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Detección y caracterización de *Staphylococcus aureus* procedentes de aninales y aguas

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CERTIFICAN

Que la tesis doctoral que lleva por título "Detección y caracterización de *Staphylococcus aureus* procedentes de animales y aguas" ha sido realizada por la licenciada en Veterinaria Dª. M. Concepción Porrero Calonge en el Departamento de Sanidad Animal de la Facultad de Veterinaria y en el Servicio de Zoonosis de Transmisión Alimentaria y Resistencia a Antimicrobianos del Centro de Vigilancia Sanitaria Veterinaria (VISAVET) de la Universidad Complutense de Madrid bajo la dirección conjunta de los que suscriben, y estimamos que reúne los requisitos exigidos para optar al título de Doctor por la Universidad Complutense.

Madrid, 23 de abril de 2014

Fdo. Lucas Domínguez   Fdo. José F. Fernández-Garayzábal   Fdo. Ana Mateos

Proyectos y contratos de investigación

El trabajo de investigación que se presenta a continuación se ha realizado en el marco de los siguientes proyectos y contratos de investigación:

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Resumen

1. Resumen

Staphylococcus aureus es un microorganismo comensal del hombre y los animales, que puede dar lugar a procesos patológicos de diferente gravedad, desde infecciones cutáneas a neumonía, endocarditis, septicemia, etc. La aparición de aislados de *S. aureus* resistentes a meticilina (MRSA) tras la captación de mecanismos de resistencia a meticilina por parte de aislados sensibles (MSSA) aumenta la gravedad de los procesos clínicos debido a las dificultades en el tratamiento terapéutico.

Aunque el hallazgo de MRSA en animales se describió ya en 1972, la detección de MRSA perteneciente a una línea genética diferenciada en animales de abasto supuso la descripción de un reservorio potencial de MRSA para la especie humana, con el consiguiente riesgo para la Salud Pública. En el presente trabajo de tesis doctoral, se ha analizado la presencia de *S. aureus* en animales de abasto, animales salvajes y aguas (agua residual y agua fluvial), con el fin de conocer la estructura genética de la población de *S. aureus* en estos reservorios y analizar su posible implicación en la epidemiología de MRSA.

Los resultados obtenidos en los diferentes estudios implican la presencia de MRSA en prácticamente la totalidad de los reservorios analizados, siendo la única excepción la colección de aislados de mastitis de pequeños rumiantes. La proporción de aislamiento de MRSA varía en función del reservorio, detectándose los mayores valores en el cerdo blanco (82,8%) y el agua residual (60,4%), seguidos del agua de rio (29,6%) y cerdo ibérico (28,3%). En el caso de los animales salvajes (buitre leonado, cabra montés, ciervo y jabalí), la proporción media de MRSA detectada fue menor del 1%, oscilando entre el 0,4% y el 5,0%. ST398 es el genotipo mayoritario detectado en MRSA en cerdo blanco, cerdo ibérico y animales salvajes. Dicho genotipo fue descrito inicialmente como adaptado al porcino, pero también como un genotipo capaz de colonizar numerosos hospedadores, tal como reflejan nuestros resultados. Las características genéticas y fenotípicas de los aislados MRSA de animales salvajes sugieren que se trata de microorganismos cuyo origen radica en los animales de abasto (ST398) o las personas (ST1), habiéndose detectado prácticamente la mitad de los animales gracias al doble muestreo empleado (muestras de fosas nasales y de piel). En el caso de las muestras de agua, tanto el agua residual como el agua fluvial presentaron como clon mayoritario de MRSA el ST125, clásicamente asociado a infecciones nosocomiales en España.

La mayoría de los aislados MRSA obtenidos presentaron *mec*A como gen de resistencia a meticilina, aunque también se han descrito aislados con el gen *mec*C, gen homólogo de *mec*A de reciente descripción. Dichos aislados provienen de animales salvajes y aguas, siendo los aislados detectados en nuestro estudio CC130 y ST425, genotipos comunes con los descritos previamente por otros autores como portadores de *mec*C. Este trabajo constituye la primera detección en España de *mec*C en aislados no clínicos de *S. aureus*.

En cuanto a los aislados MSSA, la proporción de portadores en animales salvajes varió entre el 5,0-22,9% dependiendo de la especie animal, aunque las diferencias no fueron estadísticamente significativas. Otros autores han descrito una mayor proporción de MSSA en animales de abasto que los descritos para los animales salvajes analizados, incluso teniendo en cuenta que nuestro sistema de muestreo en piel y fosa nasal ha mejorado la detección de portadores. Aunque se han encontrado gran variedad de genotipos, las líneas genéticas mayoritarias de MSSA se asociaron al hospedador de origen como es el caso de ST581 en cabra montés, ST425 en ciervo y ST2328 en jabalí. En las muestras de agua, de nuevo los aislados mayoritarios de MSSA obtenidos se habían detectado previamente en personas (ST5, ST15, ST30), aunque la proporción de los diferentes genotipos varía en función de si se trata de agua residual o agua fluvial.

Otros estudios habían detectado la presencia de *S. aureus* en aguas relacionándola con la eliminación de la bacteria por parte de individuos colonizados. En este sentido, nuestro estudio de detección de MRSA-*mec*C positivos en las muestras de agua de rio manifiesta la presencia en el agua de aislados relacionados genéticamente con los aislados detectados en animales del mismo entorno. Así, la presencia de aislados de *S. aureus* en el agua pone de manifiesto su papel potencial en la diseminación de este microorganismo, hecho que cobra especial relevancia en el caso de MRSA.

Summary

2. *Summary*

Staphylococcus aureus belongs to the normal microbiota of animals and humans and remains a public health concern as microbial agent causing infections in humans. Infections might be from mild to severe, even fatal and include skin and soft tissues infections, pneumonia, endocarditis, septicaemia, food poisoning, etc. Methicillin resistant *S. aureus* (MRSA) emerged by the integration of resistance mechanisms in methicillin susceptible *S. aureus* (MSSA), limiting therapeutic options.

Although MRSA in animals was described in 1972, MRSA detection of a specific genetic lineage in food animals led to the description of a potential MRSA reservoir for humans, with the consequent risk to public health. In this thesis, the presence of *S. aureus* in food animals, wild animals and water (wastewater and river water) has been studied in order to assess the population structure of *S. aureus* in these reservoirs and to analyse their possible role in the epidemiology of MRSA.

The results obtained in our studies revealed the presence of MRSA in almost all reservoirs tested, with the exception of the collection of isolates from small ruminant (mastitis). Proportion of MRSA isolation varies between reservoirs, detecting the highest values in standard white pig (82.8%) and wastewater (60.4%), followed by river water (29.6%) and Iberian pig (28.3%). In wild animals (Eurasian griffon vulture, Iberian ibex, red deer and wild boar), the average proportion of MRSA detected was less than 1%, ranging between 0.4% and 5.0 % depending on the animal species. ST398 is the most frequent MRSA genotype detected in white pig, Iberian pig and wild animals. This genotype was defined as pig-adapted, but can also colonize many other hosts, as reflected by our results. Genetic and phenotypic characteristics of MRSA isolates from wild animals in our study suggest that these microorganisms originate from livestock (ST398) or humans (ST1). Double sample increased the detection, as only one animal was positive to both samples tested (skin and nostrils). In the case of water samples, both the wastewater and river water showed ST125 as the most frequent MRSA clone, clone that has been associated with nosocomial infections in Spain.

Most of MRSA isolates presented *mec*A gene as resistance mechanism against methicillin. However, *mec*C (*mec*A homologue recently described) has also been found. *mec*C-MRSA isolates were detected in wild animals and wastewater. Genotypes found were CC130 and ST425, similar genotypes to those previously described by other authors as *mec*C-MRSA. This is the first detection of *mec*C-MRSA in non-clinical isolates in Spain.

Regarding MSSA, the proportion of carriers in wild animals ranged from 5.0% to 22.9% depending on the animal species, but the differences were not statistically significant. Although MSSA detection was improved taking two samples per animal (skin and nostrils), other authors have described a higher proportion of MSSA in livestock. A considerable genetic diversity was determined for MSSA in wild animals, but predominant STs were associated to host. Thus, ST581 was associated to Iberian ibex, ST425 to red deer and ST2328 to wild boar. In water samples, the most frequent genotypes of MSSA were common in wastewater and river water and have been detected in people previously (ST5, ST15, ST30); however, its proportion varies depending on the kind of water.

Other studies have detected the presence of *S. aureus* in waters related to bacterial shedding by colonized individuals. In this sense, the detection of *mec*C-MRSA in river water samples within a delimited environment reveals the presence in water of isolates genetically related with those detected in wild animals. Thus, the presence of *S. aureus* isolates in water shows its potential role in the spread of this microorganism, particularly relevant in the case of MRSA.

Introducción

3. Introducción

3.1. *Staphylococcus aureus*

3.1.1. Generalidades

Staphylococcus aureus es un microorganismo Gram positivo que pertenece al orden *Bacillales*, Familia *Staphylococcaceae*, género *Staphylococcus* (1). Dentro del género se han descrito 48 especies (http://www.bacterio.net/). Los miembros del género *Staphylococcus* son cocos inmóviles que crecen formando racimos, generalmente anaerobios facultativos catalasa positivos (2). Son capaces de crecer en un amplio rango de pHs y temperaturas (3), y *S. aureus* además, a altas concentraciones de cloruro sódico (4). La mayoría de las especies forman parte de la microbiota bacteriana existente en la piel y mucosas del hombre y los animales (primates, ungulados, carnívoros, roedores, lagomorfos, marsupiales y aves) (5, 6).

Aunque existen varias especies patógenas dentro del género, *S. aureus* es la que con mayor frecuencia causa procesos clínicos en los hospedadores que coloniza (7, 8). La localización más frecuente de *S. aureus* en la especie humana son las fosas nasales (9), aunque se han descrito otras localizaciones como la piel, el tracto gastrointestinal y la garganta (10). Aproximadamente el 50% de los individuos sanos son portadores de *S. aureus* en fosas nasales, de los cuales, entre el 20-30% son portadores persistentes (11-13). En animales, se ha descrito la presencia de *S. aureus* fundamentalmente en piel y fosas nasales, aunque también en glándula mamaria, garganta y tracto intestinal (6).

Ser portador de *S. aureus* supone un factor de riesgo para padecer una infección por este microorganismo (10, 11, 13, 14), riesgo que aumenta en los portadores persistentes (13). Factores relativos a la bacteria (capacidad de colonización, capacidad de evasión de la respuesta inmune, virulencia, etc.) y al hospedador (estado inmunitario, exposición, hábitos higiénicos, tratamientos con antimicrobianos, etc.) parecen determinantes para que un se produzca el estado de portador e incluso para que se desarrolle un proceso clínico (13, 15). En este sentido, diferentes estudios han demostrado que los aislados de *S. aureus* detectados en portadores son los mismos que los aislados responsables de los procesos clínicos (12, 16, 17).

