved in direct cases of suspected interspecies transmission showed enterotoxigenic properties (carrying *egc* clusters). As for *S. aureus* isolates from pound dogs, while MSSA ST398 and ST133 lacked all enterotoxin genes tested, the 81.3% of non-ST398/ST133 isolates were positive for at least one enterotoxin gene. Among these, more than half (53.9%) *S. aureus* isolates presented genes of the *egc*.

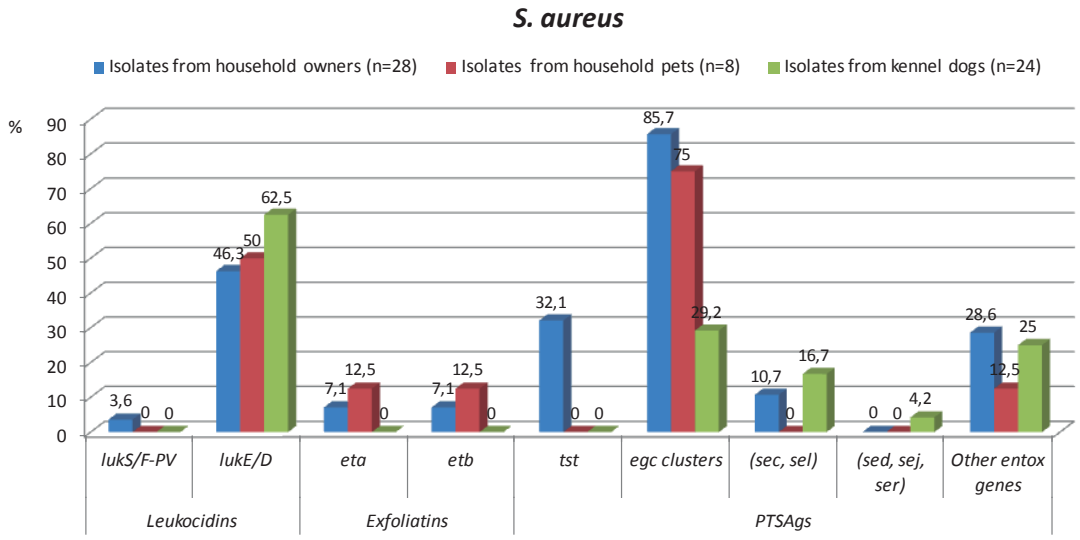

Figure 26. Comparative distribution of the virulence genes detected among *S. aureus* isolates recovered from household individuals and kennel dogs. Virulence genes within brackets are those usually physically linked, based on bibliography (Argudín, 2011). *egc* clusters refer to the presence of *seg*, *sei*, *sem*, *sen*, *seo* and/or *seu* in physical linkage. PTSAgs, pyrogenic toxin superantigen genes.

Based on these data, human-adapted *S. aureus* isolates are more prone to carry virulence genes, what should be the focus of further surveillance.

***S. pseudintermedius*.** In contrast to *S. aureus*, most *S. pseudintermedius*, with independence of the animal or human origin or with the type of animal (household, pound) carried the same virulence genes, with lower virulence gene diversity than *S. aureus*. However, it is interesting to underline the presence of the exfoliatin *expA* in 3 isolates, 2 of human origin (**Fig. 27**). The *expA* and *expB* genes were more prevalent than previous descriptions on healthy animals (Iyori et al., 2011). However, due to scarce data available on the virulence gene content of *S. pseudintermedius*, proper epidemiological comparison in animals and humans is not possible. All isolates carried the leukocidin *lukS/F-I*, the exfoliatin *siet* and enterotoxin *se-int* genes (**Fig. 27**), what evidences ubiquity of these virulence properties among commensal *S. pseudintermedius*.

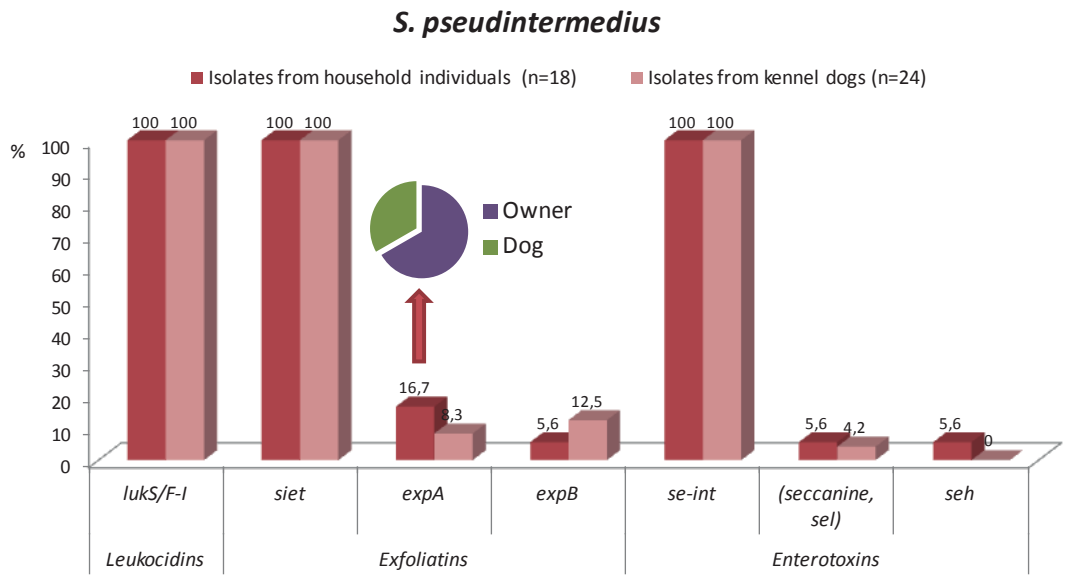

Figure 27. Comparative distribution of the virulence genes detected among *S. pseudintermedius* isolates recovered from household individuals and kennel dogs. Virulence genes within brackets are those normally physically linked, according to bibliography (Argudín, 2011). *egc* clusters refer to the presence of *seg*, *sei*, *sem*, *sen*, *seo* and/or *seu* in physical linkage.

In conclusion, due to the elevated rate of toxigenic *S. aureus* and *S. pseudintermedius* detected in both humans and their pets appropriate household infection prevention and control practices should be considered when facing community-acquired staphylococcal infections.

General characteristics of isolates from the longitudinal study

Focussing only on isolates recovered from the longitudinal study, pandemic *S. aureus* clones were observed in humans (ST45, ST121, ST30, ST398, ST5), of which ST121 and ST398 were also sporadically detected in dogs. It is important to remark that 40% (4/10) of *S. aureus* clones and 55.6% (5/9) of *S. pseudintermedius* clones were detected in both owner and pet (not necessarily at the same sampling), what highlights the possibility that prolonged direct contact between positive dog and/or owner may favour bacteria transmission in both directions.

Three *S. pseudintermedius* clones were MDR (33.3% of *S. pseudintermedius* clones) while the rest of clones from this species were only resistant to penicillin. As for *S. aureus*, two clones were MLS_B resistant, one was tetracycline resistant and the rest (70%) were only penicillin resistant. Interestingly, the 5 MSSA ST398 isolates showed inducible MLS_B resistance and harboured the *erm*(T) gene.

Elevated virulence gene content was detected in all *S. aureus* clones, with 70% of them carrying enterotoxin gene clusters (*egc*), and 50% the leukotoxin gene *lukED*. These data, together with the presence of several virulence genes among all *S. pseudintermedius* clones, enhance the importance to control possible sources for acquisition of potential pathogenic bacteria. Finally, it is interesting to remark that all representative *S. aureus* human isolates (9/9) presented the IEC genes, in comparison to the 54.2% (13/24) detected among pound dogs.

In conclusion, based on the data obtained in this work, dogs contribute to the propagation of acquired resistant and potentially virulent bacteria, therefore enhancing their spread in the human population and probably in the environment. Further surveillance on pet animals as reservoirs of antimicrobial resistance and virulence genes is warranted.

CHAPTER 3. SARM FROM SLAUGHTER PIGS AND CoPS FROM HOSPITALIZED EQUINES.

MRSA in slaughter pigs of different age groups.

An overall pig nasal occurrence of 35% was detected in this study. Positive animals came from 5 of the 6 farms of origin of the tested pigs. Since only 4 animals were sampled from the negative farm, underestimation of the real occurrence in this holding may have also occurred. These values are high, bearing in mind that La Rioja does not account with a high pig density. However, latter studies on MRSA in pigs in Spain revealed much higher nasal carriage rates for pigs at slaughter (83-90%) in regions with also low pig density (**Fig. 28**).

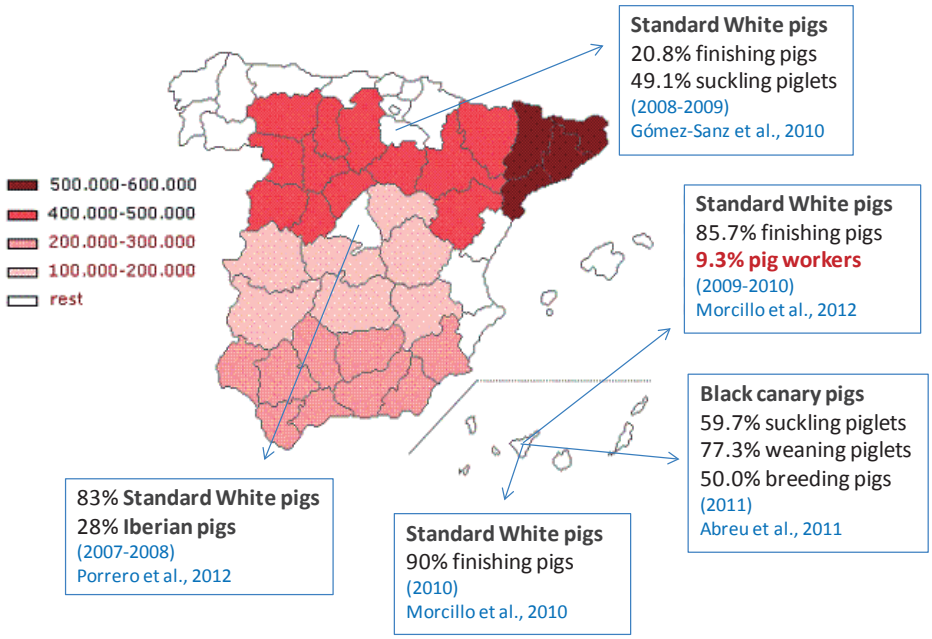

Figure 28. Geographic distribution of the total number of pigs per county in 2008 (according to Ministerio de Agricultura, Alimentación y Pesca) in addition to information on the MRSA occurrence detected among pigs and humans in the published reports in this country.

Highly variable data on LA-MRSA ST398 occurrence at the farm or at the slaughterhouse are available in different countries (from 0% to 91%), what depends on multiple factors (Crombé et al., 2013). Studies from Ireland Switzerland, Brasil, Singapur, Japan, Corea, and Malasia (Aquino et al., 2012; Baba et al., 2010; Horgan et al., 2010; Huber et al., 2010; Lim et al., 2012; Neela et al., 2009; Sergio et al., 2007) detected less than 5% of positive animals; in contrast, Canada reported rates of 26% (Khanna et al., 2008), Croatia of 35.3% (Habrun et al., 2011) and some studies in The Netherlands and Germany have recovered LA-MRSA ST398 in more than 70% of pigs sampled (Tenhagen et al., 2009; van Duijkeren et al., 2007). In many occasions, colonization rates also vary within the same country. In this sense, studies conducted in

Denmark, Belgium, The Netherland, Germany and the USA have detected LA-MRSA ST398 rates that vary from the complete absence at farm level to 90% (Cuny et al., 2012; Denis et al., 2009; Guardabassi et al., 2007; Lewis et al., 2008; Molla et al., 2012; Pletinckx et al., 2011; Smith et al., 2008; Tenhagen et al., 2009; van Duijkeren et al., 2007; van Duijkeren et al., 2008b). These MRSA levels depend on multiple factors, as will be indicated below.

Carriage values in the current study were significantly higher among piglets (49.1%) than among adult swine (20.8%). (Smith et al., 2008) already reported high nasal carriage rates among piglets (100%) than among finishing pigs (36%) in US. Latter reports have shown age related differences in MRSA prevalence (Broens et al., 2011b; Crombé et al., 2012; Pletinckx et al., 2013; Weese et al., 2011), what seems to be highly influenced by the MRSA carriage status of the sow (Pletinckx et al., 2013; Verhegghe et al., 2013). Recent studies have shown that 100% of piglets coming from positive sows were MRSA carriers, while variable values were detected among piglets from negative sows (Verhegghe et al., 2013; Weese et al., 2011). In addition, a recent study reported that transmission rates were higher in pre-weaning pigs compared to post-weaning pigs which might be explained by an age-related susceptibility or the presence of the sow as a primary source of MRSA CC398 (Broens et al., 2012).

Higher nasal carriage rates among piglets than in adult swine have also been detected in Spain among an autochthonous pig breed (black canary pig) (**Fig. 28**) (Abreu et al., 2011). In that case, nasal samples were collected at a farm in which breeding conditions and feeding of animals are better than in most pig farms on the Island, since black canary pig is protected native species in danger of extinction. To this respect, it is interesting to mention a recent study performed in our country that detected remarkably lower carriage rates among Iberian pigs normally raised outdoors (28%) than among intensively farmed standard white pigs (83%) (Porrero et al., 2012). These data lead to the conclusion that pig density and living conditions are important factors that influence the MRSA carriage status of pigs. In addition, lower human contact occurs when pigs live in an open environment, in comparison to intensively high density reared pigs, where personnel has frequent contact with the animals, which apparently favours the exchange of microbiota from human to animal and vice versa. On the other hand, that study observed that both pigs raised extensively and those from intensively farmed animals all carried LA-MRSA ST398, although antimicrobial resistance was lower in the former group. Interestingly, another recent study performed in Germany, detected neither MRSA nor *S. aureus* in pigs raised in an alternative system (Cuny et al., 2012). In contrast to farms with conventional fattening methods, these alternative farms are usually smaller and pigs are kept on floors with straw

bedding, with sufficient room for running of the animals. Further, there is no administration of antibiotics to pigs with body mass >25 kg and are closed with respect to imports from conventional systems. These requirements may have prevented LA-MRSA introduction. Unfortunately, no data was available in our study on the pig size of the herds or on any potential factor that may have influenced on the MRSA carriage.

Trade of animals between farms or to the slaughterhouse has been also reported as an increased factor for MRSA ST398 acquisition (Broens et al., 2011b; Espinosa-Góngora et al., 2012), where cross-contamination can occur between animals sharing transport or lairage areas of slaughterhouses. Also, it is clear that the environment (fomites, surfaces, airborne via ventilation systems) play a role in the transmission of MRSA in the farm and the slaughterhouse, since similar ST398 clones have been detected in farmers, animals, and environmental samples (Espinosa-Góngora et al., 2012; Fessler et al., 2012; Friese et al., 2012; Friese et al., 2013; Pletinckx et al., 2013; Schulz et al., 2012). It is interesting to mention a recent study that detected MRSA up to 150m downwind of MRSA-positive farms (Schulz et al., 2012). This study observed high proportion of LA-MRSA-positive samples within the main downwind direction of the barns suggesting that the dispersion into the environment is strongly influenced by wind direction. Proximity to other farm-animals, such as cattle (Crombé et al., ,2013) or presence of rodents has been also associated to high LA-MRSA carriage rates in pigs (Van de Giessen et al., 2009). On the other hand, the prevalence of LA-MRSA ST398 positive pets residing on farms, as well as its possible role as MRSA ST398 vectors is to date unknown, what warrants further research. Yet, a recent study reported LA-MRSA ST398 among 2 dogs living in LA-MRSA ST398 positive farms, in which pigs were also positive (Fessler et al., 2012). These data would support the hypothesis that dogs are prone to carry the *S. aureus* isolates of in-contact environments. In the present study, all animals tested came from production holdings so direct contact to other livestock may be discarded; however, no information was obtained on the possible influence of other animal species (especially rodents, but also dogs or cats) along the production chains or in the surroundings of the productions systems.

All in all, LA-MRSA carriage values in swine depend on external multiple factors (Crombé et al., 2013) that should be taken into account when designing, analysing and comparing studies. Some of the factors that influence the LA-MRSA carriage in swine are summarized below:

- Age and type of animals (suckling, weaning, breeding, finishing)
- Density and size of pig herds

- Life style of the animals (extensive versus intensive production)
- Animal trade
- Sampling location (farms, slaughterhouses)
- Low hygiene level
- Geographic location
- Environmental exposure (Air, soil, fomites, surfaces)
- Proximity to other animals (especially other livestock)
- Contact with LA-MRSA positive humans or animals (in special rodents, but also pets).

Most of our MRSA isolates belonged to the ST398 lineage (91% of isolates) with the remaining strains being ST1379/CC97 (**Fig. 29**). The European baseline study conducted in 2008 (EFSA, 2009b) showed that 92% of MRSA detected in dust samples were ST398 whereas 8% of strains were non-ST398, with ST1/CC1 and ST97/CC97 as the most frequently recovered. However, in the European survey, MRSA CC97 was not detected among isolates from Spanish holdings (neither breeding nor production holdings), with ST1/CC1 as the unique non-ST398 lineage in this country. The lineage CC97 is closely associated with cattle, and MRSA belonging to this lineage have been detected worldwide, in most cases being responsible for bovine mastitis (Aires-de-Sousa et al., 2007; EFSA, 2009b; Monecke et al., 2011; Pantosti, 2012; Smith et al., 2005; Sung et al., 2008). Additionally, the first description of CC97 in pigs was reported on MSSA causing a swine infection in France (Armand-Lefevre et al., 2005). This lineage has been also detected as MRSA on clinical isolates in pigs in Germany (Meemken et al., 2010), from healthy slaughter pigs in Italy (Battisti et al., 2010) and very recently among healthy farm pigs in Senegal (10.9%) (Fall et al., 2012). Remarkably, one publication detected one MRSA CC97 on a rat living on a pig farm in Denmark (Van de Giessen et al., 2009), what suggests contamination of the rodent from the in-contact environment. Further, MRSA CC97 has been recently described as causative agent of food poisoning (Sobral et al., 2012) and among Chinese butchers in a wet market (Boost et al., 2012). Although this lineage is a well-known LA-MRSA lineage, it has been detected in several occasions in human clinical samples in different countries (Chung et al., 2004; Ellington et al., 2008; Feil et al., 2003; Köck et al., 2013; Menegotto et al., 2012 IGE; Sung et al., 2008) and also very recently as MSSA among healthy individuals in the community in Spain at low frequencies (Argudín et al., 2013; Lozano et al., 2011a). These data point to the suggestion that CC97 exhibits an apparent EHSG.

As stated above, contact between pigs and dairy cattle along the production chain may be discarded. Moreover, MRSA CC97 strains came from animals of two different production holdings and were sampled at different periods. Based on these data, the presence of non-ST398 MRSA strains, and of CC97 in particular, is of great interest given that other potentially pathogenic lineages are possibly being underestimated in terms of human and animal health implications.

Intra-lineage differences were detected among MRSA ST398 from piglets and those from adult swine. The vast majority of MRSA ST398 isolates recovered from piglets (93.3%) belonged to the *spa* type t011, while it was present in half of MRSA ST398 positive adult swine (see figure below). In addition, data analysis of PFGE macrorestriction fragments showed that PFGE-profiles seemed to cluster according to their age group and in line with their SCC*mec* cassette (**Fig. 29**).

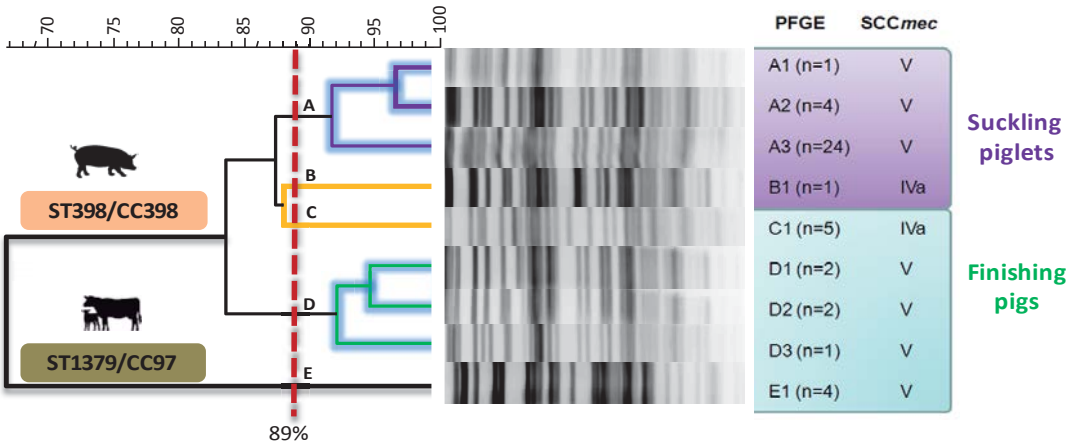

Figure 29. Dendrogram that shows the PFGE clonal relation of the MRSA strains investigated. The colonized animal age group, ST and SCC*mec* type of strains is also indicated.

These results point to the suggestion that distinct MRSA ST398 clones could be better adapted to different animal ages. Both MRSA ST398 positive piglets and adult swine that came from the same holding (farm 2) clustered separately; suggesting that PFGE clustering results may be not due to a farm-dependent lineage.

Several reports have shown a closer genomic background within ST398 isolates with the same SCC*mec* type (SCC*mec* IVa or SCC*mec* V) after digestion with *Cfr9I* enzyme, which also suggest that MSSA ST398 strains could have acquired the SCC*mec* cassette at different stages and then evolve and diversify (Argudín et al., 2010; van Wamel et al., 2010). This represents the first study that detects this association using the *ApaI* enzyme.

MDR was predominantly present in strains from adult swine, both MRSA ST398 and CC97 (**Fig. 30**). These data could respond to a longer exposure time to conditions of selective pressure (such as the use of antimicrobials) among MRSA from adult swine than among those recovered from piglets, and would evidence the quick acquisition gene capacities of both clonal lineages (CC398 and CC97).

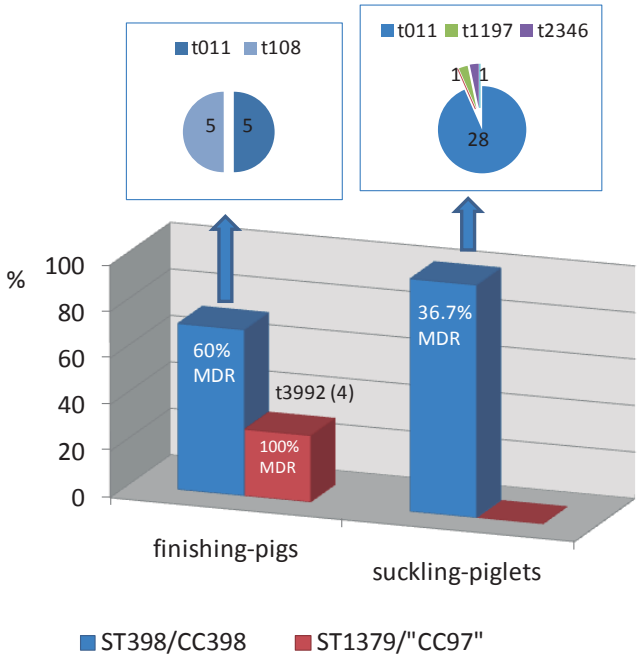

Figure 30. Distribution of the 14 MRSA from finishing-pigs and the 30 MRSA from suckling-piglets in relation to the lineage detected, *spa* types present and the rate of MDR (resistance to at least three classes of antimicrobials) per group.

High diversity of AMR profiles was observed among adult swine, while isolates from piglets showed a more homogenous pheno- a genotype of resistance to the tested agents (**Table S7**). The unique common characteristic in all 44 isolates was the presence of tetracycline resistance what is a common trait among *S. aureus* of animal origin. To date, virtually all LA-MRSA ST398 investigated present tetracycline resistance (Crombé et al., 2013), and it has been indicated as a good molecular marker to identify LA-MRSA ST398 (Lozano et al., 2012c). Interestingly, resistance to aminoglycosides and thrimethoprim/sulphametoxazol was only detected among MRSA from adult swine (**Table S7**). Not only the phenotype of resistance was associated to the age of positive animals, but also some antimicrobial resistance genes [*tet*(L), *erm*(T), *aacA-aphD*, *aadD*, *aphA3*, *dfrA, dfrG* and *dfrK*] were only detected among MRSA isolates from adult swine. Antimicrobial resistances (AMR) resistances observed among isolates reflect

the extensive use of the correspondent antimicrobial agents in food-producing animals (EMA, 2010b).

Although the *tet*(L) tetracycline resistance gene is not common among MRSA isolates (Kadlec et al., 2009c; Kadlec et al., 2012a), it is relatively frequent among LA-MRSA ST398 (kadlec et al., 2012a), what is in line with our results from adult swine, with half of strains being positive. Given that it is normally plasmid located, plasmid-mediated transference of this gene under conditions of selective pressure may be favoured. On the other hand, practically all MRSA ST398 to date exhibit *tet*(M) (Kadlec et al., 2012a; Lozano et al., 2012c; McCarthy et al., 2012; van dendriese et al., 2013). Given that this gene is transposon located, its plausible location on the chromosomal DNA would ensure maintenance due to vertical transference.

Published data on MRSA from livestock have reported high rates of erythromycin-clindamycin resistance (Battisti et al., 2010; Crombé et al., 2013; Kadlec et al., 2009a; Kadlec et al., 2012a; Mulders et al., 2010; Witte et al., 2007). Nevertheless, due to the absence of D-test analysis, there is scarce data on the prevalence of either constitutive or inducible. Consequently, the constitutive expression of MLS_B resistance observed in this study is noteworthy, since it may be of especial interest for treatment of infections caused by these organisms. It is interesting to remark that four MLS_B resistant isolates harbored the *erm*(T) gene. This gene has recently been detected for the first time in staphylococci in LA-MRSA ST398 of porcine origin, being located in a plasmid (pKKS25) which also carried the *tet*(L) and *dfrK* genes (Kadlec et al., 2010a) (**Fig. 31**). Although all positive strains also harboured the *tet*(L) gene, they were susceptible to trimethoprim and lacked the *dfrK* gene. In addition, specific PCR analysis using primers that flanked both *tet*(L) and *erm*(T) (**Fig. 31**) yielded a PCR-fragment of approximately 1.5 kb smaller than the one achieved in pKKS25, what suggests the presence of a different but related genetic environment (see **chapter 4**).