3.1.2. Patologías producidas por *S. aureus*

S. aureus se describió por primera vez en el año 1881 como causante de infecciones en heridas quirúrgicas y bacteriemia (18, 19). En la actualidad, sigue siendo uno de los agentes microbianos que con mayor frecuencia se asocia con procesos invasivos en

personas (20, 21). Aproximadamente el 90% de los procesos clínicos que causa son infecciones en piel y mucosas aunque también puede producir cuadros más graves como endocarditis, neumonía, bacteriemia, síndrome del shock tóxico e intoxicaciones alimentarias (10, 22). En animales, son frecuentes las mastitis en rumiantes y conejos (23-25), procesos cutáneos en cerdos, animales de compañía y conejos (2, 25, 26) y artritis en aves (27).

Numerosos factores de colonización y/o virulencia así como la capacidad de *S. aureus* de evadir la respuesta inmune o de adaptarse al entorno han sido estudiados con el fin de diferenciar cepas patógenas de aquellas que no lo son (7). Dichos estudios concluyen que todos esos elementos han de considerarse de forma conjunta para comprender la patogenia de *S. aureus*, ya que los factores de patogenicidad son detectados también en aislados de individuos asintomáticos y en otras especies del género *Staphylococcus* consideradas no patógenas. Los mecanismos de regulación de la expresión génica son determinantes para permitir la adaptación de *S. aureus* al entorno y por tanto podrían influir en la expresión de factores de patogenicidad, pero de nuevo, dichos mecanismos están presentes tanto en *S. aureus* como en otros estafilococos no patógenos. En principio, cualquier *S. aureus* podría dar lugar a un proceso invasivo, aunque algunos clones son más frecuentes que otros (12).

De forma breve y sin pretender hacer una revisión exhaustiva, se presenta un resumen de factores relativos al microorganismo (Tabla 1) y factores relativos al hospedador (Tabla 2), que de alguna manera pueden favorecer que se produzca una infección por *S. aureus* (7, 28-30).

3.1.3. Factores de patogenicidad relativos al microorganismo

- Factores implicados en la adhesión

S. aureus presenta una serie de proteínas de superficie que le permiten anclarse a las proteínas del hospedador como fibronectina, plasminógeno, colágeno, proteínas implicadas en la captación de hierro, etc. Dichas adhesinas parecen estar implicadas en la colonización de las fosas nasales, la capacidad de este microorganismo para formar *biofilms* o el metabolismo del hierro, pero su presencia varía en los diferentes aislados clínicos de *S. aureus* y algunas de ellas están presentes también en aislados no clínicos. Se considera también que los ácidos teicoicos de la pared bacteriana participan en la adherencia de la bacteria en el epitelio nasal durante la colonización (15).

- Enzimas extracelulares y otras proteínas

Las exoenzimas tipo nucleasas, proteasas y lipasas son fundamentales para el crecimiento bacteriano ya que permiten la obtención de nutrientes y componentes necesarios para la síntesis de biomoléculas. Se trata en general de enzimas conservadas (*housekeeping*) que son necesarias para permitir la supervivencia de la bacteria en condiciones desfavorables como las que pueden producirse durante una infección. Además, algunas de ellas pueden participar en la formación de *biofilms* (nucleasas, proteasas), la inactivación de proteínas generadas como parte de la respuesta inmune del hospedador (proteasas) o la colonización (lipasas).

Se ha estudiado especialmente el elemento catabólico móvil de arginina (*arginine catabolic mobile element* o ACME), una ruta de degradación de arginina que está presente en un elemento genético móvil además de en el cromosoma, y que es muy frecuente en *Staphylococcus epidermidis*. Ha sido detectado en algunas cepas de *S. aureus*, entendiéndose que podía suponer una mejora en su capacidad de colonización y así, haber favorecido el éxito de clones altamente prevalentes (31-34).

- Factores que les permiten evadir la respuesta inmune del hospedador

Los primeros elementos a considerar para evitar la respuesta inmune del hospedador son las barreras mecánicas que establece *S. aureus* tanto al intervenir en la coagulación sanguínea como al formar *biofilms* (35). La coagulasa secretada por *S. aureus* es capaz de unirse a la protrombina y dar lugar a un cambio conformacional que conduce a la conversión de fibrinógeno en fibrina. Por otro lado, el factor bacteriano que se une al factor de von Willebrand tiene también un efecto en la coagulación mediante interacción con la protrombina y conversión de fibrinógeno en fibrina (36). En ambos casos, *S. aureus* forma microcolonias adheridas a una estructura tipo malla que lo protege de los mecanismos de defensa del hospedador, del mismo modo que en la formación de *biofilms*. Otro mecanismo de defensa consistiría en la internalización de la bacteria por parte de las células del sistema inmune o células endoteliales mediante proteínas de unión a fibronectina o adhesinas (22).

También es capaz de producir diferentes proteínas que interfieren en la respuesta inmune del hospedador al impedir la opsonización (proteína A) y la fagocitosis (proteína extracelular de unión al fibrinógeno), inhibir la quimiotaxis, (proteína inhibidora de la quimiotaxis CHIPS) y el complemento (factor inhibidor del complemento SCIN), destruir péptidos antimicrobianos (estafiloquinasa), etc. Otros factores que interfieren en la respuesta inmune del hospedador son las toxinas con efecto citolítico sobre células del

sistema inmune como las fenol-soluble modulinas, la α-toxina (o α-hemolisina) y las leucotoxinas (37) o la estafiloxantina, pigmento presente en *S. aureus* que favorece la resistencia a la oxidación de la bacteria y evita la actividad de los neutrófilos (10). Además, *S. aureus* es capaz de sintetizar una capsula de naturaleza polisacarídica que impide la fagocitosis además de participar en la formación de *biofilms*.

- Producción de toxinas

Determinados cuadros clínicos producidos por *S. aureus* están relacionados con la secreción de proteínas que actúan como toxinas como es el caso de los cuadros digestivos por la producción de enterotoxinas, el síndrome de piel escaldada por la producción de toxinas exfoliativas (ETA-ETD) o el síndrome del shock tóxico producido por la toxina del mismo nombre (*toxic shock syndrome toxin* o TSST). Estas toxinas son además consideradas superantígenos, es decir, son capaces de producir la proliferación masiva de linfocitos T y así la producción de citoquinas, lo que conduce al síndrome del shock tóxico. Otras exotoxinas son las hemolisinas (α, β, γ y δ) y la leucocidina de Panton-Valentine (*Panton-Valentine leukocidin* o PVL), que son toxinas citolíticas. La toxina PVL ha sido relacionada con casos de neumonía necrotizante y forunculosis (31), pero diferentes estudios aplicando modelos animales parecen aportar resultados discordantes, por lo que su papel en la patogenia de *S. aureus* es controvertido (10).

- Otras características de *S. aureus* que le confieren ventajas en caso de infección

Aunque en los microorganismos anaerobios facultativos en general existen variantes de colonias de pequeño tamaño (*small-colony variants* o SCV), estas morfologías se han asociado con infecciones recurrentes causadas por *S. aureus* en el hombre (7). Las SCV presentan un metabolismo ralentizado (38) que les permite sobrevivir en presencia de antimicrobianos e incluso de toxinas producidas por otros microorganismos presentes en el lugar de la infección, como *Pseudomonas aeruginosa* en los casos de fibrosis quística (7).

S. aureus presenta resistencia a moléculas con actividad antibiótica natural como resistencia a lisozima debido a la composición de su pared bacteriana. Adicionalmente, en caso de presencia de antimicrobianos en el entorno, los determinantes de resistencia a antimicrobianos pueden suponer una ventaja competitiva para los microorganismos resistentes sobre aquellos que no lo son. *S. aureus* es capaz de obtener material genético (como los determinantes de resistencia a antimicrobianos) mediante transferencia genética horizontal, lo que incrementa su capacidad de adaptación al entorno. También

la formación de *biofilms* protege a la bacteria de los antimicrobianos presentes en el ambiente.

Tabla 1. Factores relativos al microorganismo		
Factores de virulencia y/o colonización		**Mecanismo de acción**
Estructura del microorganismo	Adhesinas	Implicadas en la adhesión a matrices y/o células del hospedador
	Ácidos teicoicos	Implicados en la adhesión y en la resistencia a lisozima
Síntesis de enzimas extracelulares y otras proteínas	Nucleasas, proteasas y lipasas	Permiten la obtención de nutrientes y componentes necesarios para la síntesis de biomoléculas
	ACME (*arginine catabolic mobile element*)	Se ha sugerido la presencia de este elemento como una ventaja en la capacidad de colonización de *S. aureus*
Mecanismos de evasión de la respuesta inmune	Coagulasa (*coa)*, factor de von Willebrand (*vWbp*)	Desencadenan la formación del coágulo de manera que se constituye una barrera física que impide actuar a los elementos del sistema inmune frente al microorganismo
	Capacidad de formar *biofilms*	La estructura del *biofilm* ralentiza el metabolismo bacteriano, impidiendo el efecto de algunos antimicrobianos. Además se dificulta la actuación de los diferentes elementos del sistema inmune y de los antimicrobianos
	Proteínas de unión a fibronectina o adhesinas	Internalización en células del sistema inmune o del endotelio vascular
	Cápsula polisacarídica	Inhibe la fagocitosis y participa en la formación de *biofilms*
	Proteína A (*spa*)	Inhibe la opsonización
	Proteína extracelular de unión al fibrinógeno	Inhibe la fagocitosis
	Proteína inhibidora de la quimiotaxis (*chips*)	Inhibe la quimiotaxis
	Factor inhibidor del complemento (*scin*)	Inhibe el complemento
	Estafiloquinasa (*sak*)	Destrucción de péptidos antimicrobianos
	Estafiloxantina	Pigmento que favorece la resistencia a la oxidación de la bacteria y evita la actividad de los neutrófilos
Toxinas	Enterotoxinas (SE)	Cuadros digestivos (superantígenos)
	Toxinas exfoliativas (ETA-ETD)	Síndrome de piel escaldada (superantígenos)
	Toxina del síndrome del shock tóxico (TSST-1)	Toxicidad endotelial y síndrome del shock tóxico (superantígenos)

Tabla 1. Factores relativos al microorganismo		
Factores de virulencia y/o colonización		**Mecanismo de acción**
	Toxinas citolíticas: fenol soluble modulinas (PSM), hemolisinas (*hla*, *hlb*), leucocidinas (*luk*D, *luk*E, *luk*F, *luk*M)	Lisis de células del sistema inmune y eritrocitos
Otras propiedades	SCV (*small colony variants*)	Les permite sobrevivir en presencia de antimicrobianos e incluso de toxinas producidas por otros microorganismos presentes en el lugar de la infección
	Resistencia a antimicrobianos	En caso de presencia de antimicrobianos en el entorno, los determinantes de resistencia a antimicrobianos pueden suponer una ventaja competitiva

3.1.4. Factores de riesgo relativos al hospedador

La capacidad de dar lugar a un cuadro clínico está también influenciada por la mayor o menor sensibilidad del hospedador, habiéndose determinado factores de riesgo relacionados con la presencia de *S. aureus* (12, 39). En términos generales, presentar otras patologías, tener hábitos higiénicos deficientes, convivir con una persona que es portador o la alta densidad de individuos en un entorno con frecuente contacto físico son factores que favorecen ser portador de *S. aureus* (12, 29, 30). Otros aspectos como la edad, el sexo y la raza pueden también suponer un factor de riesgo, aunque los resultados difieren en función de la población en estudio (10, 12, 13, 29).