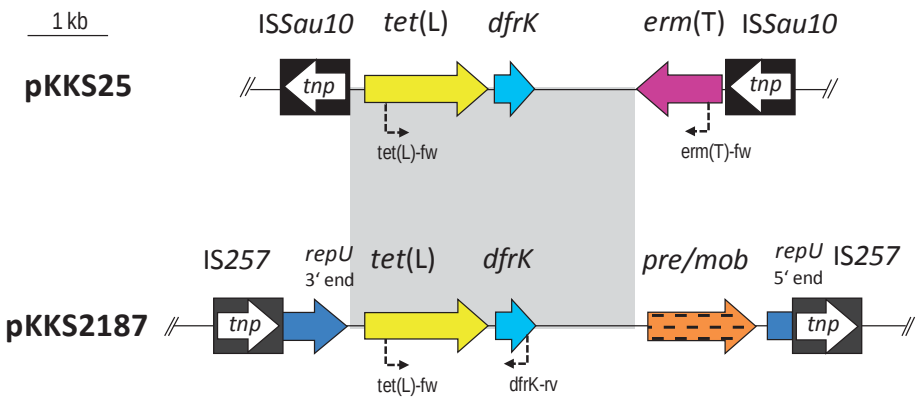

Figure 31. Schematic representation of the sequenced fragments of plasmids pKKS25 [carrying the *erm*(T) + *dfrK* + *tet*(L) clustered genes] y pKKS2187 (clustered *dfrK* + *tet*(L)] (figure adapted from Kadlec et al., 2010a, Kadlec et al., 2010c).

On the other hand, both thrimethoprim/sulphametoxazol resistant strains exhibited the *dfrK* gene in physical linkage to the *tet*(L) gene, as detected by PCR-mapping based on a previous plasmid description (pKKS2187) of another MRSA ST398 porcine isolate (Kadlec et al., 2010a) (**Fig. 31**). In addition, the *str* gene had been only detected in *S. aureus* ST398 in a recent whole genome sequencing study on LA-MRSA ST398 of porcine origin (Schijffelen et al., 2010).

PCR analysis of the IEC genes on one representative MRSA strain per PFGE and *spa* type revealed an animal adaption, since none of the isolates was positive for any of the genes. In general, LA-MRSA ST398 showed low virulence gene content. Although these strains normally harboured fewer virulence determinants than other MRSA lineages (Crombé et al., 2013; Köck et al., 2013), it is important to take into account that several deep-sited infections caused by LA-MRSA ST398 among pig farmers have also occurred (Lozano et al., 2011d). Interestingly, the presence of the exfoliatin *eta* and the leukocidin *lukE/D* genes, as well as the haemolysin *hlgv*, was homogeneous in the four MRSA CC97 isolates. LukED is closely associated with *S. aureus* that cause bovine mastitis and classically seldom detected in pigs (Battisti et al., 2010; Yamada et al., 2005), what points to a possible bovine origin of these isolates and evidences an homogeneous genetic background among unrelated CC97 strains. In addition, a great prevalence of these genes has been reported among clinical human *S. aureus* isolates (Vandenesch et al., 2003) and its pathogenic properties have been recently reviewed considering it important for lethal *S. aureus* blood-stream infections (Alonzo et al., 2012). These data remark the higher pathogenic potential of CC97 isolates in comparison with those ST398 recovered. Further, the *lukE/D* gene has been detected in half of *S. aureus* strains of animal origin in this thesis, with lower rates among human isolates (36.1%), suggesting a remarkable preference towards *S.*

aureus of animal origin. Worryingly, the PVL gene has been recently detected in MRSA CC97 isolates among pigs in Dakar, Senegal (Fall et al., 2012).

Few data are available on the presence enterotoxin genes in MRSA strains of livestock (Argudín et al., 2011; Kadlec et al., 2009a; Vanderhaeghen et al., 2010). Although the presence of these genes was negative in the current study, in-depth virulence analysis should be conducted on *S. aureus* from livestock in order to elucidate their enterotoxigenic properties, due to the potential impact on human health throw food poisoning infections.

The presence of MRSA ST398 among pigs at slaughter in Spain is revealed in this study. In addition, the detection of several CC97 MDR MRSA strains with higher virulence properties than those observed among ST398 reveals that swine can be also carriers of other potentially pathogenic MRSA lineages typically associated to other farm-animals. The resistance gene acquisition capacities of all isolates studied (CC398 and CC97) is of particular significance since both clonal lineages can colonize various animal and human hosts. Epidemiological studies on the circulating lineages in diverse animal ecosystems should be conducted, in order to elucidate the transmission routes of MRSA lineages among animals and humans, as well as its rates of colonization and infection. Similarly, age of animals must be considered when designing and comparing studies, in order to estimate the most relevant animal ages in terms of animal and human implications, on which comprehensive studies on MRSA should be addressed.

CoPS in hospitalized equines

Eight CoPS isolates were investigated in this work. Although the epidemiological burden of this study is limited due to the reduced number of animals tested (n=39), an overall *S. aureus* occurrence of 20.5% (8/39) and 5.1% for *S. pseudintermedius* (2/39) was detected in clinical samples of horses along the whole period (2005/2011). Of these isolates, six *S. aureus* and both *S. pseudintermedius* were kept and further studied. A total of 10.3% of animals sampled exhibited MRSA ST398, and represents to date its first description in horses in Spain. The majority of strains were isolated from infections related to the skin and mucosa (**Table S8**), what gives evidence of the pathogenic properties of MRSA ST398 in this animal species. In fact, MRSA ST398 has mainly been reported in equine clinics to date (Couto et al., 2012; Cuny et al., 2008; Haenni et al., 2010; Hermans et al., 2008; Sieber et al., 2011; van den Eede et al., 2009; Weese et al., 2010a). A LA-MRSA ST398 carriage rated of 10.9% was detected among horses admitted to a

Belgian veterinary clinic, what is line with the data reported here (van den Eede et al., 2009). Although LA-MRSA ST398 in pigs is predominantly present as commensal, horses might be more susceptible to MRSA ST398 infection, what needs further surveillance. Limited data is available at farm level among healthy horses, with carriage rates of 0-11% (van den Eede et al., 2012; van den Eede et al., 2013). However, very low (0.5%) or even absent farm level carriage rates of MRSA ST398 haven been reported in some European countries (Busscher et al., 2006; Vengust et al., 2006), and Canada (Burton et al., 2008).

Curiously, the first detection of MRSA ST398 in animals in the UK was reported in a horse (from a clinical sample of a postoperative wound) imported from Spain (Loeffler et al., 2009). However, real origin of that MRSA ST398 isolate cannot be exerted based on the precedence of the animal, since the animal already lived in the UK for the previous 9 months, and many other epidemiological aspects should be taken into account (trade of animal, farm in the UK, possible acquisition at the clinic, etc).

The first description of MRSA ST398 in Spain was reported in food samples of pig origin recovered in 2008 (Lozano et al., 2009), and several reports have been later described on swine and people in contact to these animals in this country (Abreu et al., 2011; Gómez-Sanz et al., 2010; Lozano et al., 2011c; Morcillo et al., 2010; Morcillo et al., 2012; Porrero et al., 2012). Therefore, the strain recovered in 2005 represents the earliest case of MRSA ST398 in Spain to date, and evidences a clear adaptability to be present in non-porcine animal species. The horse carrying this strain came from a horse farm located very close to animal husbandries and to a slaughterhouse. In addition, all positive animals came from one of the regions in Spain with highest pig-density (Aragón, see **Fig. 28**), what may suggest swine as the source of acquisition. Unfortunately, epidemiological data on possible risks factors for acquisition of these bacteria (contact to livestock or pig farmers, living close to a pig farm, etc) could not be established in the rest of positive animals.

The absence of the IEC genes in all strains (ST398, ST1, ST1660) suggests animal adaptation of these isolates. Further, the MSSA ST1660 detected is an uncommon lineage that was described for the first time in a borderline-oxacillin-resistant *S. aureus* strain from a clinically diseased horse in Switzerland in 2006 (Sieber et al., 2011), sharing epidemiological homology to the detection of this study. Alternatively, as indicated before in this thesis, isolates belonging to CC1 have been previously detected and shown to be highly adapted to different animal species

(Pantosti, 2012). Again, the absence of the IEC seems to evidence an animal adaptation, suggesting that this isolate was not transmitted from a human source.

Regarding *S. pseudintermedius*, lineage ST68 is the predominant MRSP lineage in North America in dogs, and to date it has not been detected among MSSP isolates (Bannoehr et al., 2012). In Europe, MRSP isolates of this lineage have only been detected in Portuguese dogs (Couto et al., 2011). In the present work, the single *mec*A-positive *S. pseudintermedius* was ST68 representing its first description in horses, what suggests a less restricted geographic and animal host spectrum than expected. Background of the patient could neither be determined and therefore role of dog contact cannot be ruled out. However, the normal *S. pseudintermedius* occurrence among equidaes has not been investigated, and they presence might have been also overlooked. The MSSP recovered from a donkey, belonged to an MLST lineage (ST184) described for the first time in a pound dog in this thesis (see **chapter 2**). Again, due to the limited data available on the occurrence and geographic distribution of MSSP lineages, epidemiological evaluations are not recommended. The natural nasal or skin microbiota of donkey has not been investigated, and, to the best of our knowledge, *S. pseudintermedius* had been recovered from single donkey to date (Ruscher et al., 2009).

Epidemiological burden of this study is limited, but the proportion of *mec*A-positive isolates (5/8, 62.5%) is elevated. Interestingly, *mec*A-positive *S. pseudintermedius* ST68, strain C5337, was phenotypically susceptible to both oxacillin and cefoxitin by both disk diffusion (**Fig. 32**) and agar dilution methodologies (**Table S9**).

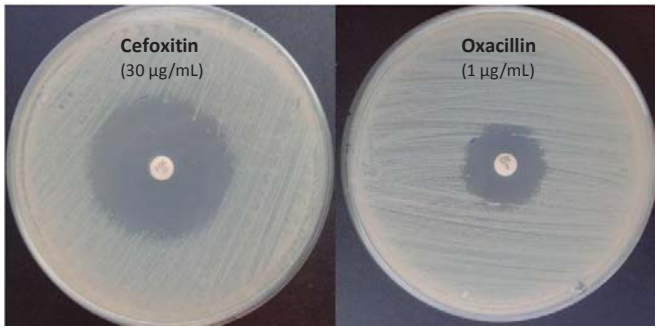

Figure 32. Antimicrobial resistance phenotype observed to cefoxitin (35 mm) and oxacillin (22 mm) following CLSI guidelines (CLSI, 2012).

Several reports have indicated a low reliability of cefoxitin disk diffusion tests to determine the methicillin resistance phenotype of *S. pseudintermedius* using interpretive guidelines recommended for human *S. aureus* and CoNS isolated from dogs (Bemis et al., 2012).

(Weese et al., 2009) reported a MRSP canine infection that was masqueraded as MSSP, but confirmed as MRSP after subsequent evaluation and observation of a MDR profile. Authors suggested that certain MRSP strains may be falsely identified as methicillin susceptible on the basis of results of testing for cefoxitin susceptibility because cefoxitin may not induce the *mec*A gene as reliably in *S. pseudintermedius* as it does in *Staphylococcus aureus*. To overcome this unhelpful aspect in the diagnosis of methicillin-resistance in *S. pseudintermedius*, a recent study has suggested an epidemiological breakpoint for cefoxitin zone diameter of ≤30 mm for resistant and ≥31 for susceptible, after analyzing a large *S. pseudintermedius* collection from clinical samples of dogs recovered between 2006 and 2011 (a total of 1,146 isolates) (Bemis et al., 2012). However, strain C5337 remained not only susceptible to cefoxitin under these criteria, but also to oxacillin, which is still considered a reliable marker of methicillin resistance in this species.

The fact that neither the *mec*A gene nor its promoter region presented mutations that could explain this atypical phenotype is surprising. In addition, the absence of the complete *mec*I/*mec*R1 regulatory region of SCC*mec*V_T should be indicative of a higher *mec*A expression level; however, the detection of the entire *bla*I/*bla*R1 regulatory genes was confirmed and speculated to be implicated, at least in part, in the stringent regulation of *mec*A expression in our strain. Moreover, other factors (different mutations on surface proteins, lower permeability, etc) or additional regulatory elements might affect the *mec*A expression in C5337 (McCallum et al., 2010), what warrants further in-depth investigations.

It is important to remark that all strains resistant to penicillin, gentamicin, or trimethoprim-sulfametoxazole were recovered from animals that had received previous treatment with those antimicrobials, what may have harboured the acquisition or survival or the recovered isolates. Risk factors for MRSA colonization and infection in horses have been, albeit preliminary, been investigated. Administration of ceftiofur or aminoglycosides was associated with acquisition of MRSA during hospitalization (Weese et al., 2010). In addition, antimicrobial administration within 30 days has been considered a risk factor for community-associated horse colonization, among others (Weese et al., 2010).

The thrimethoprim resistance gene *dfrK* was first described in a porcine MRSA ST398 in a plasmid (pKKS2187) (Kadlec et al., 2009c), and it has later been predominantly detected in plasmids of different sizes (Kadlec et al., 2009d; Kadlec et al., 2010a; Kadlec et al., 2012a). Rarely, this resistance gene has been solely detected in the chromosome as part of the novel

Tn*559* (Kadlec et al., 2010b; Kadlec et al., 2012a; López et al., 2012). Therefore, the presence of the entire and conserved Tn*559* (**Fig. 33**) among the three *dfrK*-positive MRSA ST398 strains is remarkable.

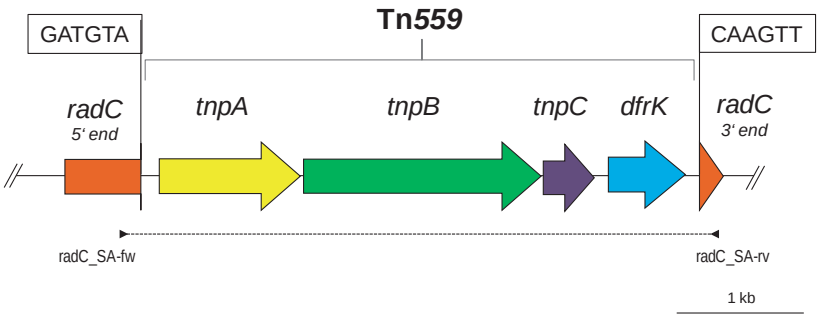

Figure 33. Transposon Tn*559* detected in 3 of the 4 MRSA ST398 isolates investigated in this study. Pair of primers designed to amplify the whole *radC*-in-integrated Tn*559* are also displayed.

To date, the Tn*559* had been only detected in a single porcine MSSA ST398 strain (Kadlec et al., 2010b), in several MRSA ST398 from poultry/poultry products in Germany (Fessler et al., 2011) and in some enterococcal strains in Spain (López et al., 2012); thus, the detection of the Tn*559* in MRSA ST398 from horses is a novel observation, and reflects the antimicrobial resistance gene acquisition capacity of this MRSA lineage in different animal species. Primary target site for integration of Tn*559* is designated *att559*, and is located within the chromosomal *radC* gene (Kadlec et al., 2010b). To date, the limited reports that have addressed its presence and location have detected it within this chromosomal *radC* gene, which codes for a DNA repair protein. Primers designed in this study were useful to amplify the complete *radC*-in-integrated Tn*559*, and evidences the preferable chromosomal location within the bacterial genome of the 3 isolates. Whether this preferential location also occurs within the chromosomal DNA of other staphylococci is unknown, but the identical location within a collection of enterococcal strains suggests a preferable location with independence of the bacterial species and genus (López et al., 2012). The detection of circular forms indicates functional activity and therefore the possibility of exchange between bacteria. Further molecular surveillance is then needed to evaluate their dissemination among and between different bacteria. Curiously, MRSA ST398 strain recovered in 2005 was susceptible to trimethoprim and negative for this gene, what might suggest a relatively recent acquisition or appearance of the *dfrK* gene.

Once again, the low occurrence of virulence genes detected in the MRSA ST398 isolates investigated is in line with previous reports (Crombé et al. 2013; Köck et al., 2013). Likewise, the

presence of the leukocidin *lukS/F-I*, the exfoliatin *siet* and the enterotoxin *se-int* genes in both *S. pseudintermedius* isolates is in agreement with the virulence gene content detected among *S. pseudintermedius* isolates investigated in this thesis, and, although very limited data on the occurrence of the different virulence genes is available to date (Ben Zakour et al., 2011; Ben Zakour et al., 2012; van Duijkeren et al., 2011a), its presence might be ubiquitous for this bacterial species. Further studies are required to gain knowledge on the virulence gene content and virulence properties of *S. pseudintermedius*.

The fact that all four MRSA ST398 isolates exhibited closely related patterns, regardless they were isolated in different years from unrelated horses, is remarkable, and points to a clonal spread of these clone among these animals. Since horses came from the same geographical region, the possible clonal spread of a single clone cannot be discarded. To tackle this possibility, further epidemiological studies on different animal species of the same area are guarantee. The possibility that horses acquired these isolates at the clinic, what may have been favoured due to the antimicrobial treatment administered, cannot be discarded. Unfortunately, samples from the environment of the clinic, from fomites or other devices or from the veterinarians could not be analysed.

The first description of MRSA ST398 in horses in Spain and one of the possibly earliest MRSA ST398 isolates in this country is reported in this thesis. This is also the first report on *S. pseudintermedius* ST68 in horses and the first detection of this American associated lineage in Europe in a non-canine animal species. The detection of the Tn*559* in MRSA ST398 from horses is a novel observation, and reflects the antimicrobial resistance gene acquisition capacity of this MRSA lineage in different hosts. The finding of a *mec*A-positive *S. pseudintermedius* strain that shows susceptibility to oxacillin and cefoxitin enhances the need for testing the presence of the *mec*A gene in routine analysis to avoid treatment failures. Given that the first detection of a suspected horse-to-human transmission of MRSA ST398 has been reported (van Duijkeren et al., 2008c), and due to the close contact of horses and owners, a potential threat for humans in contact to positive horses exits.

GLOBAL SUMMARY OF ALL *S. aureus* ST398 ISOLATES RECOVERED IN THIS THESIS.

A total of 57 *S. aureus* isolates of the lineage ST398 were recovered and investigated in this thesis. ST398 was the predominant lineage detected and, although the majority of isolates were isolated from slaughter pigs, it was the unique lineage present in humans, dogs, horses and pigs, reflecting a notable EHSG (**Table 6**). This EHSG character has been only recently addressed (Vinzce et al., 2012), since, as indicated in the previous chapter, *S. aureus* ST398 has been traditionally considered a livestock associated lineage, with swine as the main host.

Table 6. *S. aureus* ST398 isolates detected and relevant characteristics observed in relation to the origin of the samples.

Animal species	No isolates	Type	*spa* type (no isolates)	AMR in 100% of isolates	AMR gene or gene combination	IEC genes
Swine	40	MRSA	t011 (33), t108 (5), t1197 (1), t2346 (1)	**TET**	*tet*(K)+***tet*(M)**+*tet*(L) (4), *tet*(K)+***tet*(M)** (23), ***tet*(M)**+*tet*(L) (3), *tet*(K) (10), ***tet*(M)** (4)	-
Horse	4	MRSA	t011 (4)	**TET-GEN**	*tet*(K)+***tet*(M)** (4), *aacA-aphD* (4)	-
Dog	8	MSSA	t034 (4), t5883 (2), t108 (1), t1451 (1)	-	-	***scn*** (1), ***chp*** (1)
Human	5	MSSA	t1451 (4), t571 (1)	**ERY-CLIi**	***erm*(T)** (5)	***scn*** (5), ***chp*** (5)

Only isolate from dog with *spa* type t1451 harboured any of the IEC genes (show in red).

Five and 4 different *spa* types were detected among the 13 MSSA and the 44 MRSA ST398 isolates, respectively, showing remarkably higher diversity among MS isolates. All *spa* types clustered together with the default cost of 6, reflecting *spa* relatedness, with t011 as the predicted founder (CC011) (**Fig. 34**). Additionally, although this *spa* type was detected in the majority of isolates (n=37), it has only been present in LA-MRSA isolates (**Table 5**). Only *spa* type t108, typical of MRSA from swine, was detected in both types of isolates. However, t034 (detected here only in MSSA) is one of the most common lineages detected among MRSA from swine (EFSAb, 2009) and its presence in MSSA isolates is so far highly uncommon. In this way, it is important to remark the presence of both *spa* types t108 and t034 in canine isolates. In contrast, t571 and t1451 have been shown as the typical *spa* types present in MSSA ST398 isolates of human origin, specially t571 (Bhat et al., 2009; David et al., 2013; Jimenez et al., 2011; Lozano et al., 2011a; McCarthy et al., 2012; Mediavilla et al., 2012; Price et al., 2012; Rasigade et

al., 2010; Valentin-Domelier et al., 2011; Vandendriessche et al., 2011). *spa* type t034 had been previously detected in MRSA from dogs (Floras et al., 2010; Nienhoff et al., 2009; Witte et al., 2007), but this work describes the first detection of MSSA t034 and t108 in this animal species.

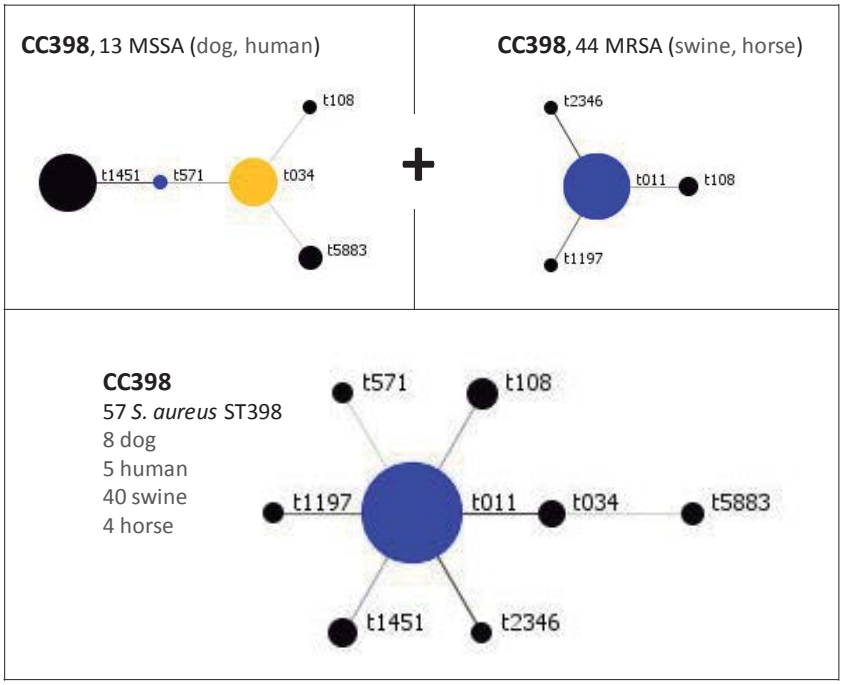

Figure 34. eBURP cluster analysis of the different *spa* types detected among *S. aureus* ST398 isolates, in relation to their methicillin resistance profile (MRSA, MSSA).

In contrast to the high clonal diversity observed among the rest of *S. aureus* lineages, *S. aureus* ST398, both MRSA and MSSA, seem to have a clonal distribution (**Fig. 35**), with independence of the methicillin resistance profile or origin of the isolate. With this respect, slightly higher clonal diversity was observed among swine isolates (**Fig. 35**). Low discriminatory power of the *ApaI* enzyme might be hypothesised; however, this enzyme achieved optimum discriminatory power when all MSSA from pound dogs were analysed (personal observation).

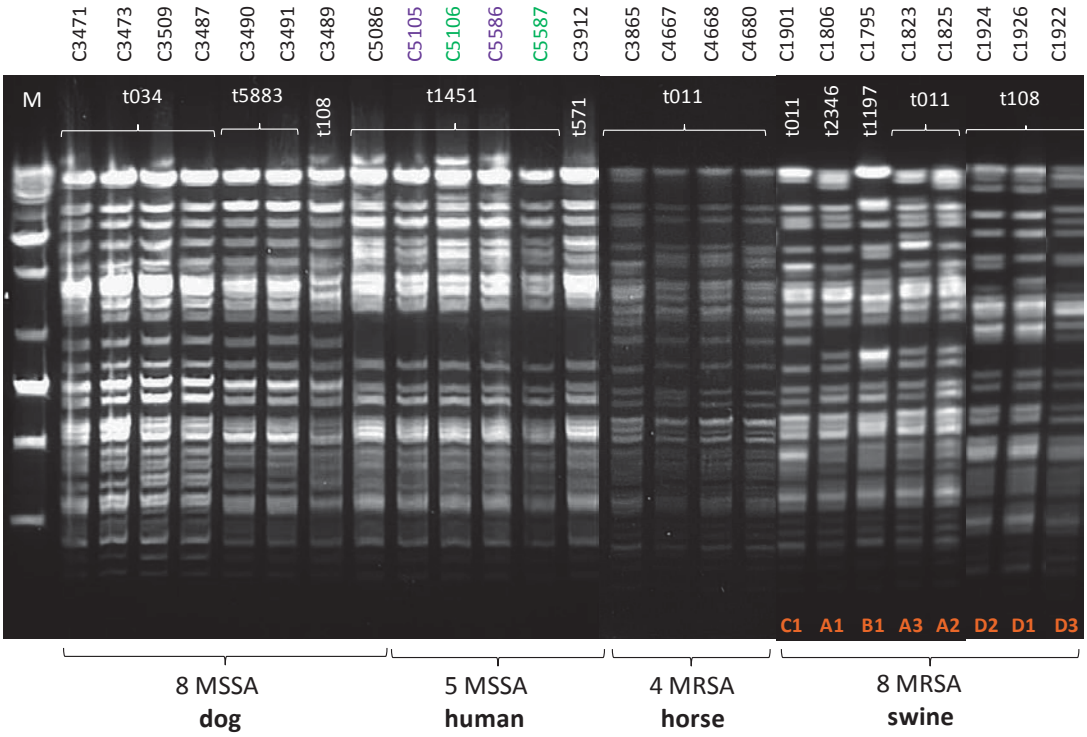

Figure 35. PFGE profile of all MSSA ST398 isolates, all MRSA ST398 from horses and one representative swine isolate per PFGE band pattern. It should be noted that isolates C5105 and C5586 correspond to two consecutive isolates (3 months separate in time) from the same owner, as it also occurs with C5106 and C5587. M, Low Range Molecular marker (New England Biolabs).

It is interesting to remark that all human and one canine MSSA ST398 isolates exhibited resistance to MLS_B (inducible clindamycin resistance) and carried the *erm*(T) gene (**Table 6**). In contrast, all MRSA isolates (horses, swine) showed tetracycline resistance and presented different combinations of tetracycline resistance determinants, with all of them carrying the transposon located *tet*(M) gene (**Table 6**). As suggested in the former chapter, the ubiquity of this gene among MRSA ST398 of different origins may suggest a chromosomal location. Likewise, the ubiquity of the *erm*(T) in these MSSA ST398 isolates suggests that this gene might be also located within the chromosomal DNA of this sub-lineage, what would ensure its maintenance.