En relación a las infecciones, el mayor mecanismo de defensa del hospedador frente a las infecciones por *S. aureus* son las barreras primarias (piel y mucosas), por lo que la pérdida de su integridad supone un riesgo de infección (10). Los implantes vasculares se han identificado como un factor de riesgo para sufrir endocarditis ya que constituyen una superficie que se recubre por proteínas del hospedador (tipo fibrinógeno o fibronectina), creando la base que permite la colonización de la bacteria (22). Así, prácticas médicas como cirugías, implantes, hemodiálisis, etc., aumentan el riesgo de infecciones por *S. aureus*. Deficiencias del sistema inmune (ya sea por enfermedades que afectan a la respuesta inmune por neutrófilos, a un proceso subyacente como el síndrome de inmunodeficiencia adquirida o a tratamientos médicos inmunosupresores como los que suelen aplicarse a enfermos oncológicos o trasplantados) constituyen también factores de riesgo en las infecciones por este patógeno (19). Otros procesos clínicos como la diabetes, el fallo renal, cuadros cardiacos, neurológicos o respiratorios se han relacionado con una mayor frecuencia de procesos invasivos (19, 22).

En el caso de los animales, los factores de riesgo de colonización o infección por *S. aureus* serían similares a los definidos para personas. Ser portador o tener contacto estrecho con portadores, sufrir un proceso clínico subyacente, cirugías recientes e implantes suponen un riesgo de sufrir infecciones por *S. aureus,* aunque la mayoría de los estudios están orientados a colonización o infección por *S. aureus* resistentes a meticilina (*methicillin-resistant S. aureus* o MRSA) (40, 41).

Tabla 2. Factores relativos al hospedador

Hospedador	**Factores de riesgo**
Hombre	Pérdida de integridad de las barreras primarias (piel y mucosas) Proceso clínico primario (que implique un sistema inmune deficiente y/o la pérdida de las barreras primarias y/o la colocación de implantes) Hospitalización Practicas higiénicas deficientes Estrecho contacto con portadores
Animales de compañía y caballos	Pérdida de integridad de las barreras primarias (piel y mucosas) Convivencia estrecha con portadores Procesos clínicos subyacentes Cirugías recientes, implantes
Animales de abasto	Portadores Convivencia estrecha con portadores

3.2. Resistencia a antimicrobianos

3.2.1. Generalidades

El descubrimiento de los antimicrobianos supuso un punto de inflexión en el tratamiento de las infecciones producidas por bacterias al disminuir su morbilidad y mortalidad (42). Desafortunadamente, y en poco tiempo tras su aplicación, se han ido describiendo mecanismos de resistencia a todas las clases de antimicrobianos (30, 43), limitándose las opciones terapéuticas. En teoría, la ineficacia de los antimicrobianos frente a los microorganismos patógenos conduciría a los tiempos anteriores a su descubrimiento, con el consiguiente aumento de la morbilidad y mortalidad (44) pero también dificultaría prácticas médicas que se realizan actualmente como por ejemplo la intubación de pacientes o tratamientos oncológicos, cuyo éxito depende en gran medida del uso de antimicrobianos (42, 45).

Aunque se ha descrito la presencia de microorganismos resistentes a antimicrobianos en entornos donde aparentemente no hay exposición (46) o en muestras prehistóricas de ADN (47), el uso de antimicrobianos es uno de los mecanismos que favorece el desarrollo (48, 49) y la selección de resistencias a antimicrobianos (43, 50).

Los antimicrobianos son sustancias que inhiben el crecimiento bacteriano, de manera que se produce resistencia a un antimicrobiano cuando una bacteria no se inhibe en presencia del mismo (43). La resistencia a antimicrobianos puede producirse mediante adquisición de material genético extra (transferencia genética horizontal) o por mutaciones (30), aunque hay microorganismos resistentes a antimicrobianos de forma natural (42). Generalmente, estos microorganismos con resistencia intrínseca son microorganismos presentes en el medioambiente y se consideran el origen de los determinantes de resistencia de los microorganismos patógenos (42).

El papel de los determinantes de resistencia a antimicrobianos en la naturaleza se ha descrito básicamente como un mecanismo de supervivencia en presencia del antimicrobiano: hay microorganismos que producen antimicrobianos (antibióticos) limitando el crecimiento de los competidores, con lo que la resistencia permite sobrevivir al microorganismo que genera el antimicrobiano y constituye una ventaja competitiva frente a otros microorganismos del entorno (42). Adicionalmente, se ha visto que a concentraciones bajas los antimicrobianos podrían tener funciones de comunicación (*quorum sensing*) entre las bacterias y los mecanismos de resistencia participarían de estas actividades de comunicación (49). Otros mecanismos de resistencia (como las β-lactamasas), podrían haber surgido como funciones metabólicas en la bacteria, pero la presencia de antimicrobianos ha conducido a que se consoliden como un mecanismo de resistencia (42).

Así, aunque las resistencias a antimicrobianos son anteriores al uso generalizado de los mismos, las actividades humanas tienen un efecto en su evolución (42). La liberación de antimicrobianos (compuestos en muchos casos de difícil degradación) y la diseminación de bacterias con determinantes de resistencia (potencialmente seleccionadas por el uso de antimicrobianos en el entorno humano y/o animal) aumentan la presión selectiva y las posibilidades de intercambio de determinantes de resistencia.

Existen determinados entornos donde se favorece la transferencia genética horizontal como pueden ser los *biofilms*, el intestino, las plantas de tratamiento de aguas residuales, etc. La captación de material genético adicional que confiera resistencia a antimicrobianos junto con la presión selectiva que pueda darse en el individuo para tratar otros procesos, constituyen un mecanismo de selección de cepas comensales resistentes (51, 52). De este modo, los microorganismos oportunistas que generan enfermedades en individuos con el sistema inmune comprometido pueden ser componentes de la microbiota del hospedador pero también tener un origen medioambiental.

La interacción entre microorganismos comensales, medioambientales y patógenos es posible tanto en el hospedador como fuera de él, favoreciéndose la diseminación de determinantes de resistencia a antimicrobianos entre las diferentes poblaciones bacterianas. En otras ocasiones, como es el caso de *S. aureus*, la propia bacteria puede actuar como comensal o como patógeno, tal como se ha descrito anteriormente.

3.2.2. Resistencia a antimicrobianos en *S. aureus*

Uno de los primeros antibióticos de uso clínico que se descubrió fue la penicilina, lo que produjo un cambio sustancial en el tratamiento de las infecciones por *S. aureus* (51, 53). Sin embargo, los primeros casos de resistencia a penicilina fueron descritos poco después de su aplicación clínica (50, 51). Las infecciones por *S. aureus* resistente a penicilina afectaron inicialmente a individuos hospitalizados, pero poco más tarde alcanzaron al resto de la población (53). En términos generales, las resistencias a antimicrobianos en *S. aureus* se han descrito primero en ambientes hospitalarios, pero se han diseminado posteriormente a la comunidad (51, 53).

La sustitución de la penicilina por penicilinas semi-sintéticas resistentes a la penicilinasa (como oxacilina y meticilina) amplió de nuevo las opciones terapéuticas hasta la aparición de MRSA (54). La diseminación de clones con resistencia a meticilina (nomenclatura que se ha mantenido para denominar este mecanismo de resistencia aunque la meticilina ya no se usa en clínica) supuso un gran impacto en el tratamiento de las infecciones por *S. aureus*, ya que la resistencia a meticilina limita el uso de β-lactámicos en general, siendo los β-lactámicos el tratamiento de elección de infecciones por este patógeno (32, 55).

La problemática de las infecciones por MRSA aumentó con la aparición de cepas multi-resistentes que, además de la resistencia a β-lactámicos, acumulaban mecanismos de resistencia a otros antimicrobianos como estreptomicina, tetraciclina, eritromicina y gentamicina (30). También la resistencia a quinolonas apareció rápidamente tras su aplicación terapéutica, y se ha descrito que el uso de estos antimicrobianos selecciona cepas de *S. aureus* con resistencia a quinolonas entre los microorganismos comensales, constituyéndose así un reservorio de cepas resistentes (51, 52). La vancomicina quedó entonces como el tratamiento de elección para las infecciones por MRSA, pero rápidamente se produjeron las primeras descripciones de cepas de *S. aureus* con reducida sensibilidad a vancomicina (*vancomycin intermediate S. aureus* o VISA) y cepas altamente resistentes (*vancomycin resistant S. aureus* o VRSA) (30). Otros antimicrobianos empleados en el tratamiento de infecciones por MRSA son linezolid, tigeciclina, daptomicina, telavancina, nuevos betalactámicos como la ceftarolina, tetraciclinas de

efecto prolongado, clindamicina y rifampicina (10, 32). Desafortunadamente, la eficacia frente a estos compuestos va en disminución, habiéndose descrito resistencias también a los antibióticos de última generación como el linezolid y la daptomicina (10, 32).

La mupirocina y el ácido fusídico son antimicrobianos empleados en la descolonización nasal de individuos portadores para prevenir la infección y la transmisión de MRSA, pero la eficacia de su uso es variable y se ha relacionado con un desarrollo rápido de resistencias (51, 56).

3.2.3. Resistencia a meticilina

La resistencia a meticilina implica resistencia a β-lactámicos mediante la síntesis de una proteína de unión a penicilina (*Penicillin Binding Protein* o PBP) alterada (PBP2a o PBP2´), que presenta baja afinidad por la molécula del antimicrobiano β-lactámico (57). Las PBPs son enzimas de la familia de las serin-proteasas que participan en la transpeptidación necesaria para la creación de puentes interpeptídicos que se establecen entre las cadenas de peptidoglicano de la pared celular (57). En cepas sensibles, la unión covalente de los β-lactámicos en el lugar de acción de la enzima impide que pueda realizar su función, y así se produce la lisis bacteriana por inhibición de la síntesis de la pared celular (32). En las cepas resistentes, la PBP2a sigue cumpliendo sus funciones y por tanto se mantiene la integridad de la pared y la viabilidad de la bacteria (30, 58).

La resistencia a meticilina en *S. aureus* se ha relacionado con la presencia de los genes *mec*A (57) y *mec*C, un gen homólogo de *mec*A recientemente descrito y que fue inicialmente denominado *mec*A_{LGA251} (59, 60). Ambos genes se encuentran localizados en el casete *mec* de *S. aureus* ubicado en el cromosoma (*Staphylococcal Cassette Chromosome mec* o SCC*mec*), un elemento genético móvil de transferencia horizontal (61).