One dog owner from the cross-sectional study and two owners from the longitudinal study were MSSA ST398 carriers (t571 and t1451, respectively) (See **Fig. 22** and **Fig. 23**). The last two owners exhibited this clone in two consecutive samplings (T3-T4) and were co-inhabitants of the same residence. This observation evidences the ability of this lineage to be present for prolong periods of time in humans. It is then strongly suggested that person-to-person spread of MSSA ST398 may also occur (Jimenez et al., 2011). Positive owners did not have direct contact to

rural areas or farm animals and all five isolates presented the IEC *scn* and *chp* genes, what points to a human origin or human adaptation (McCarthy et al., 2012).

As discussed in the previous chapter, the *erm*(T) gene was also detected in four MRSA ST398 from swine (constitutive clindamycin resistance profile). Complete characterization of the genetic environment of the *erm*(T) in these strains and in several of the MSSA ST398 isolates will be the focus of **chapter 4**.

Surprisingly, all seven MSSA ST398 isolates from pound dogs were susceptible to all antimicrobial agents tested, but five of them presented *spa* types closely associated to MRSA from swine and did not harbour any of the IEC genes. These data might point either to an animal origin or a possible loss of the IEC genes as a consequence of host adaptation from a human source. Although all but one of these MSSA ST398 isolates were captured in rural areas, the potential contact to livestock remains unknown. These isolates seem to constitute an intermediate link between human MSSA and swine MRSA isolates, what warrants further molecular investigations.

Low virulence gene content was homogeneous in all 57 isolates investigated. Lower virulence gene properties is a common trait among LA-MRSA ST398, while recent reports on the animal-independent MSSA ST398 have showed that this sub-lineage may harbour more virulence properties (see introduction section). Whether MSSA ST398 from canine isolates follows the same trend is to date unknown.

Surveillance on MSSA ST398 in humans and animals is scarce but essential to uncover potential primary or secondary reservoirs for this lineage, as well as their possible role in the propagation of these bacteria.

CHAPTER 4. CHARACTERIZATION OF NOVEL GENETIC STRUCTURES THAT CARRY ANTIMICROBIAL RESISTANCE GENES

Novel *erm*(T)-carrying plasmids that also carry heavy metal resistance genes.

erm(T)-carrying small integrative plasmid that also carries a cadmium resistance operon in animal-independent MSSA ST398

The genetic environment of the *erm*(T) gene was studied in one MSSA ST398 strain of *spa* type t571, named C3912, isolated from a healthy human. This gene was described for the first time in the genus *Staphylococcus* spp. in a LA-MRSA ST398 strain of porcine origin located in plasmid pKKS25 in physical linkage to the *erm*(T), the *dfrK* and the *tet*(L) genes (see **Fig. 31**) (Kadlec et al., 2010a). Given that the *erm*(T) gene was uncommon in staphylococci (Kadlec et al.,2012a) and since strain C3912 was only resistant to erythromycin (with inducible clindamycin resistance), the genetic environment of this gene as well as its location within the cell (both chromosomal and plasmid location) were investigated.

Few data was available at that time on the location of this gene, however, in addition to the previous report on plasmid pKKS25 in porcine LA-MRSA ST398 (Kadlec et al., 2010a), a couple of previous studies had revealed the presence of *erm*(T) in the chromosomal DNA of MSSA ST398 strains from human infections (Uhlemann et al., 2012; Vandendriessche et al., 2012).

Close analysis of the *erm*(T) genetic environment revealed that it was located on a small RCR plasmid (~6,2 kb), named pUR3912, in physical linkage to a functionally active cadmium resistance operon (*cadDX*). This plasmid represents the first detection of an *erm*(T) positive plasmid that also carries cadmium resistance determinants. In addition, two copies of the IS*431* element in direct repeat were found to flank the *erm*(T) gene. Only one of this IS elements (IS431_L) presented the typical 8-bp duplication at the extremes of the IS integration site, what suggested that this copy had independently integrated into a pUR3912 precursor (**Fig. 36**).

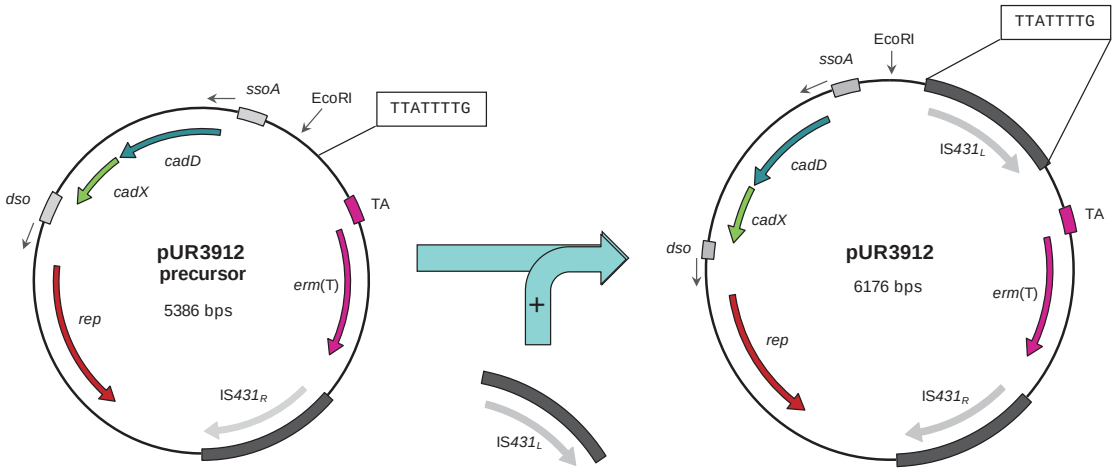

Figure 36. Graphical representation of a hypothetical pUR3912 precursor and the IS*431*$_L$ integration step to become the described novel pUR3912.

The detection of a suspected identical plasmid in two additional animal-independent MSSA ST398 isolates (one t571 and another t1451) of human origin (Lozano et al., 2011a), but not in a small collection of six *erm*(T)-carrying LA-MRSA ST398 of porcine (**chapter 3**) and human origin (Lozano et al., 2012c) suggests that plasmid pUR3912 may occur more often in the animal-independent MSSA ST398. In addition, two related owners and one unrelated dog from the longitudinal study (**chapter 1**) also carried an animal-independent MSSA ST398 strain (*spa* type t1451) that also harboured the *erm*(T) gene (with inducible clindamycin resistance). These isolates were also susceptible to the rest of antimicrobials tested, except for penicillin and revealed the presence of the cadmium resistance operon also in physical linkage to the *erm*(T) gene. These data emphasises on the possible ubiquity of this or a similar plasmid in this human adapted sub-lineage. However, the small number of *erm*(T)-positive *S. aureus* strains does not allow reliable conclusions to be drawn and warrants further molecular surveillance.

Due to the great similarity of pUR3912 and the corresponding chromosomal segment of animal-independent MSSA ST398 strain ST398NM01 [also of human origin (Uhlemann et al., 2012)], evaluation of its possible co-localization in the chromosomal DNA of the same strain was tempting. Not only pUR3912 or a pUR3912 precursor was also integrated in the chromosomal DNA of such strain, but also it was integrated within the same chromosomal integration site (a *tnp*), evidenced by the 8-bp direct repeats observed (5'-CCTTTTTC-3') in both extremes of the integration target site. In addition, it exhibited an identical reorganized structure as that detected in ST398NM01. The only difference with respect to ST398NM01 strain was a 700-bp segment in the former structure. This may indicate that either (i) more than one pUR3912-like plasmid has integrated within the chromosomal DNA at different occasions or (ii) that a rapid

microevolution that advocated to the loss or acquisition of DNA fragments (either in the chromosome or as is free extrachromosomal copy) have occurred.

Remarkably, the integrated plasmid presented a direct duplication of 64 bp that was also present in the chromosomal segment of ST398NM01 but was absent in the free pUR3912 copy (**Fig. 37**). This 64-bp direct repeat represents the plasmid sequence immediately 3' to the initiating nick site for the Rep protein. This strategic position and the length of this duplication points towards a possible abortive termination of a novel cycle of replication of the integrated RCR pUR3912. Abortive termination can occur at sequences similar but not identical to the origin of replication (Del Solar et al., 1998; Khan, 2005); however, the low level of similarity between the origin of replication (5'-ATAATA-3') and the abortive termination region is unexpected. Possible inactivation of the Rep protein or inability of the Rep to continue the original replication scheme in the integrated structure might be speculated, what could imply absence of replication of pUR3912 within the chromosomal context, when no free copies of pUR3912 coexisted in the cell.

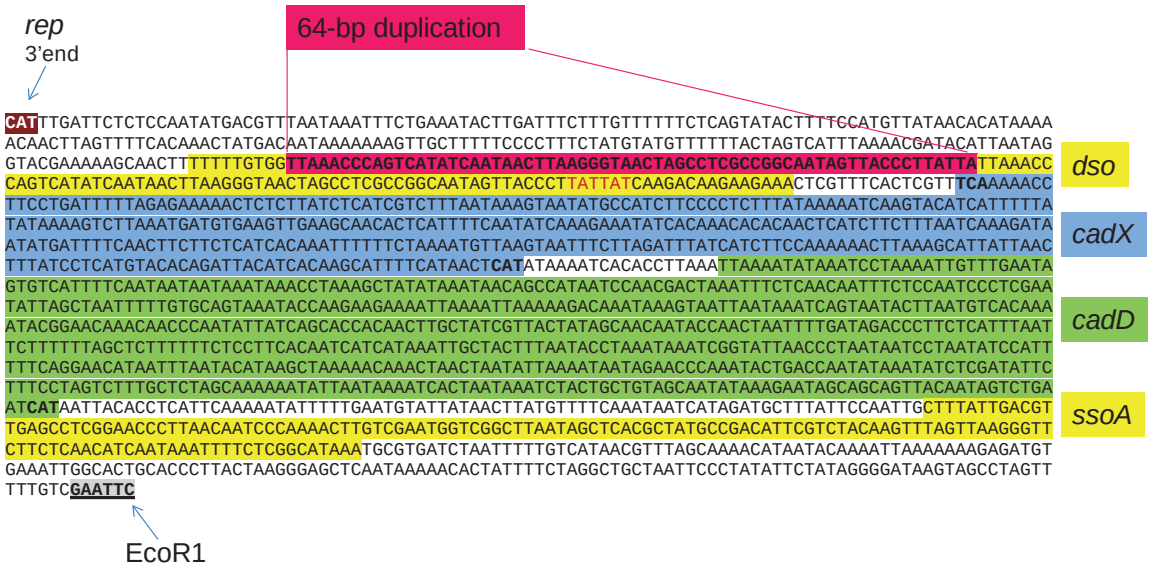

Figure 37. Selected DNA fragment of the integrated pUR3912 within the chromosomal DNA of strain C3912. The displayed fragments show the double strain origin of replication (*dso*, 153 bp) and the single strand origin of replication (of the type A) of the lagging strand (*ssoA*, 142 bp). The *dso* and *ssoA* are displayed in yellow, the cadmium resistance gene *cadD* is shown in green and the cadmium transcription *cadX* regulator in blue colour. The 3'en extreme of the the *rep* gene is shown in red and the 64-bp segment duplicated within the *dso* of pUR3912 is displayed in pink. The EcoR1 site is also indicated.

The presence of this 64-bp direct duplication might involve the production of an extended, reorganized and more instable *dso* secondary structure (**Fig. 37**). Possible incapability

of the Rep to recognize the bind and nick sites of the enlarged *dso* structure in the integrated plasmid is unknown. Further molecular approaches are needed to determine whether the integrated copy of the RCR pUR3912 is functionally active.

In addition, both MSSA ST398 strains of human origin (strains C2549 with *spa*-type t571 and C2679 with t1451) (Lozano et al, 2011a), which, based on the expected plasmid size and gene content [*erm*(T) and *cadDX*], carried plasmid pUR3912, also harboured an integrated copy of pUR3912 within the same genetic context and also presented the additional 64-bp repeat. Chromosomal integration within this IS element in unrelated ST398-t571 and -t1451 MSSA strains points towards a preferable integration site.

Long-range PCR analysis (**Fig. 38**) of the *erm*(T) region in the five *erm*(T)-carrying MSSA ST398 of human and canine origin recovered in the longitudinal study suggested that a similar but bigger *erm*(T)-carrying chromosomal segment was also located within the same integration site (*tnp*). In these isolates, the extreme carrying the 700-bp additional fragment downstream the *rep* gene (absent in ST398NM01) was about 2 kb longer. These results suggest a relative dynamism of that fragment of the plasmid, but still a clonal spread of pUR3912-like integrated plasmids in this sub-lineage.

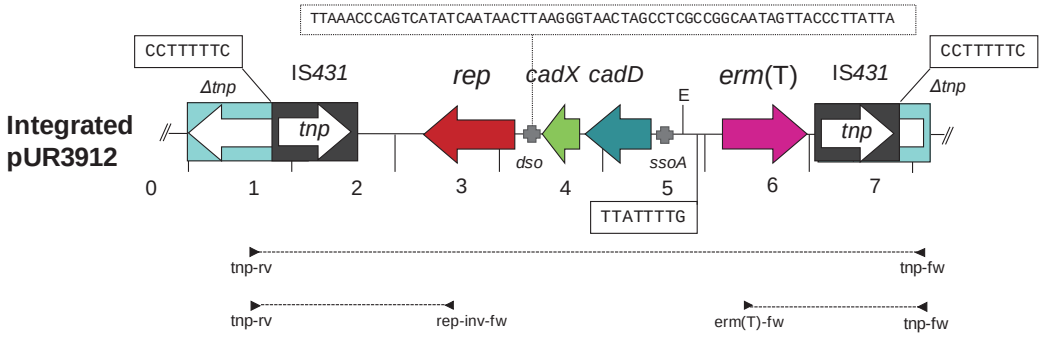

Figure 38. **A.** Integrated pUR3912 and several combination of primers employed to detect its chromosomal integration site as well as to amplify the whole cointegrate (tnp-fw + tnp-rv) within the *tnp*.