SCC*mec* consta de dos complejos: el complejo *mec*, que contiene el gen *mec* y los genes que regulan su expresión (*mec*I y *mec*R1) y el complejo *ccr* (*cassette chromosome recombinase*), que contiene el mecanismo de recombinación necesario para su integración/escisión en el cromosoma. SCC*mec* está flanqueado por unas secuencias de repetición que le permiten anclarse en una localización específica del cromosoma. Dicha localización se denomina sitio de integración de la secuencia (*Integration Site Sequence* o ISS), y está ubicada en un marco abierto de lectura (*open reading frame* u *orf*) de función desconocida (*orf*X) que está presente tanto en cepas sensibles como en cepas resistentes a meticilina (58, 62). Las características genéticas de los complejos *mec* y *ccr* y las

estructuras intermedias delimitadas por las secuencias de repetición (denominadas *Joining Regions* o J3-J1) se emplean para clasificar el SCC*mec* con fines epidemiológicos (58, 63, 64). En la actualidad se han descrito once tipos diferentes de SCC*mec* (I-XI; Tabla 3), y su clasificación está coordinada por el grupo de trabajo internacional para la clasificación del SCC*mec* (*International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements* o IWG-SCC; http://www.sccmec.org/).

Tabla 3. Tipos de SCC*mec* descritos en *S. aureus*

Tipos de SCC*mec*	**Complejo *mec***	**Complejo *ccr***
I	B (IS431-*mec*A-Δ*mec*R1-IS1272)	1 (A1B1)*
II	A (IS431-*mec*A-*mec*R1-*mec*I)	2 (A2B2)
III	A (IS431-*mec*A-*mec*R1-*mec*I)	3 (A3B3)
IV	B (IS431-*mec*A-Δ*mec*R1-IS1272)	2 (A2B2)
V	C2* (IS431-*mec*A-Δ*mec*R1-IS431)	5 (C1)
VI	B (IS431-*mec*A-Δ*mec*R1-IS1272)	4 (A4B4)
VII	C1** (IS431-*mec*A-Δ*mec*R1-IS431)	5 (C1)
VIII	A (IS431-*mec*A-*mec*R1-*mec*I)	4 (A4B4)
IX	C2* (IS431-*mec*A-Δ*mec*R1-IS431)	1(A1B1)
X	C1** (IS431-*mec*A-Δ*mec*R1-IS431)	7(A1B6)
XI	E (*bla*Z-*mec*C-*mec*R1$_{LGA251}$-*mec*I$_{LGA251}$)	8(A1B3)

Adaptado de http://www.sccmec.org/; *: IS431 dispuestas en direcciones opuestas; **: IS431 dispuestas en la misma dirección

En relación a los diferentes genes *mec*, estos se definen por comparación con el gen *mec*A de *S. aureus* N315, primer MRSA secuenciado completamente (60). La nueva nomenclatura propuesta (60) establece que diferencias inferiores o iguales al 5% mantienen el nombre del gen; diferencias entre de entre el 5-30% con la secuencia genética de *mec*A de N315, darán lugar a la determinación de alotipos, tal como se propone para *Staphylococcus sciuri* (*mec*A1; homología del 80%) y *Staphylococcus vitulinus* (*mec*A2; homología del 90%); y homologías inferiores al 70%, suponen la descripción de un nuevo *mec*A homólogo, como *mec*B de *Macrococcus caseolyticus* (homología del 62%) o *mec*C de *S. aureus* LGA251 (homología del 69%).

La captación de SCC*mec* por diferentes líneas genéticas de *S. aureus* sensibles a meticilina (*methicillin susceptible S. aureus* o MSSA) ha dado lugar a diferentes líneas genéticas de MRSA (21, 62, 65). Sin embargo, desde su descripción, se han propuesto diferentes teorías sobre los orígenes de la resistencia a meticilina en *S. aureus*. *S. sciuri* se propuso como precursor de la resistencia a meticilina en *S. aureus*, en base a la homología determinada para su PBP (87,8%) (57, 66). Además, es frecuente la presencia del gen *mec*A en este

microorganismo (57) por lo que podría haber actuado como reservorio de este mecanismo de resistencia para *S. aureus*. Por otro lado, otros autores han señalado *Staphylococcus fleurettii* (que pertenece al grupo de *S. sciuri*) como origen del gen *mec*A, ya que posee este gen localizado en el cromosoma y rodeado de genes esenciales para el crecimiento de la bacteria no relacionados con el SCC*mec* (67). Finalmente, otros estudios han sugerido la transferencia horizontal del SCC*mec* a *S. aureus* por parte de otros estafilococos coagulasa negativos basándose en diferentes evidencias: i) la alta prevalencia de *mec*A en *S. epidermidis* (alrededor del 75%) indicaría su posible actuación como reservorio; ii) la mayor homología detectada entre las secuencias del SCC*mec* tipo IV de *S. aureus* y *S. epidermidis* en relación a la homología existente para el resto de sus genomas; iii) la descripción del SCC*mec* tipo IV en *S. epidermidis* con anterioridad a su descripción en *S. aureus;* iv) la presencia intacta de la secuencia de inserción IS1272 en el SCC*mec* tipo I de *Staphylococcus haemolyticus* mientras que en *S. aureus* presenta frecuentes delecciones, lo que señalaría a *S. aureus* como un receptor secundario de dicho elemento; y finalmente, v) la aparente captación del SCC*mec* en un paciente a partir de un aislado de *S. epidermidis* resistente a meticilina (30, 58, 62, 68).

En cualquier caso, la adquisición horizontal de la resistencia a meticilina en *S. aureus* se ha producido a partir de diversos eventos genéticos independientes entre sí, tal como refleja la variabilidad de estructuras del SCC*mec* que se han encontrado en *S. aureus* (69, 70). El número de líneas genéticas de *S. aureus* que han adquirido este casete es limitado, observándose una mayor heterogeneidad en las poblaciones de aislados MSSA (21, 62, 71).

3.3. *S. aureus* resistentes a meticilina

3.3.1. MRSA en el hombre y líneas genéticas mayoritarias

Los primeros aislados de MRSA fueron descritos en el Reino Unido (54), aunque rápidamente se diseminaron a otros países (51). Inicialmente, la problemática de resistencia a meticilina en infecciones causadas por *S. aureus* estaba circunscrita a infecciones intrahospitalarias, pero posteriormente fueron aumentando las infecciones por MRSA en el resto de la población (72).

La primera descripción de MRSA en la comunidad se produjo en 1993, detectándose MRSA en individuos para los que no era reconocible ninguna interacción con ambientes hospitalarios ya que se trataba de poblaciones aborígenes de Australia (30, 55). Los factores empleados para discriminar entre los aislados hospitalarios y de la comunidad (Tabla 4) era que presentaban líneas genéticas diferentes, tipos de SCC*mec* distintos y

patrones de resistencia a antimicrobianos no coincidentes (las cepas de hospital presentaban además de resistencia a β-lactámicos resistencia a antimicrobianos de otras clases). Además, los aislados de la comunidad se relacionaron con la presencia de PVL (30, 73).

De este modo surgieron los conceptos MRSA asociado a hospitales (*Hospital Associated*-MRSA o HA-MRSA) y MRSA asociado a la comunidad (*Community Associated*-MRSA o CA-MRSA). Posteriormente esta terminología fue definida por el centro para la prevención y control de enfermedades de Estados Unidos (*Centers for Disease Control and Prevention* o CDC) (10, 29) de manera que se entienden como infecciones hospitalarias (HA-MRSA o *Health-care Associated* MRSA) aquellas que se producen a partir de las 48h de admisión en un hospital; infecciones en la comunidad con un origen hospitalario (*Health-care Associated Community Onset* MRSA o HACO-MRSA) cuando la infección se produce antes de la admisión en el hospital o antes de las primeras 48h post-admisión pero el paciente presenta factores de riesgo tales como historia de hospitalización previa, cirugía, diálisis o residencia prolongada en un centro de rehabilitación-hospitalización (*health care facilities*); y como infecciones de la comunidad (CA-MRSA) cuando la infección se produce previamente al ingreso en el hospital o en las primeras 48h, pero no se identifican los mencionados factores de riesgo.

Teniendo en cuenta que estas definiciones de HA-MRSA y CA-MRSA están basadas en factores relacionados con la clínica (32), y aunque hay clones claramente asociados a un entorno concreto, resulta cada vez más difícil mantener esta diferenciación (29, 62, 74, 75). Algunos elementos que contribuyen a ello serían el aumento de las resistencias a antimicrobianos no β-lactámicos en clones categorizados como CA-MRSA (76), las discrepancias en la presencia de PVL en CA-MRSA (71, 74, 77-79) y la colonización de los ambientes hospitalarios por parte de cepas de la comunidad (76, 80, 81). Finalmente, en el año 2005 (82) se describieron los MRSA asociados a animales de abasto (*Livestock Associated* MRSA o LA-MRSA), que igualmente presentaban características diferenciales de los HA-MRSA y los CA-MRSA (Tabla 4), pero cuyo mayor impacto fue el hecho de que se hubiera establecido un reservorio de MRSA en animales de abasto (83).

Tabla 4. Características generales de diferenciación entre aislados de MRSA

HA-MRSA	Líneas genéticas frecuentemente asociadas: ST5, ST22, ST36, ST45, ST239, ST250 Historia de hospitalización o intervención quirúrgica reciente Resistencia a β-lactámicos y otras clases de antimicrobianos SCC*mec* tipo I, II, III No relacionado con una mayor frecuencia de PVL

Tabla 4. Características generales de diferenciación entre aislados de MRSA	
CA-MRSA	Líneas genéticas frecuentemente asociadas: ST1, ST8, ST59, ST80, ST93 Sin historia de hospitalización pero contacto estrecho entre individuos y prácticas higiénicas inadecuadas Resistencia a β-lactámicos SCC*mec* tipo IV y V Relacionado con una mayor frecuencia de PVL
LA-MRSA	Líneas genéticas frecuentemente asociadas: ST5, ST9, ST97, ST130, ST398 Portadores, uso de antimicrobianos Resistencia a β-lactámicos y otras clases de antimicrobianos SCC*mec* tipo IV y V No relacionado con una mayor frecuencia de PVL

MRSA: *methicillin resistant S. aureus*; HA-MRSA: *Hospital Associated*-MRSA; CA-MRSA: *Community Associated*-MRSA; LA-MRSA: *livestock Associated*-MRSA; SCC*mec*: *Staphylococcal Cassette Chromosome mec*; PVL: Panton-Valentine leukocidin

Diferentes estudios (29, 31, 32, 53, 55, 84) han esquematizado la evolución de la epidemiología de los clones mayoritarios de MRSA, clasificándolos en base a la técnica de MLST (*Multilocus Sequence Typing*) y el tipo de SCC*mec*. MLST se basa en la secuenciación de un fragmento de aproximadamente 450 pares de bases de siete genes conservados (85) obteniéndose un perfil alélico denominado secuencia tipo (*Sequence Type* o ST). Aislados con pequeñas variaciones en su perfil alélico (comparten entre 5 y 6 alelos) se agrupan en complejos clonales (CCs).