Moreover, a recent study that analyzed a collection of different *S. aureus* ST398 isolates revealed that 95% of human-associated MSSA ST398 carried the *cadDX* operon, in contrast to only 25% of pig-associated MRSA ST398 isolates investigated (McCarthy et al., 2012). Unfortunately, the location of *cadDX* in those isolates was not investigated. The same study reported ϕ3 bacteriophage, which carries the human-specific IEC genes *scn, sak* and/or *chp*, as the best genetic marker of the human-specific ST398 clade. As expected, PCR analysis of the IEC

genes revealed the presence of both *scn* and *chp* in all eight isolates. As plasmid pUR3912 is neither conjugative nor carries mobilization genes, co-resident plasmids that carry the respective genes for horizontal transfer are needed. Such plasmids have so far not yet been detected in the strains that carry pUR3912-like plasmids. This may point towards limited options for horizontal transfer of pUR3912 to other MRSA/MSSA lineages, including the animal-associated ones, under the current conditions. Further epidemiological studies are warranted to elucidate whether pUR3912 and/or its chromosomal co-integrate are preferentially present in the animal-independent ST398 MSSA clade.

The IS*6* family, to which IS*431* belongs, gathers an extensive group of ISs (http://www-is.biotoul.fr/is.html), including the IS*431*-closely related IS*257*, IS*sau10* and IS*26*. These IS elements have been shown or suggested to mediate replicon fusions and/or chromosomal integration processes (Firth et al., 1998), and many ISs of this family have been found as part of compound transposons (Mahillon et al., 1998). The existence of two copies of IS*431* in the same orientation flanking the integrated structure and the presence of 8-bp direct repeats at their external integration site suggests that in the event of losing the *rep* gene, this segment would become a transposon-like structure, suggesting that integration via these ISs may evolve other MGEs. The widespread distribution of these elements in genetic structures carrying antimicrobial resistance determinants evidences that they play an essential role in the emergence of staphylococcal resistance. Moreover, the location of these elements in small plasmids, such as the RCR pUR3912, facilitates its mobilization and maintenance under selective conditions.

The ability of pUR3912 to integrate into the chromosome of different MSSA ST398 isolates via ISs, as well as its possible co-localization within the cell, ensures persistence and dissemination due to both vertical and horizontal transference. These data highlights the important role of insertion sequences in the acquisition, maintenance and dissemination of antimicrobial and/or metal resistance genes. In addition, further molecular surveillance studies are warranted to elucidate whether pUR3912 and/or its chromosomal co-integrate are preferentially present in the animal-independent ST398 MSSA clade.

erm(T)- *carrying MDR plasmids that also carry cadmium and copper resistance determinants in LA-MRSA ST398*

Three large novel MDR plasmids (~30 kb) that also carried cadmium and copper resistance determinants in LA-MRSA ST398 strains of animal (pUR1902) and human (pUR2940 and pUR2941) origin were described in this work. This is the first report of plasmids from *S. aureus* ST398 carrying both cadmium and copper resistance determinants and the first *erm*(T)-carrying plasmid that also possesses copper resistance genes.

Since the first description of MRSA ST398 in pigs in 2005 (Armand-Lefevre et al., 2005; Voss et al., 2005), MRSA strains of this lineage have been shown to have an outstanding capacity to acquire and maintain multiple and diverse antimicrobial resistance genes as well as MGEs, such as plasmids and transposons (Kadlec et al. 2012a). The *erm*(T) gene, in spite of its recent description in staphylococci, has been already detected in a number of LA-MRSA ST398 strains of both animal and human origin (Argudín et al., 2011; Fessler et al., 2010; Fessler et al., 2011; Kadlec et al., 2010a; Lozano et al., 2012c). In contrast to LA-MRSA ST398, the animal-independent MSSA ST398 is susceptible to the majority of antimicrobials tested, showing in most cases only resistance to erythromycin and inducible clindamycin resistance, in addition to penicillin (Chen et al., 2010; Lozano et al., 2012c; Mediavilla et al., 2012; Price et al., 2012; Rasigade et al., 2010; Uhlemann et al., 2012; Valentine-Domelier et al., 2011; Vandendriessche et al., 2011), what contrasts with the MDR and constitutive resistance profiles observed in *erm*(T)-carrying LA-MRSA ST398 strains.

DNA plasmid comparison analysis with deposited sequences on GenBank as well as with that of pUR3912, revealed that the 3 novel MDR plasmids were mosaic structures evolved from the reorganization and recombination of already known plasmids from *S. aureus* but also from other staphylococci (*S. epidermidis*, *S. warneri*) and other Gram-positive bacteria (*Lactococcus lactis*) (**Table S11**). In addition, the relatively high percentage of identity that pUR1902 exhibited with relation to pUR2940 and pUR2941 suggests that a pUR1902-like plasmid might be the ancestor of the more diversified pUR2940 and pUR2941 plasmids.

It is interesting to remark that the three novel plasmids also presented a region of homology with the recently described pUR3912. This similar region (sharing >80% identity) included the cadmium resistance operon and the *ssoA* region of the RCR pUR3912, which also shared homology to the plasmid pUB101 of *S. aureus* (**Fig. S7**). Given that the *ssoA* region is only

essential for RCR plasmids, its detection in large mobilizable plasmids indicate that this region has been acquired as a result of inter-plasmid recombination processes.

The phylogenetic relationships of the *erm*(T) gene with all deposited sequences on GenBank/EMBL database revealed that those from plasmid pUR3912 and strain ST398NM01 (both MSSA-t571 ST398), although they shared 98.9% or 98.8% identity with those of pUR2940, pUR2941 and pUR1902, constituted a separate branch (**Fig. 39**) due to the presence of several nucleotide substitutions.

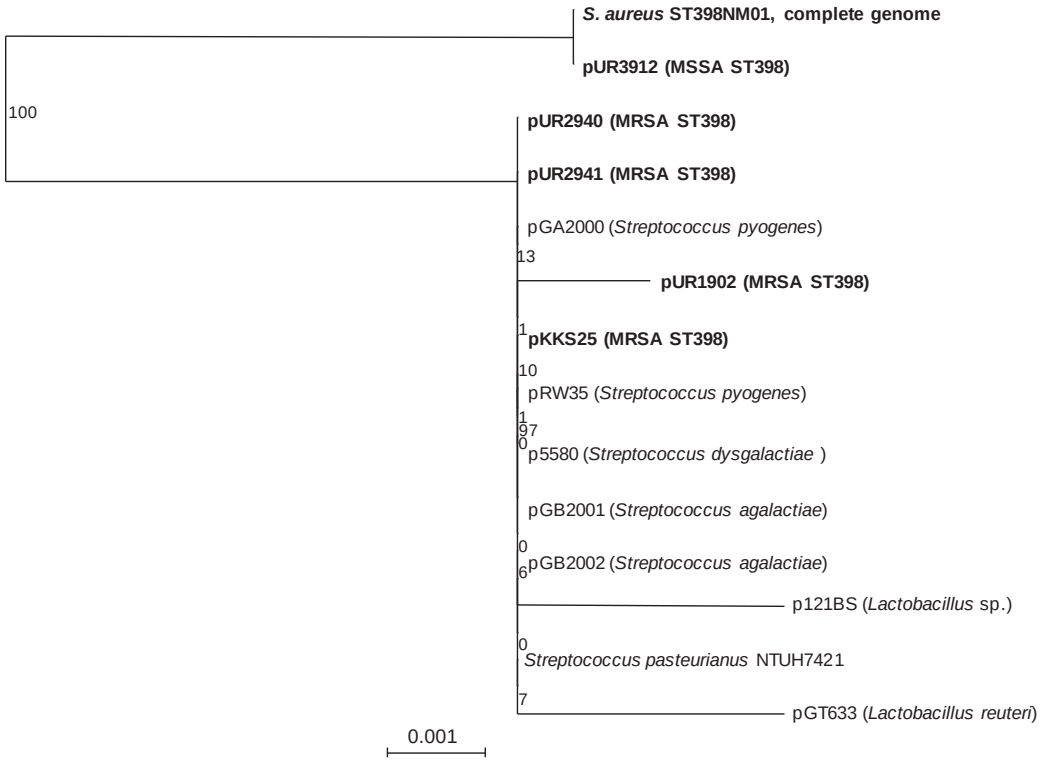

Figure 39. Phylogenetic relationships of the *erm*(T) gene. Sequences were aligned using MUSCLE, and the trees were built with PhyML using a general time reversible (GTR) model. The numbers at the tree branches are percentage bootstrap values indicating the confidence levels. The bar length indicate the number of substitutions per site. In bold, *erm*(T) gene detected in *S. aureus* ST398. Nucleotide sequences of *erm*(T) were found under the following accession numbers: CP003045 (*Staphylococcus aureus* ST398NM01); HE805623 (pUR3912); HF583292 (pUR2940); HF583290 (pUR2941); JF308631 (pGA2000); HF583291 (pUR1902); FN390947 (pKKS25); EU192194 (pRW35); HE862394 (p5580); JF308630 (pGB2001); JF308629 (pGB2002); AF310974 (p121BS); AY894138 (*Streptococcus pasteurianus* NTUH 7421); M64090 (pGT633).

Moreover, a 56/58-bp region that was found to flank the *erm*(T) gene immediate up- and downstream regions of the three novel plasmids was also detected in the correspondent part of some streptococcal plasmids (pGB2002, pGB2001, pGA2000, p5580 and pRW35), but not in pUR3912 or in ST398NM01. We speculate that this homologous region, flanked by large non-

homologous segments, may be responsible for the integration of the *erm*(T)-carrying segment in a precursor of these LA-MRSA ST3987 MDR plasmids. All these data point either to a different origin of *erm*(T) in the animal-independent MSSA ST398 isolates or a divergent evolution of this gene after adaptation to the different sub-lineages or hosts.

The presence of cadmium resistance determinants in plasmids of *S. aureus* of different lineages is relatively common (Crupper et al., 1999; Holden et al., 2004; Malachowa et al., 2010; Schwarz et al., 2011). Likewise, genes involved in copper resistance, especially *copA* and *mco* or variants (*copB*, *copC*), have been detected in different staphylococcal species and other Gram positive bacteria, such as *Listeria monocytogenes* or *Macrococcus caseolyticus* (Baba et al., 2009; Diep et al., 2006; Gill et al., 2005; Gilmour et al., 2010; Highlander et al., 2007; Holden et al., 2004; Sitthisak et al., 2005; Takeuchi et al., 2005; Zhang et al., 2003). In LA-MRSA ST398, cadmium, copper and other heavy metal resistance genes had been only detected in the chromosome, either within the SCC*mec* V(5C2&5)$_C$, which carries the cadmium/zinc resistance gene *czrC*, or in the novel SCC*mec* IX and X, which carry cadmium (*cadDX*), copper (*copB-mco*) and arsenic (*arsRBC* and *arsDARBC*) resistance elements (Cavaco et al., 2010; Li et al., 2011).

The phylogenetic relationships of the cluster *copA-mco* gene with all sequences deposited in GenBank/EMBL that shared >80% similarity were also evaluated. The *copA-mco* cluster of the three novel plasmids exhibited the highest degree of homology with those detected in the recently described SCC*mec* IX and X, among others (**Fig. 40**), These data suggests a plausible acquisition from a common source, if not a direct recombination between precursors of such structures. The possibility that these heavy metal resistance determinants and the novel described plasmids may have originated from such SCC*mec* elements or vice versa cannot be ruled out, and underlines the capacity of this lineage to acquire, recombine and exchange genetic elements.

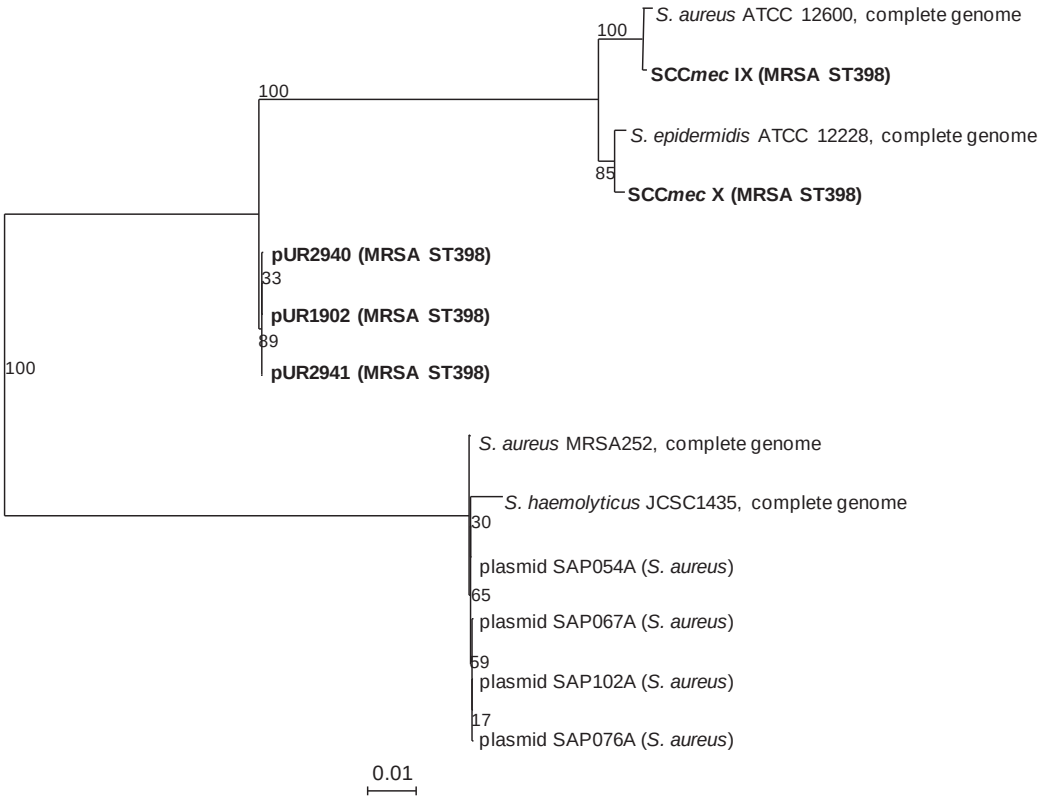

Figure 40. Phylogenetic relationships of the gene cluster *copA-mco*. Sequences were aligned using MUSCLE, and the trees were built with PhyML using a general time reversible (GTR) model. The numbers at the tree branches are percentage bootstrap values indicating the confidence levels. The bar length indicate the number of substitutions per site. In bold, *copA-mco* cluster detected in *S. aureus* ST398. Nucleotide sequences of cluster *copA-mco* and its homologues (>95% coverage, >80% identity) were found under the following accession numbers: AY259130 (*S. aureus* ATCC 12600); AB505628 (SCC*mec* IX); AE015929 (*S. epidermidis* ATCC 12228); AB505630 (SCC*mec* X); HF583292 (pUR2940); HF583291 (pUR1902); HF583290 (pUR2941); BX571856 (*S. aureus* MRSA252); AP006716 (*S. haemolyticus* JCSC1435); GQ900477 (plasmid SAP054A); GQ900483 (plasmid SAP067A); GQ900496 (plasmid SAP102A); GQ900427 (plasmid SAP076A). Given that plasmid SAP019A (GQ900385) was identical to SAP054A and plasmids SAP075A (GQ900486), SAP078A (GQ900430) and SAP077A (GQ900428) were identical to SAP102A, they were not included in the phylogenetic tree for a better display of the results.

Cadmium is highly toxic to all animal species. It is frequently found as contaminant of food and feed materials, due to its natural (abrasion and erosion of rocks and soils, etc.) and anthropogenic (fossil fuels power generation plants, by-product of the extraction of other metals, cement production, cadmium products, etc.) occurrence in the environment and the resulting levels of cadmium in soils and plants (**Fig. 41**). Elevated concentrations of cadmium in feedstuffs may also occur due to the application of sewage sludge or phosphate fertilizers with high levels of cadmium in agricultural soils (EFSA, 2004).

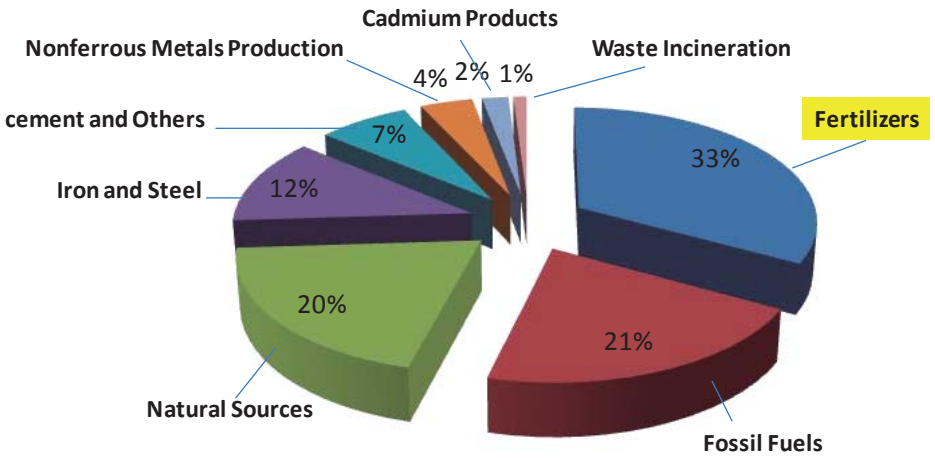

Figure 41. Relative contributions of different sources to human cadmium exposure (Regoli, meeting of UNECE task force on heavy metals, 16 March 2005, Berlin) (http://www.cadmium.org/pg_n.php?id_menu=15)

Even though the accepted maximum levels of cadmium in feedstuff for livestock are regulated (EFSA, 2004), the possibility that such intake might be in part responsible for the selection of cadmium resistance determinants in the present bacterial population of swine cannot be excluded. In humans, smoke of cigarettes is an additional important source of cadmium (Ashraf et al., 2012). However, the major route of cadmium intake for the non-smoking and non-occupationally-exposed population is also through ingestion of contaminated food (including food of animal origin) and water (EFSA, 2004; Ciobanu et al., 2012). This exposure might also select for cadmium resistance in transferable elements in the *S. aureus* human population. In contrast, copper and zinc are essential trace elements for animals and humans (EC, 2003a; EC, 2003b). In addition, as previously indicated, they are added as feed supplements for livestock, particularly pigs, in great quantities to increase the daily growth rate of piglets (up to 8-10 weeks of age), to prevent gastrointestinal infections and to limit and control the cases of post-weaning wanting and wasting (Cavaco et al., 2010; EC, 2003a; EC, 2003b; Hasman et al., 2006). The presence and maintenance of genetic elements that carry genes for copper and zinc resistance/tolerance in LA-MRSA ST398 is most probably favoured by the extensive use of copper and zinc in pig production.

Although the *copA-mco* genes of pUR1902 and pUR2941 increased the MIC values for copper only by one dilution, it should be noted that these MIC differences observed are in the >1,000 µg/ml range. Unlike for antimicrobial agents, there are no approved breakpoints to

assess microbial tolerance or resistance to copper, zinc or similar compounds (Hasman et al., 2006). The truncation of the *mco* gene in pUR2940, which is involved in oxidation of copper ions, implied preservation of the $CuSO_4$ MIC values despite the presence of the *copA* gene. Higher intracellular copper levels have been observed in a *S. aureus* ATCC12600 mutant with nucleotide substitutions within the *mco* gene, suggesting that inactivation of *mco* has an additional negative effect upon either *copA* expression or CopA functionality (Sitthisak et al., 2005).

All three novel plasmids were MDR, carrying tetracycline and MLS_B resistance genes, as well as kanamycin/neomycin or trimethoprim resistance genes. Due to the elevated use of antimicrobial agents in the animal sector, in particular tetracyclines, macrolides, lincosamides and aminoglycosides (Kadlec et al. 2012; Prescott, 2006), the co-selection of structures carrying both antimicrobial resistance and heavy metal resistance genes may favour their maintenance and dissemination under selective conditions. In addition, the fact that all these resistance genes are plasmid-located facilitates their spread under the selective pressure imposed by the use of one of them.

The presence of several copies of the recently described IS*Sau10* element in the sequenced region of all plasmids is relevant, and these IS*Sau10* copies are most probably responsible for the structural organization of such mosaic plasmids. In these terms, **Fig. 42** shows a plausible evolutionary pathway of the pUR1902 sequence deduced from previously described plasmids and involving IS*Sau10*. This model suggests that part of a pKKS627-like plasmid and pGB2002 and a piece of pSW49 and pUB101 have recombined to form the hypothetical structures 1 and 2, respectively. Then, IS*Sau10* copies have integrated within *repU* (structure 1) and *rep49* (structure 2) and formed the 8-bp direct repeats present in the final pUR1902. These latter structures may have undergone homologous recombination of both IS copies to form a unique sequence closely related to that of pUR1902. A final recombination step between both IS*Sau10* that flank the *aadD* gene may have lead to a change of the orientation of the sequence between both IS elements, finally resulting in the *erm*(T)-flanking region detected on plasmid pUR1902.

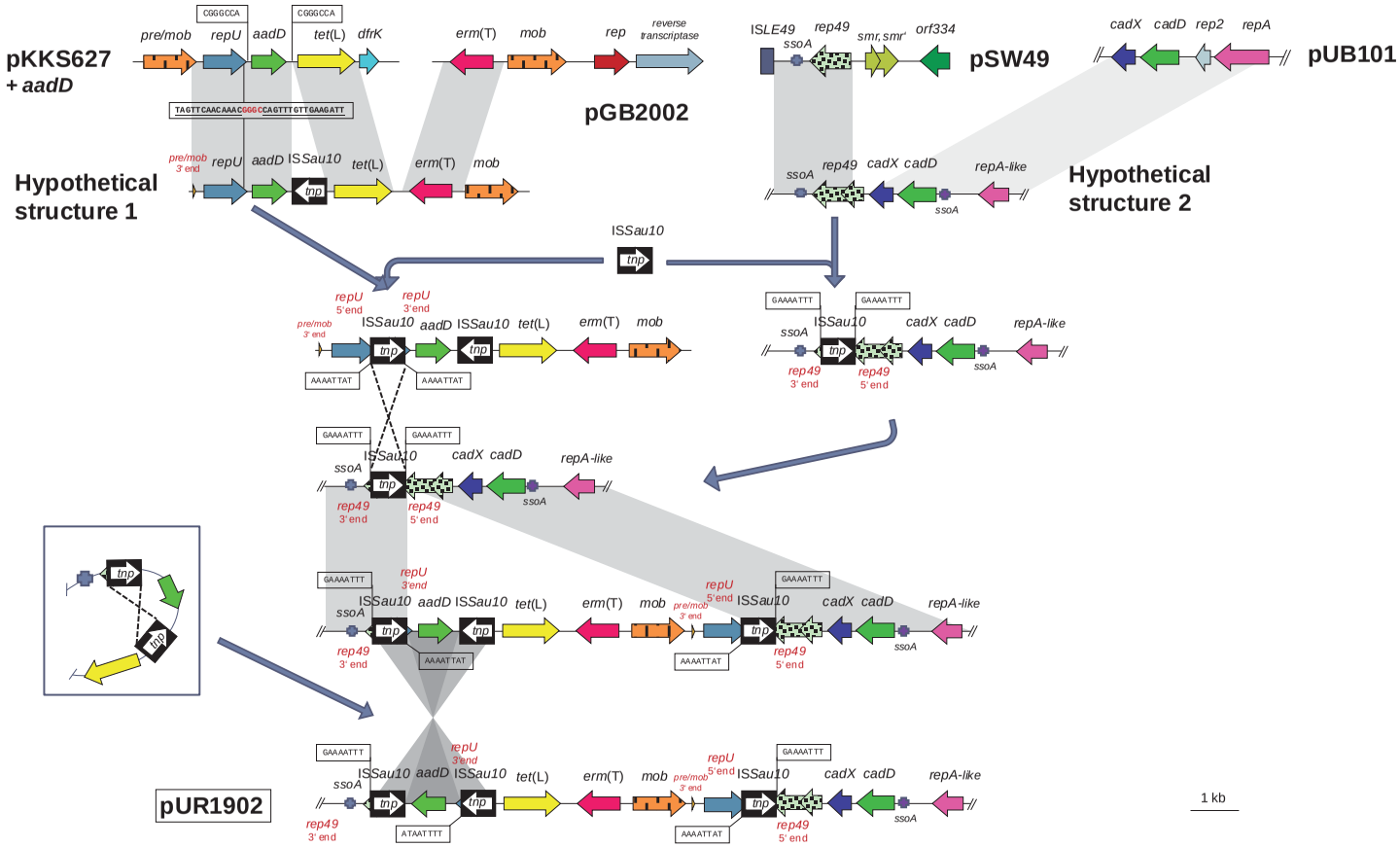

Figure 42. Potential evolutionary pathway followed by the *erm*(T) flanking region of pUR1902 (accession no. HF583291) from several putative precursors [pKKS627 (FN390948), pGB2002 (JF308629), pSW49 (AM040730) and pUB101 (AY373761)]. The arrows indicate the positions and directions of the transcription of the genes. The 5' and 3' end of the truncated *rep49* and *repU* genes are shown. The different SSO regions detected are indicated as *ssoA*. The IS*Sau10* copies are displayed as black boxes with the white arrow indicating the transposase gene *tnp*. The 8-bp direct target site duplications at the extremes of IS*Sau10* integration sites within the *rep* genes are shown within boxes and underlined. The regions of >90% homology are displayed in dark gray shaded while those between 80-90% similarity are shown in faint gray shaded. A distance scale in kilobases (kb) is displayed in the lower right corner.