El primer MRSA descrito resultó ser ST250-CC8-SCC*mec* tipo I, se detectó en el Reino Unido poco después de la introducción del uso clínico de la meticilina y se conoce como clon “arcaico”. En los años 80, se produjo una pandemia de MRSA en hospitales apareciendo otros STs pertenecientes al mismo complejo clonal (CC) como el ST247 (“*Iberian clone*”; SCC*mec* tipo II) y el ST239 (“*Brazilian/Hungarian clone*”; SCC*mec* tipo III) así como otros clones denominados “*NewYork/Japan clone*” (ST5-CC5-SCC*mec* tipo II), “*Pediatric clone*” (ST5-CC5-SCC*mec* tipo IV), EMRSA-15 (ST22-CC22-SCC*mec* IV) y EMRSA-16 (ST36-CC30-SCC*mec* II). Ya en los años 90 se produjo la aparición de clones CA-MRSA, siendo el primer clon ST1-CC1-SCC*mec* tipo IV (WA-1 o USA400), sustituido después, principalmente en Estados Unidos, por el clon ST8-CC8-SCC*mec* IV (USA 300). Otros clones prevalentes son el ST45-SCC*mec* II y el ST80-SCC*mec* IV en Europa, el ST59-SCC*mec* IV y V en Asia y el ST93-SCC*mec* IV en Australia.

La situación actual refleja que hay HA-MRSA distribuidos globalmente, pertenecientes a los complejos clonales CC5, CC8, CC22, CC30 y CC45 (80), con algunos STs responsables también de cuadros en la comunidad como ST5, ST8, ST22 y ST36 (53, 74). Algunos clones presentan alta frecuencia pero con una distribución geográfica limitada,

aparentemente debido a la mayor o menor movilidad de la población (21, 86). En relación a los CA-MRSA, en Estados Unidos la mayoría de las infecciones son producidas por ST8 (29), mientras en Europa se observa una mayor heterogeneidad (71). Sin embargo, recientemente se ha observado un aumento en la detección de CA-MRSA ST8, superando en términos de frecuencia la detección de aislados ST80, clon clásicamente relacionado con CA-MRSA en Europa (71). En determinadas áreas además, parece que están cobrando mayor importancia determinadas líneas genéticas de LA-MRSA como ST130 y ST398 (87, 88). En relación a las líneas genéticas más frecuentes en España, destaca el ST125 en HA-MRSA (21, 89) y el ST8 en CA-MRSA (90, 91). Las infecciones causadas por ST8 en la comunidad en España se han relacionado en términos generales con población foránea (78, 90, 92) aunque parece que la proporción de ST8 está aumentando en el sur de Europa y en algunas zonas de España (71, 77). En cuanto a los LA-MRSA, infecciones por ST398 se han relacionado con el sector porcino (93, 94) mientras que en los recientes casos de ST130 detectados no en todos los casos se han identificado vínculos epidemiológicos entre los pacientes y actividades ganaderas (95, 96).

La frecuencia de las infecciones causadas por MRSA difiere entre estudios debido a variaciones en el diseño de los mismos y a la población analizada (80). Así, hay localizaciones con una frecuencia de infecciones hospitalarias superior al 50% como Sudamérica y Asia, lugares con prevalencia entre el 25-50% como China, Australia, África y el sur de Europa y otras áreas con baja prevalencia (0-25%) como países del norte de Europa (80, 97). En relación a las infecciones en la comunidad, los porcentajes difieren igualmente entre localizaciones oscilando entre un 8-64% en Estados Unidos, y estimándose una prevalencia menor en Europa (81). En general, las infecciones por CA-MRSA son más frecuentes en países con baja prevalencia de HA-MRSA (20, 80). En España, se estima que el 29,2% de las infecciones hospitalarias por *S. aureus* son MRSA (89), mientras que los datos publicados para las infecciones en la comunidad indican porcentajes más bajos (17,2%) (98).

3.3.2. MRSA en los animales y riesgo de zoonosis

La presencia de MRSA en animales fue descrita por primera vez en 1972, en un aislado de mastitis de rumiantes (99). Sin embargo, los casos de mastitis por MRSA se consideran esporádicos (41, 100). En el caso de animales de compañía, la diseminación de HA-MRSA en personas se relacionó con una mayor proporción de infecciones por MRSA en perros y gatos (41). Tanto en rumiantes como en animales de compañía, las infecciones por MRSA se han considerado infecciones puntuales producidas por aislados de origen humano (32, 101, 102), y debidas a un contacto persistente entre individuos (41, 99, 101). Sin embargo

en el caso de los caballos, las líneas genéticas de MRSA detectadas presentan características propias de la especie equina, y los casos clínicos detectados en personas se han asociado fundamentalmente a actividades profesionales del mundo del caballo (103).

La gran diferencia en la presencia de MRSA en animales y el consiguiente riesgo de zoonosis se produjo con la aparición de casos clínicos en personas relacionados con el sector porcino (82, 104), ya que supuso la primera descripción de un reservorio de MRSA en animales de abasto (40, 105). Se trataba de casos clínicos o portadores en individuos que trabajaban en granjas de cerdos y/o sus familias, y en todos los casos se detectaron aislados MRSA ST398, es decir, MRSA pertenecientes a una línea genética diferenciada (83). Estos primeros datos condujeron al estudio de la presencia de MRSA en cerdos en diferentes países (41, 83, 106), describiéndose la predominancia de aislados ST398 en animales sanos en Europa y Norteamérica (107). Se realizó también un estudio estandarizado de prevalencia en la Unión Europea en 2008 (108), indicando frecuencias de detección muy diferentes entre países (desde ausencia de MRSA hasta un 46% de granjas positivas) pero en general, con una gran homogeneidad con respecto a las líneas genéticas obtenidas (fundamentalmente ST398). Contrariamente a los rumiantes, animales de compañía y caballos, la mayoría de los animales positivos eran portadores (41). Además de la adaptación de este clon al porcino (83), MRSA ST398 se ha descrito en otras especies de animales domésticos, peri-domésticos y salvajes (107). Además, se ha investigado su presencia en alimentos de origen animal, y aunque se ha detectado, se considera que la transmisión por contacto es el mecanismo más frecuente y por tanto constituye un riesgo profesional (109, 110).

La gran diseminación del LA-MRSA ST398 planteó la necesidad de conocer su origen. Los primeros datos apuntaban un origen porcino puesto que ST398 MSSA fue detectado en cerdos (111, 112). Sin embargo, estudios posteriores de secuenciación masiva sugieren que el origen ancestral del CC398 es humano, habiéndose adaptado posteriormente al ganado porcino (113).

Otros clones de MRSA para los que se han observado saltos inter-especies y que definen a los animales de abasto como reservorio de MRSA han sido el CC5 (en avicultura), el CC97 y el CC130 (ambos en bovino). En el caso de los aislados que producen clínica en pollos de engorde (*broilers*), estudios de secuenciación masiva indican que el CC5 tendría también un origen ancestral humano, aunque los sistemas de producción avícola parecen haber favorecido la diseminación de esta línea clonal una vez adaptado al pollo (27). En el caso del ganado bovino, el CC97 es un genotipo cuya frecuencia de aislamiento está

aumentando en personas y su origen es bovino de acuerdo con estudios de secuenciación masiva (114). Finalmente el CC130 es una de las líneas genéticas en las que se ha detectado con mayor frecuencia el gen *mecC* tanto en personas como en animales, habiéndose analizado recientemente la potencial transmisión entre pequeños rumiantes y personas (115).

La capacidad de adaptación de MRSA a más de un hospedador, incluyendo la especie humana, junto con una alta prevalencia de MRSA en determinadas especies animales, como por ejemplo en porcino, supone un riesgo para la población al actuar los animales de abasto como reservorio de MRSA (32). Es necesario por tanto estudiar la presencia de MRSA en animales y el medioambiente para conocer el grado de diseminación de estos y otros clones de MRSA.

Objetivos

4. Objetivos

En el presente trabajo de tesis doctoral, se ha analizado la presencia de *S. aureus* en animales de abasto y animales salvajes, así como en agua residual y fluvial, con el fin de analizar su papel en la epidemiología de MRSA. Para ello se han desarrollado los siguientes objetivos:

4.1. Detección y caracterización de *S. aureus* en animales de abasto

La detección de MRSA en animales de abasto en diferentes países de Europa, especialmente en porcino, derivó en la necesidad de conocer la situación en España. La gran diseminación de ST398 como línea genética asociada al sector porcino reveló la falta de conocimiento de las líneas prioritarias presentes en otros hospedadores.

Así, dentro del presente objetivo se han desarrollado los siguientes estudios:

- Detección y caracterización de MRSA en cerdo ibérico, publicado en 2012 (Porrero, MC *et al.*, *Detection of methicillin-resistant Staphylococcus aureus in Iberian pigs*. 2012 *Lett Appl Microbiol*, 54: 280-285).
- Detección y caracterización de MRSA en granjas de reproductoras de porcino, publicado en 2009 (EFSA, *Analysis of the baseline survey on the prevalence of methicillin-resistant Staphylococcus aureus* (MRSA) *in holdings with breeding pigs, in the EU*. 2008 *EFSA Journal*, 7: 1-82).
- Caracterización de *S. aureus* aislados de mastitis de pequeños rumiantes, publicado en 2012 (Porrero, MC *et al. Clonal diversity of Staphylococcus aureus originating from the small ruminants goats and sheep*. 2012 Vet Microbiol, 156: 157-161).

4.2. Detección y caracterización de *S. aureus* en animales salvajes de vida libre

La presencia de MRSA en animales de abasto criados en extensivo puso de manifiesto el riesgo de transmisión de MRSA a los animales salvajes de vida libre. Además, no había estudios sobre portadores de *S. aureus* en animales salvajes de vida libre, y por tanto no había datos sobre los genotipos más frecuentes de *S. aureus* presentes en esta población.

Como parte del objetivo 2 se han realizado los siguientes trabajos:

- Detección y caracterización de MRSA en animales salvajes de vida libre, publicado en 2013 (Porrero, MC, *et al. Methicillin resistant Staphylococcus aureus* (MRSA) *carriage in different free-living wild animal species in Spain*. 2013 *Vet J*, 198: 127-130).
- Detección y caracterización de MSSA en animales salvajes de vida libre, enviado para su publicación en 2014 (Porrero, MC, *et al. Carriage of Staphylococcus aureus in free-living wild animals*. 2014 *Appl Environ Microbiol*).
- Presencia de *mec*C en *S. aureus* de animales de abasto, animales salvajes y aguas residuales, publicado en 2014 (Porrero, MC, *et al. Staphylococcus aureus carrying mecC gene in animals and urban wastewater, Spain.* 2014 *Emerg Infect Dis*, 20: 899-901).

4.3. Detección y caracterización de *S. aureus* en agua residual y fluvial

La presencia de cepas MRSA-*mec*C en animales ubicados en un mismo entorno condujeron al análisis de agua fluvial como un posible mecanismo de diseminación de MRSA. Por otro lado, *S. aureus* ha sido detectado anteriormente en aguas residuales y aguas de uso recreativo, entendiéndose que el origen de las cepas detectadas son aquellos individuos colonizados en contacto con las aguas. La comparación de los aislados de *S. aureus* obtenidos a partir de agua residual y agua fluvial permitiría estudiar diferencias en la proporción de MSSA y MRSA así como los genotipos más frecuentes detectados en diferentes nichos ecológicos.

El objetivo número 3 ha dado lugar a los siguientes trabajos:

- Presencia de MRSA-*mec*C en una finca de caza: posible papel del agua en su diseminación, enviado para su publicación en 2014 (Porrero, MC, *et al*. *Detection of mecC-MRSA isolates in river water: a potential role for water in the environmental dissemination*. 2014 *Environ Microbiol*).
- Diversidad genética y genotipos predominantes de *S. aureus* en aguas residuales y en agua de rio, artículo pendiente de enviar para su publicación en 2014 (Porrero, MC, *et al. Staphylococcus aureus* genetic lineages found in river water and urban effluents. 2014 *Appl Environ Microbiol*).

Trabajos de investigación

5. Trabajos de investigación

5.1. Detección y caracterización de MRSA en cerdo ibérico

Numerosos estudios determinaron la presencia de LA-MRSA ST398 en porcino y personas relacionadas con el sector porcino (106). Los productos cárnicos del cerdo ibérico se consideran productos de alta calidad que tienen una gran demanda en el mercado internacional (116, 117). La producción de cerdo ibérico diferencia animales de montanera (pasan la última fase de engorde en la dehesa durante el periodo comprendido entre noviembre y febrero) y de cebo (sin la fase final en la dehesa), y aunque la montanera es más apreciada, también se comercializan productos obtenidos de animales de cebo (118). Las diferencias en la raza y los sistemas de producción (extensivo o intensivo) junto con la alta prevalencia de MRSA en porcino, hacían necesaria la realización de un estudio de detección de MRSA en cerdo ibérico, comparando animales de montanera y animales de cebo con el cerdo blanco.

Como parte de los programas de vigilancia de resistencias a antimicrobianos en microorganismos de origen animal realizados por la Red VAV (119); (http://www.vigilanciasanitaria.es/vav/), se tomaron muestras de animales de raza ibérica (montanera y cebo) y de cerdo blanco en diferentes mataderos. La proporción de MRSA detectada en cerdo ibérico fue inferior a la que presentaba el cerdo blanco (28% y 83% respectivamente), aunque no se encontraron diferencias estadísticamente significativas entre animales de montanera (25%) y de cebo (32%). Los aislados obtenidos fueron caracterizados mediante secuenciación de la fracción variable del gen *spa* (*spa typing*), MLST y resistencia a antimicrobianos. La mayoría de los aislados pertenecieron al genotipo ST398-t011 tanto en cerdo ibérico como en cerdo blanco, aunque la proporción de resistencia a antimicrobianos no β-lactámicos en cerdo ibérico fue menor que en cerdo blanco ($P<0,05$).

A pesar de las diferencias en la frecuencia de detección de MRSA, CC398 se reveló como el clon mayoritario también en cerdo ibérico, lo que sugiere una población propia de MRSA, en cuanto a líneas genéticas se refiere, tanto en cerdo blanco como en cerdo ibérico. El hecho de que animales criados en extensivo presentaran MRSA, permitió la identificación del posible riesgo de diseminación de MRSA a otros animales de vida libre.

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ORIGINAL ARTICLE

Detection of methicillin-resistant *Staphylococcus aureus* in Iberian pigs

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Abstract

Aims: Iberian pigs are bred in Spain for the production of high-value dry-cured products, whose export volumes are increasing. Animals are typically reared outdoors, although indoor farming is becoming popular. We compared carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in Iberian pigs, raised indoors and outdoors, with intensively farmed Standard White pigs.
Methods and Results: From June 2007 to February 2008, 106 skin swabs were taken from Iberian pigs and 157 samples from SWP at slaughterhouses in Spain. We found that Iberian pigs carried MRSA, although with a significantly lower prevalence (30/106; 28%) than SWP (130/157; 83%). A higher prevalence of indoor Iberian pigs compared with animals reared under outdoor conditions was not significant; however, all but one positive indoor Iberian pig samples were detected from one slaughterhouse. Overall, 16 different *spa* types were identified, with t011 predominating in all three animal populations. A subset of isolates was characterized by MLST. Most of these belonged to ST398. MRSA isolates from Iberian pigs presented a higher susceptibility to antibiotics than those isolated from SWP.
Conclusions: Despite limited contact with humans, pigs raised outdoors are colonized by an MRSA population that genetically overlaps with that of intensively farmed pigs, although antimicrobial resistance is lower.
Significance and Impact of the Study: To our knowledge, this is the first detection of MRSA in food animals raised in free-range conditions.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) presents a major health problem in humans as it is a common cause of nosocomial infections, displaying a complex epidemiology (Tacconelli 2009). The problem is partly caused by hospital-acquired infections, but MRSA infections acquired in the community can also pose serious health risks (Vozdecky 2009). MRSA can be isolated from a variety of animal species, including pets and food-producing animals, which may become colonized as a result of zooantroponotic transfer (Epstein and Price 2009). Such animals can potentially serve as a reservoir for human infection (Cuny *et al.* 2010). MRSA can cause veterinary clinical cases, and in addition, just like humans, asymptomatic animal carriers can be the source of recurrent infections in animal hospitals or in humans (Leonard *et al.* 2006; Juhasz-Kaszanyitzky *et al.* 2007; Graveland *et al.* 2010).

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The detection of MRSA carriage in Dutch families associated with pig farming (Voss *et al.* 2005) raised awareness of the porcine host as a potential source for infection and led to multiple investigations of MRSA in swine and humans (mainly farmers and veterinarians) (Catry *et al.* 2010). Those studies revealed that there was a remarkably high rate of MRSA carriers in swine and humans in contact with pigs, with one MRSA sequence type, ST398, being dominant for this livestock-associated MRSA.

In most described investigations, reared pigs were kept at high density and personnel had frequent contact with the animals, which apparently favours the exchange of microflora from human to animal and *vice versa*. In contrast, Iberian pigs (a breed of *Sus scrofa domesticus* indigenous to the Mediterranean area) produced in Spain are mainly reared outdoors, with limited contact with humans. These animals, locally known as '*montanera*', are of economic importance as their products are highly valued on the international market; export of their meat totalled approximately 18 000 tons in 2010. The animals are adapted to the Mediterranean environment, and their exploitation is linked to the preservation of the Dehesa or Mediterranean forest. In response to an increased demand, this breed is now also being raised indoors, producing meat that is denominated '*cebo*'. In view of the ongoing discussion on MRSA carriage in swine, its dependence on farming conditions and potential dissemination to wildlife, we investigated MRSA carriage in Iberian pigs and compared this with the carriage of Standard White pigs reared conventionally (indoor housing on farrow-to-finish farms and breeding farms, typically housing 250–300 sows and fattening units containing more than 500 animals; the fattening phase includes both continuous and all-in/all-out management systems). The genetic diversity of MRSA in Iberian pigs was determined and compared with isolates from SWP.

Materials and methods

From June 2007 to February 2008, 263 skin swabs (Amies transport medium; Copan Italia SpA, Brescia, Italy) were taken from finishing pigs at 19 Spanish slaughterhouses. The slaughterhouses were selected according to the Commission Decision 2006/668/EC and had been responsible for 62% of the pork produced in Spain in 2005 (1969 539·8 tons; Merino 2006). Six slaughtered Iberian pigs (IP) exclusively, while 13 almost exclusively handled SWP. Only one IP sample was taken from a slaughterhouse that handled both types of animals (house G) from which SWP samples were also obtained (Table 1).

Within the IP samples, a distinction was made between animals from outdoors *montanera* production (IPm) and indoors *cebo* production (IPc).

In the described period, 106 IP (56 IPm and 50 IPc) from 7 slaughterhouses and 157 SWP from 13 slaughterhouses were sampled in the lairage areas. Sample size per slaughterhouse was proportional to their relative contribution in pork production, and when multiple samples were taken per slaughterhouse, they were collected on several visits. Animals from each slaughter batch were kept in separate lairage rooms, and SWP were never mixed with IP. One skin sample was taken at random per animal and per slaughter batch; all sampled animals originated from different farms, so that only one animal per farm was tested. A surface of approximately 1 cm^2 of skin was swabbed at the base of the ear. Following the procedures advised by EFSA (EFSA, 2007), the swabs were sent to the laboratory immediately after sampling, kept at room temperature and processed within 1 week upon receipt.

Skin swabs were cultured in salty BHI broth (Brain Heart Infusion [Difco Laboratories, Le Pont de Claix, France], supplemented with 6·5% NaCl) for 18 h. Subsequently, an overnight incubation in selective broth with 75 mg l^{-1} aztreonam and 5 mg l^{-1} ceftizoxime was performed. Finally, the broth was subcultured onto selective chromogenic plates (Brilliance MRSA Chromogenic Agar plate; Oxoid, Wesel, Germany), and after 24–48 h of incubation at 37°C, the plates were checked for growth. Suspected colonies were confirmed as MRSA by multiplex-PCR (CRL-Antimicrobial Resistance protocol; http://www.crl-ar.eu/200-mrsa_-_baseline_study.htm).

All MRSA isolates were characterized by *spa* typing, according to a standard protocol (http://spaserver.ridom.de) (Harmsen *et al.* 2003). A selection of strains was further characterized by multilocus sequence typing (MLST) including at least one isolate per *spa* type, following published protocols (http://www.mlst.net) (Enright *et al.* 2000). New spa types were submitted to GenBank, with accession numbers FJ785819·1, FJ865403·1, FJ865405·1 and FJ865404·1 for t4871–t4874, FJ865406·1 for t4884 and FJ865407·1 for t4885. New MLST types were nominated as ST1965, ST1966, ST1967, ST1968 and ST1969 and submitted at http://www.mlst.net.

Antimicrobial susceptibility testing was performed according to the recommended methods (Clinical and Laboratory Standards Institute (CLSI) 2007) and as listed in the Supplementary Table S1. Tests for resistance to chloramphenicol (CHL), quinupristin–dalfopristin (SYN), oxacillin (OXA), tetracycline (TET), amoxicillin (AMX), lincomycin (LIN), vancomycin (VAN), trimethoprim (TMP), ciprofloxacin (CIP), streptomycin (STR), erythromycin (ERY) and penicillin (PEN) were carried out by broth microdilution (Treck Diagnostic plates). Resistance to fusidic acid (FUS), linezolid (LZD), mupirocin (MUP), gentamicin (GEN), cefoxitin (FOX) and sulphafurazole (SF) was tested by disc diffusion. The

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Table 1 MRSA isolated from different breeds of pigs, per slaughterhouse

Breed	Slaughterhouse	No of samples	Positive MRSA		Spa type (number)
			Number	(%)	
IPm	A	1	1	–	t011 (1)
	B	3	2	67	t011 (2)
	C	7	1	14	t1451 (1)
	D	8	3	38	t011 (3)
	E	17	1	6	t011 (1)
	F	20	6	30	t011 (5), t108 (1)
Total IPm	6 slaughterhouses	56	14	25	3 different spa types
IPc	A	1	0	–	
	B	22	15	68	t011 (13), t108 (1), t4871 (1)
	C	7	0	0	
	E	1	0	0	
	F	18	0	0	
	G	1	1	–	t1197 (1)
Total IPc	6 slaughterhouses	50	16	32	4 different spa types
SWP	H	3	0	0	
	I	6	6	100	t011 (5), t108 (1)
	J	7	7	100	t011 (6), t1451 (1)
	K	7	7	100	t011 (6), t034 (1)
	L	8	7	88	t011 (5), t108 (2)
	M	10	10	100	t011 (8), t108 (2)
	N	12	12	100	t011 (10), t108 (2)
	O	12	3	25	t011 (3)
	P	14	14	100	t011 (11), t899 (1), t1456 (2)
	Q	16	15	94	t011 (8), t108 (4), t1451 (3)
	R	16	12	75	t011 (9), t108 (2), t4885 (1)
	S	18	18	100	t011 (11), t108 (3), t1344 (1), t4872 (1), t4873 (1), t4874 (1)
	G	28	19	68	t011 (15), t127 (1), t693 (1), t1451 (1), t4884 (1)
Total SWP	13 slaughterhouses	157	130	83	14 different spa types

break-points/cut-offs applied are shown in the Supplementary Table S1.