As observed in pUR3912, IS*Sau10*-related IS*257* and IS*431* have been shown to be responsible for the co-integration of small plasmids within larger plasmids or within the chromosomal DNA (Firth et al., 1998; Leelaporn et al., 1996; Schwarz et al., 2011). The different copies of IS*Sau10* disrupted diverse *rep* genes of small plasmids as well as the *mco* gene of pUR2940. It has been suggested that the truncation of the *rep* genes in the co-integration process of different plasmids is important for the maintenance of the replication system of the original replicon (Schwarz et al., 2011). In addition, the presence of two IS*Sau10* copies in the same orientation with the typical 8-bp direct target site duplications at both ends and bracketing a sequence that closely resembles a small *erm*(C)-carrying plasmid (pNE131) in pUR2940, strongly suggested that its integration was IS*Sau10*-mediated. IS elements play an important role in the evolution of multidrug resistance plasmids because of their capacity to integrate, split, and undergo homologous recombination with related IS elements. Interestingly, several SSO regions, characteristic of small plasmids that replicate via the rolling circle mechanism, were observed in all novel plasmids at different positions. This observation additionally evidences the acquisition and reorganization capacities of LA-MRSA ST398 to form mosaic plasmid structures. However, only continuing molecular epidemiological studies will be able to uncover the stability of such novel plasmids.

In conclusion, this is the first report of *S. aureus* ST398 plasmids carrying both cadmium and copper resistance determinants and to the best of our knowledge, the first description of staphylococcal multidrug resistance plasmids that also carry cadmium and copper resistance genes. The co-location of multiple heavy metal and antimicrobial resistance genes underlines the flexibility of *S. aureus* ST398 to adapt to the selective conditions imposed by the use of antimicrobial agents and heavy metals. Moreover, the location of these resistance genes on MGEs, such as plasmids or SCC*mec* structures, and the presence of IS elements facilitate their co-selection, persistence, reorganization and dissemination, which in turn warrants further continuous surveillance.

The observation that *erm*(T) is present either on a small free or chromosomal integrated plasmid (animal-independent MSSA ST398 isolates) or on larger plasmids that also carry *tet*(L) (in LA-MRSA ST398 isolates), suggests that the *erm*(T) gene has been acquired at independent occasions by animal-independent MSSA and LA-MRSA ST398 strains that are adapted to different hosts, what opens that door to further molecular analysis on the evolution of this highly adapted ST398 lineage in different hosts.

Novel *fexAv* gene variant that only confers chloramphenicol resistance in a canine *S. pseudintermedius*.

Since the first description of the *fexA* gene from a bovine *S. lentus* isolate (Kehrenberg et al., 2004), this gene has been detected - either alone within the conserved transposon Tn*558* or in combination with the *cfr* gene in other Tn*558* variants - in *S. aureus* and several coagulase negative staphylococci from healthy and diseased bovine, swine, equine and human samples (Argudín et al., 2011; Fessler et al., 2010; Lozano et al., 2012d; Kadlec et al., 2009a; Kehrenberg et al., 2006; Shore et al., 2010; Wang et al., 2012). Recently, it has been detected in a *Bacillus* isolate from swine faeces and in environmental pollutants from swine feedlots in China (Dai et al., 2010; Li et al., 2012), what suggests its ability to reside on soils, environmental surfaces or bacterial communities adapted to those conditions.

In **chapter 2**, one chloramphenicol- and penicillin-resistant *S. pseudintermedius* isolate recovered from a healthy household dog was positive for the *fexA* gene. This strain, named C2719, belonged to ST20 - lineage that has already been detected in a household dog in this thesis -, and harboured the leukocidin *lukS/F-I*, the exfoliatin *siet* and the enterotoxin *se-int* virulence genes. Surprisingly, chloramphenicol and florfenicol microdilution assays revealed that this strain was resistant to cloramphenicol but susceptible to florfenicol, even though it lacked any of the *cat* genes described for staphylococci. Further molecular evaluation of the *fexA* gene and its genetic environment revealed that strain C2719 possessed a novel *fexA* variant, named *fexAv*, enclosed within transposon Tn*558*, which only conferred chloramphenicol resistance. Tn*558* was, as expected for this type of transposons (to which the *dfrK*-carrying Tn*559* belongs), integrated within the chromosomal *radC* gene of *S. pseudintermedius* C2719, and represented the first detection of this transposon in *S. pseudintermedius*.

Further molecular experiments based on information provided by (Braibant et al., 2005) on FloR protein of *Salmonella*, suggested that any of two surrounding nucleotide substitutions that differed from the prototype *fexA* gene of *S. lentus* (Kehrenberg et al., 2004), were implied in the absence of resistance to florfenicol (**Fig. S8**). Site-directed mutagenesis (SDM) on both amino acids substitutions of FexAv (Gly33Ala/Ala37Val) to revert them back to the prototype *fexA* of *S. lentus* revealed that both amino acids were important for the efflux of florfenicol from the cell of *E. coli* Hb101, given that when they

were reverted separately (either Ala33Gly or Val37Ala) the initial MIC values to florfenicol were preserved (MIC 4 μg/ml) but they increased in 4-fold dilution when both mutations were reverted (MIC 16 μg/ml). The parallelism found on the mutated FloR protein suggests that these amino acids play a role in substrate recognition.

Tn*558* presented an additional semi-conserved amino acid substitution (Gly321Ala) in the transposase gene *tnpA* (nucleotide G962C). Since both transposase genes *tnpA* and *tnpB* are essential for transposition and no circular intermediates (indicative of its functional activity) of Tn*558* were detected, it is suggested that the inability of Tn*558* to transpose might be due for the presence of such amino acid substitution within TnpA. However, this is pure speculative and further molecular evaluation is needed for conclusive assertions.

This work reports the first detection of a *fexA* gene and Tn*558* in *S. pseudintermedius*, and the first description of *fexAv* that only confers chloramphenicol resistance. Given that phenicols are not licensed to be administered to dogs, the presence of a *fexAv* that does not confer florfenicol resistance might imply a favourable situation for the *fexAv*-carrying *S. pseudintermedius* strain C2719 under absence of phenicol selective pressure. Further, the chromosomal location of this transposon as well as its inability to transposase means that Tn*558* will be, a priori, preserved in the chromosomal DNA of strain C2719. This observation might support the idea of an adaptive advantage under absence of selective pressure. Whether these mutations occurred once Tn*558* was located in a phenicol-free molecular ecosystem or was the response to random mutations remains unknown.

Continued molecular surveillance on antimicrobial resistance determinants in *S. pseudintermedius* is required to detect possible antimicrobial resistance genes with distinct functional activities and to evaluate potential novel points of action against antimicrobial resistance.

Conclusions

CONCLUSIONS

1. Humans and dogs are the natural hosts for *S. aureus* and *S. pseudintermedius*, respectively; nevertheless, both bacterial species can be present in both animal hosts. The *S. aureus* carriage in household dogs is predominantly sporadic, while dog-owners can be persistent carriers of *S. pseudintermedius*. Dogs contribute to the propagation of *S. aureus* and *S. pseudintermedius* at the household.

2. Higher antimicrobial resistance profiles and virulence gene content are observed among *S. aureus* from household individuals (pets and owners) than those from kennel dogs. On the other hand, higher MRSP nasal carriage rates are observed in kennel dogs.

3. The first detection of the clonally spread European MRSP clone ST71 in Spain is reported, what evidences its broad geographic distribution.

4. High MRSA nasal carriage rates are detected in slaughter pigs (49.1% in piglets and 20.8% in adult swine), representing the first study of this characteristics in our country. A total of 71.4% of MRSA from adult swine and all MRSA from piglets belonged to the ST398 lineage. Due to the differences in prevalence, antimicrobial resistance profile and MRSA lineages detected in adult swine and piglets, age of animals should be considered when designing and comparing studies.

5. The detection of several MRSA CC97, traditionally closely associated to cattle, in swine shows that pigs can be also carriers of MRSA of lineages associated to other livestock.

6. The first detection of MRSA ST398 in horses and of *S. pseudintermedius* ST68 in equines is described in this work. One of the MRSA ST398 was recovered in 2005, being coincident with the first detections in Europe and its subsequent expansion, and represents the oldest ST398 MRSA strain detected in our country to date.

7. Differences were observed in the antimicrobial resistance profile (β-lactams, tetracyclines, MLS_B), the *spa* types and the presence of the IEC genes in the *S. aureus* ST398 strains recovered from pigs and horses, humans with not contact to livestock

and dogs. MSSA ST398 strains from pound dogs exhibit common characteristics to the porcine MRSA ST398 and human MSSA ST398 strains.

8. The association of antimicrobial resistance genes and genes that confer resistance to heavy metals in the same mobile genetic elements can facilitate its selection and dissemination.

9. The first description of a plasmid that carries MLS_B in addition to cadmium resistance genes [*erm*(T) + *cadDX*] in *S. aureus* ST398 strains is reported. The co-location of this plasmid as its free form as well as integrated within the chromosomal DNA ensures its maintenance and dissemination.

10. Different genetic environments were described for the gene cluster *erm*(T) + *cadDX* in *S. aureus* ST398, being related to the genetic (β-lactams resistance, IEC) and epidemiologic background (human/animal) of the isolates. These data appear to show a predisposition of this lineage to acquire distinct MGEs that carry both resistant determinants and show their dynamic character.

11. The first description of the *fexA* gene and the transposon Tn*558* in *S. pseudintermedius* is reported. A novel variant (*fexAv*) that only confers chloramphenicol resistance was revealed and evidenced the presence of 2 punctual nucleotide substitutions that seem to be implicated in the absence of florfenicol resistance.

General conclusion:

This work deepens in the population structure of *S. aureus* and *S. pseudintermedius*, their colonization dynamics in different hosts and their adaptation ability to evolve under conditions of selective pressure. Direct contact between pets and humans is relevant for the epidemiology of both bacterial species. Due to the high capacity of both microorganisms to acquire, maintain and (in *S. aureus*) mobilize antimicrobial resistance genes, further molecular surveillance is essential to monitor their progress over time.

CONCLUSIONES

1. El perro y el hombre son hospedadores naturales de *S. pseudintermedius* y *S. aureus* respectivamente, no obstante las dos especies bacterianas pueden encontrarse en las fosas nasales de ambos hospedadores. Los perros de hogares son mayoritariamente portadores esporádicos de *S. aureus* y sus dueños pueden ser portadores de *S. pseudintermedius* de forma persistente. Los perros pueden considerarse un eslabón en la cadena de transmisión de *S. aureus* y *S. pseudintermedius* en el hogar.

2. Se observan mayores tasas de resistencia a antimicrobianos y de genes de virulencia en las cepas *S. aureus* de individuos de hogares (dueños, mascotas), con respecto a aquellas aisladas de perros de perrera. Por otro lado, se detecta un mayor porcentaje de SPRM en perros de perrera que en los procedentes de hogares.

3. Se detecta por primera vez en España el clon europeo emergente SPRM ST71, lo que evidencia su amplia distribución geográfica.

4. Se observa una elevada prevalencia de SARM en cerdos en matadero (49,1% en lechones y 20,7% en cerdos adultos) tratándose del primer estudio de este tipo en nuestro país. El 71,4% de las cepas de cerdos adultos y todas las cepas procedentes de lechones pertenecieron al linaje ST398. En base a las diferencias detectadas en prevalencia, perfil de resistencia y linajes de MRSA aislados de cerdos adultos y lechones, la edad del animal debería tenerse en cuenta a la hora de diseñar y comparar estudios.

5. .La detección de varias cepas CC97, tradicionalmente asociado a vacuno, demuestra que los cerdos también pueden ser portadores de SARM asociados a otras especies de animales de producción.

6. Se detecta por primera vez en España SARM ST398 en caballos y *S. pseudintermedius* ST68 en equinos. Una de las cepas SARM ST398 fue aislada en el año 2005, coincidiendo con las primeras detecciones en Europa y su posterior expansión, y representa la cepa SARM ST398 más antigua detectada en nuestro país hasta la fecha.

7. Se observan diferencias en el perfil de resistencia a antimicrobianos (β-lactámicos, tetraciclina, MLS_B), en el tipo *spa* y en la presencia de genes del IEC entre las cepas

S. aureus ST398 obtenidas de cerdos y caballos, las de humanos sin contacto con animales de granja y las detectadas en perros. Las cepas SASM ST398 de perros presentan características propias de las cepas SARM ST398 de porcino y de SASM ST398 de humano.

8. La asociación de genes de resistencia a antimicrobianos y a metales pesados en los mismos elementos genéticos puede facilitar su selección y diseminación.

9. Se describe el primer plásmido portador de genes de resistencia a antibióticos MLS_B y a cadmio [*erm*(T) + *cadDX*] en cepas *S. aureus* ST398. La presencia de este plásmido tanto en forma libre como integrado en el cromosoma asegura su mantenimiento y facilita su diseminación.

10. Se describen entornos genéticos diferentes para los genes *erm*(T) + *cadDX* en cepas *S. aureus* ST398, siendo dependientes del historial genético (resistencia a β-lactámicos, IEC) y epidemiológico (humano/animal) de éstas. Esto parece indicar una predisposición de este linaje para adquirir EGMs portadores de ambos determinantes genéticos y evidencia el carácter dinámico de éstos.

11. Se detecta por primera vez el gen *fexA* y el transposón Tn*558* en *S. pseudintermedius* Así mismo, se describe por primera vez una variante del *fexA* que confiere solo resistencia cloranfenicol y que presenta dos mutaciones puntuales que parecen estar implicadas en la ausencia de resistencia a florfenicol.

Conclusión general:

Este trabajo profundiza en la estructura poblacional de *S. aureus* y *S. pseudintermedius*, su dinámica de colonización en diferentes hospedadores y su capacidad de evolución para adaptarse a condiciones de presión selectiva. Se evidencia que el contacto directo entre animales y humanos es relevante para la epidemiología de ambas especies bacterianas. La elevada capacidad de ambos microorganismos para adquirir, mantener y (en *S. aureus*) movilizar genes de resistencia a antimicrobianos hace esencial continuar con estudios de vigilancia para controlar su evolución en el tiempo.

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