Associations between MRSA carriage, antimicrobial resistance and individual variables (breed and management system) were assessed using the Pearson chi-square test or the Fisher exact test with the software SPSS 19.0 (IBM, New York, USA). With only one slaughterhouse that included IP ($N = 1$) and SWP ($N = 28$) samples, the study design did not allow the analysis of the effects of individual slaughterhouses.

Results

Of the 263 samples, 160 were MRSA positive (61% of the tested samples; CI 95%, 55–67%). Positive samples originated from all slaughterhouses analysed except for one (house H, see Table 1). A comparison between IP and SWP revealed that the prevalence of MRSA was significantly lower in IP ($P < 0{\cdot}001$), where 30 of 106 samples were positive (28%) compared with 83% of the SWP samples (130/157). The difference between outdoor-raised IPm (25%; 14/56) and indoor-raised IPc (32%; 16/50) was not statistically significant. However, all but one of the isolates derived from IPc came from the same slaughterhouse, while four of the six slaughterhouses did not produce any positive samples.

Most of the MRSA strains belonged to *spa* type t011 (122 of 160, 76%). This type was most frequently isolated from both SWP and IP animals (Table 2). Less common were t108 and t1451, which together were found in 24 samples (15%), again isolated from both breeds of pigs. One *spa* type was found twice (t1456, from SWP in house P) and 12 types were detected only once, including six novel *spa* types that had not been previously described. These were designated t4871 (08-16-02-25-46-24-24-25), t4872 (08-16-02-25-34-24-25-34-24-25), t4873 (08-02-25-34-24-25-17-25), t4874 (08-16-02-25-34-24-24-25-34-24-25), t4884 (08-16-284-25-34-24-25) and t4885 (08-16-02-285-34-24-25). The novel *spa* types were mostly from SWP but t4871 was isolated from IPc. It originated from slaughterhouse B that

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Table 2 Summary of spa typing data for all isolates

			Spa type			
Breed	Number of samples	MRSA positive (%)	t011	t108	t1451	Other
SWP	157	130 (83)	97	16	5	12
IPc	50	16 (32)	13	1	0	2
IPm	56	14 (25)	12	1	1	0
IPc + IPm	106	30 (28)	25	2	1	2
Total	263	160 (61)	122	18	6	13*

*These include t1456 (found in two animals), t034, t127, t1344, t693, t899, and five novel spa types for SWP, and t1197 and 1 novel spa type for IPc.

reported the highest frequency of MRSA-positive IP animals; the other *spa* types identified in IP slaughtered at B are shown in Table 1. Between one and six *spa* types were identified per slaughterhouse; most diversity was found in slaughterhouses G and S; whereas the latter processed SWP exclusively, house G also slaughtered IPc.

In total, 99% of the isolates could be grouped around closely related *spa* types, whereas one isolate from a SWP (from house G) had a different repeat pattern (t127). Two novel *spa* types were identified that contained novel repeats (repeat 284 found in t4884 and repeat 285 in t4885), which were most probably derived from previously described repeats (02 and 25, respectively), because they differ from those in one single base. Therefore, the corresponding *spa* types can be clustered within the t011 group. The new patterns detected were not associated with a specific breed, production system or slaughterhouse.

A subset of isolates ($n = 36$) belonging to various *spa* types and obtained from different slaughterhouses was further characterized by MLST. Of these isolates, the majority belonged to sequence type (ST) 398 ($n = 29$), a single isolate was ST1, and five new ST-types were detected. These were ST1965 (3-35-263-2-26-20-39), ST1966 (3-35-264-2-26-20-39), ST1967 (217-35-19-2-26-20-39) and ST1968 (3-35-19-2-26-20-223), which were all single isolates, and ST1969 (3-35-19-2-26-20-224), derived from two animals. These novel ST-types all belonged to CC398 and were the result of novel alleles detected for *arc*C (ST1967), *glp*F (ST1965 and ST1966) and *yqi*L (ST1968 and ST1969).

Heterogeneity of *spa* types was observed within the 29 ST398 isolates. This sequence type included 16 isolates of *spa* t011 and 13 isolates of 12 other *spa* types. Limited heterogeneity of the *spa* types within this ST has already been recorded in the MLST database, although six *spa* types we found had not been previously registered for ST398. The single ST1 isolate belonged to *spa* type t127. The remaining four t011 *spa* types from our pig isolates produced novel and different MLST types (ST1966, ST1968 and ST1969), suggesting a genetic heterogeneity in the studied populations. Some of these were from slaughterhouses where t011-ST398 genotypes had also been found.

Resistance against 18 antibiotics was tested for all 160 isolates. All isolates were resistant against PEN, AMX, TET and TMP, and all but one against FOX. No resistance was observed against SF, VAN, LZD and MUP. For the other tested antimicrobials, the findings are presented in the supplementary Table S1. The highest resistance was detected towards OXA and LIN (156 and 145 resistant isolates, respectively) and to a lesser extent to ERY and CIP. Resistance percentages detected against GEN, SYN, LIN, CIP and ERY were significantly higher in SWP than in IP ($P < 0{\cdot}05$).

Discussion

During a European baseline survey, Spanish holdings reported the highest prevalence of MRSA in pigs of all investigated countries, with 46·0% in breeding and 51·2% in production holdings (EFSA, 2009). These results were based on dust samples taken from holdings that represent 80% of the national breeding capacity. In the present study, in which swab samples were taken from individual animals (from slaughterhouses that collectively were responsible for 62% of the national pork production in 2005), 61% resulted in positive samples. The different sampling method may have been responsible, although differences in culture conditions may also have contributed to the observed difference. For the first time, IP were analysed separately, and from these animals, MRSA was found at much lower prevalence than from SWP.

Numerous studies have been published on the presence of MRSA in pigs at herd level or for individual animals. Data at herd level varied from 11% of farms in the Netherlands (Van Duijkeren *et al.* 2008) to 38% of finishing pig holdings in Italy (Battisti *et al.* 2010). An average of 22·8% was reported for MRSA-positive holdings with breeding pigs in the EU (EFSA, 2009). At individual level,

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De Neeling *et al.* (2007) reported 39% of positive samples in finishing pigs, and a German study identified between 49 and 70% of sampled pigs as positive (Tenhagen *et al.* 2009). In spite of the different approaches, all these studies showed a considerable prevalence of MRSA in farmed pigs.

None of the previous studies were focused on animals reared outdoors. The Iberian *montanera* pigs that were sampled in this study are farmed and managed differently to most commercial production systems. These differences include (i) a lower density of farmed animals; (ii) a constrained replacement policy, whereby production crosses are limited, so to conserve the IP breed; (iii) that, related to this, the exposure to intensively housed, putatively positive pigs is limited; (iv) that different antimicrobial application programmes to those common in SWP facilities are used because it is not possible to apply antimicrobial treatments through drinking water or medical feeding; and (v) limited human contact, occurs during life in an open environment. Any of these differences can, individually or in combination, explain the lower prevalence found for IP than for SWP.

There are different ports of entry for MRSA in IP populations; transmission through the production pyramid is one of these, and a strong association between the prevalence of MRSA-positive holdings and the volume of imported breeding pigs has been identified (EFSA, 2010). For the production of IP animals, commercial crosses are allowed with Duroc animals and farms may obtain external males for breeding programmes. This practice could potentially introduce MRSA in the IP production system, although the effect is probably limited compared to SWP breeding practices. Unfortunately, data on breeding crosses were not registered during this study.

The MRSA isolates were genotyped by *spa* typing, and a subset was also characterized by MLST. *Spa* typing identified an overlap in *spa* types between IP and SWP with t011 most frequently found, followed by t108. These *spa* types were also reported as dominant in other studies (e.g. De Neeling *et al.* 2007; Pomba *et al.* 2009; Gomez-Sanz *et al.* 2010), although the dominant *spa* type can vary between countries: Battisti *et al.* 2010 reported t899 as the most frequent *spa* type in pigs in Italy, while t034 was found to predominate in Germany (Tenhagen *et al.* 2009). The majority of slaughterhouses in our study resulted in multiple *spa* types, indicating that a high diversity of MRSA types exists in the examined pig populations. MLST typing confirmed that ST398 was the most prevalent ST-type detected, and all but one isolate belonged to CC398. The MLST data further demonstrated the genetic relatedness of nearly all detected isolates, which suggests an indigenous population of MRSA on pigs, both in IP and in SWP.

Cross-contamination can occur between animals sharing transport or lairage areas of slaughterhouses, and this cannot be ruled out in our study. However, this would not have influenced the outcome, as only one sample was taken per slaughter batch, and all batches were handled separately during transport and slaughter. Notably, IP and SWP are never transported together because production is well separated. The finding of different rates of antimicrobial resistance supports the absence of contamination during transport or in the slaughterhouse.

The higher demand of IP products has resulted in farmers switching from SWP to IP production. This was the case for most of the farm samples derived from slaughterhouse B, which produced 17 positives of 25 samples and most of the positive samples in IPc. As MRSA is persistent in the environment, it is possible that the strains detected on these IP were carried over from previous SWP production.

The use of antimicrobials is a likely cause of MRSA dissemination because of selective pressure. The antimicrobial resistance of SWP MRSA isolates determined here mostly confirmed data collected by others, but remarkably lower resistances were detected for IP isolates for GEN, SYN, LIN, CIP and ERY. Because our study did not include the collection on data of antimicrobial use on the farms, it could not be investigated if lower selection pressure because of differences in medication practices could be the basis for these differences.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Antimicrobial susceptibility testing for MRSA.

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5.2. Caracterización de *S. aureus* aislados de mastitis de pequeños rumiantes

La predominancia del genotipo ST398 en el sector porcino y la posterior detección de este genotipo en gran variedad de hospedadores (107) llevaron a la necesidad de analizar *S. aureus* de diferentes orígenes para conocer los clones mayoritarios de este microorganismo en otras especies animales. Aunque había estudios que incluían aislados de pequeños rumiantes indicando la predominancia del CC133 (120-123), el número de aislados incluidos era limitado, y además esta aproximación nunca se había realizado con aislados procedentes de España.

Como resultado de un estudio anterior de mastitis en pequeños rumiantes, disponíamos de una colección de *S. aureus* procedentes de ovejas (n=220) y cabras (n=47) aislados en el periodo comprendido entre 1995-2009. Estos aislados fueron analizados mediante PCR para identificar la presencia de genes de resistencia a meticilina, y caracterizados mediante *spa typing*, MLST y resistencia a antimicrobianos.

Los resultados obtenidos indicaban que todas las cepas eran MSSA, y presentaban una gran diversidad genética (en base al número de tipos *spa* y STs), mayor en cabras que en ovejas. Las líneas genéticas predominantes fueron el ST522 y ST133, líneas genéticas previamente detectadas en rumiantes (120-123), aunque en el caso de ST522 destacaba una mayor frecuencia de detección en nuestra colección (123). En general, las resistencias a antimicrobianos fueron bajas (menores del 20%) aunque las cabras presentaban una mayor proporción de cepas resistentes a penicilina, sulfamidas y kanamicina que las ovejas. Una mayor resistencia a penicilina fue asociada con cepas del CC9, CC22 y CC25, líneas genéticas previamente relacionadas con porcino y personas (124, 125).

El hecho de que la distribución de las líneas genéticas detectadas en el estudio no presentara cambios sustanciales a lo largo del tiempo para ovejas ni cabras, sugiere cierta estabilidad en la población de *S. aureus* causante de mastitis en pequeños rumiantes en el centro de España.

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Clonal diversity of *Staphylococcus aureus* originating from the small ruminants goats and sheep

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ABSTRACT

Staphylococcus aureus is an important pathogen in humans and many animal species. The prevalence of different clonal types in animal species remains largely unknown. We analyzed 267 *S. aureus* from intramammary infections in goats (47) and sheep (220) by *spa* typing, multi-locus sequence typing (MLST) and antimicrobial susceptibility. The most frequent *spa* types in goats were t337 ($N = 9$), t759 ($N = 6$) and t1534 ($N = 5$). Sheep isolates mainly belonged to *spa* types t1534 ($N = 72$), t2678 ($N = 29$) and t3576 ($N = 20$). Eighteen novel *spa*-types were observed; two from goat strains, 13 from sheep and three in both species. The majority of the goat strains grouped in MLST CC133 ($N = 10$) and ST522 ($N = 10$), followed by CC9 ($N = 9$), while the majority of the sheep strains were of ST522 ($N = 108$) followed by CC133 ($N = 86$) and CC130 ($N = 11$). Nine new MLST types were detected; three in goat and sheep isolates (ST1739, ST1758 and ST1780), two identified in goats only (ST1740 and ST2061) and four in sheep only (ST1742, ST1743, ST1781 and ST2011). Strains showed resistance below 20% against penicillin and tetracycline; a strong association between CC-types and penicillin resistance was observed. No resistance was detected to cefoxitin, quinupristin-dalfopristin, rifampicin and vancomycin. This study suggests that ST522 is the most common *S. aureus* clone associated with small ruminants followed by CC133.

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1. Introduction

Staphylococcus aureus is a frequent coloniser of many animal species, including humans. *S. aureus* is also an important opportunistic pathogen causing a wide range of different infections in humans and other animal species (Quinn et al., 2000).

Early studies indicated that *S. aureus* from different reservoirs were host-specific (Devriese and Oeding, 1976; Devriese, 1984). This has since been confirmed using modern molecular technologies (Sung et al., 2008; Hasman et al., 2010). Thus, CC97 seems to predominate among isolates from bovine mastitis, CC5 among isolates from infections in poultry and CC30 and CC398 among isolates from pigs (Sung et al., 2008; Lowder et al., 2009; Hasman et al., 2010). A number of studies have also examined a limited number of isolates from the small ruminants, goats and sheeps (Jørgensen et al., 2005; Aires-de-Sousa et al., 2007; Ben Zakour et al., 2008; Smyth et al., 2009). In these studies a single clonal complex (CC133) where found to be responsible for the majority of intramammary infections.

It is generally agreed that currently MLST is the method that gives most reliable information about the phylogenetic clustering of *S. aureus* isolates (Feil et al.,

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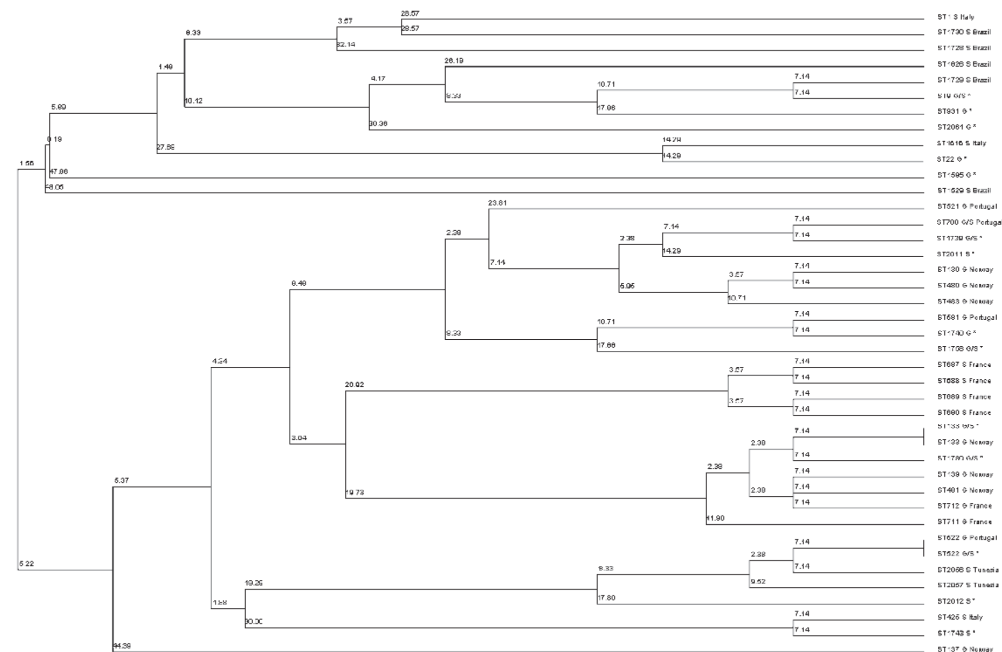

Fig. 1. UPGMA dendrogram based on pair-wise differences in the allelic MLST profiles created at http://saureus.mlst.net/sql/uniquetree.asp?. For each ST type, the animal of isolation is listed (G=goat, S=sheep). Country of origin is also listed in cases where the ST type was not detected as part of this study (which are marked with an *). A variation in one of the seven alleles lead to a relative distance of 7.14 in the dendrogram.

2003; Turner and Feil, 2007). However, the discriminatory power is still relatively low compared to other typing methods such as PFGE and *spa* typing. Therefore, the most widely used method today for first line typing and epidemiological studies is *spa* typing (Frenay et al., 1996). This method allows easy and rapid characterisation of isolates and at the same time gives robust evidence for the phylogenetic relationship, since *spa* types are normally associated to specific MLST types (Strommenger et al., 2006).

The clonal diversity of *S. aureus* among small ruminants in Spain has to our knowledge never been examined. The aim of this study was to investigate the clonal diversity, as determined by *spa* and supportive MLST typing, of 267 *S. aureus* isolates from sheep and goats in Spain, as well as their antimicrobial susceptibility.

2. Materials and methods

2.1. Bacterial isolates

A total of 267 clinical *S. aureus* isolates were collected between 1995 and 2009 from mammary infections in sheep (220) and goats (47) in the centre of Spain. The sheep isolates originated from 43 farms in seven regions and the goat isolates from nine farms in three regions. All isolates were identified as *S. aureus* based on multiplex-PCR (CRL-Antimicrobial Resistance protocol, http://www.crl-ar.eu/200-mrsa_-_baseline_study.htm).

2.2. Antimicrobial susceptibility testing

All isolates were tested for antimicrobial susceptibility using broth microdilution according to CLSI guidelines (CLSI, 2006) towards cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, penicillin, quinupristin-dalfopristin, rifampin, streptomycin, sulfamethoxazole, tetracycline, tiamulin, trimethoprim and vancomycin (Microtiter EUST plate, Treck). The interpretation of the quantitative data was performed according to EUCAST (http://www.eucast.org/mic_distributions/).

2.3. *spa* typing and multilocus sequence typing

spa typing and multilocus sequence typing (MLST) were performed as previously described (Hasman et al., 2010).

2.4. Statistical analysis

Comparison between percentages of resistance and animal species were assessed using the Fisher exact test (software SPSS 19.0; IBM, New York, USA). Differences were considered significant when $p < 0.05$.

Table 1
spa-Types, multi locus sequence typing clonal complexes (CCs) and singletons among 267 *Staphylococcus aureus* isolates from mastitis in Spanish sheep and goats.

Goats			Sheep		
CC/ST*	ST	*spa* (number of isolates)	CC/ST*	ST	*spa* (number of isolates)
CC8	ST931	t008 (3)			
CC9	ST9	t337 (9)	CC9	ST9	t3446 (1)
CC22	ST22	t005 (2)			
CC25	ST1595	t078 (1), t759 (6), t2124 (1)			
CC97	**ST2061**	t1236 (1)			
CC130	**ST1739**	t528 (1)	CC130	**ST1739**	t528 (1), t1403 (2)
			CC130	**ST2011**	t1532 (1), t1773 (5), t3570 (2)
CC133	ST133	t2678 (3), t3495 (1), **t7304** (3)	CC133	ST133	t544 (4), t2678 (29), t3495 (3), t4560 (1), t5592 (11), **t7294** (2), **t7296** (1), **t7297** (1), **t7298** (1), **t7300** (1), **t7304** (17), **t7306** (4), **t7310** (1)
CC133	**ST1780**	**t7302** (1), **t7303** (2)	CC133	**ST1780**	t1166 (1), **t7302** (7), **t7307** (2)
CC581	**ST1740**	**t7301** (1)			
			CC1742	**ST1742**	**t7309** (6)
			CC1742	**ST1781**	t1534 (1)
ST522	ST522	t1534 (5), t3576 (4), **t7295** (1)	ST522	ST522	t899 (2), t1534 (71), t2677 (1), t3576 (20), **t7295** (1), **t7299** (3), **t7305** (1), **t7308** (8), **t7311** (1)
			ST1743	**ST1743**	t3573 (3)
ST1758	**ST1758**	t5733 (2)	ST1758	**ST1758**	t5733 (1)
			Non-typeable		t026 (1), t537 (1), t817 (1)

CC, clonal complex; ST*, singleton (MLST not associated to any CC) (http://www.mlst.net/); bold letter, new ST or *spa* type.

3. Results

A total of 267 *S. aureus* isolates originating from sheep and goats in Spain were included in this study (complete details given in Table S1). None of the isolates were found to be *mec*A positive. Among these isolates, 46 different *spa* types were identified. Subsequently, MLST analysis was performed on representative isolates from the predominant (more than two isolates within a *spa* type) of these *spa* types. Based on this, sequence types and clonal complexes (CCs) were inferred through the MLST database (http://www.mlst.net/) using the eBURST algorithm (Fig. 1). This resulted in grouping the *spa* types into nine different clonal complexes (CCs) as well as three singletons according to the http://www.mlst.net database/. Three sheep isolates were non-typeable by MLST (Table 1).

The caprine isolates ($N = 47$) clustered into eight clonal complexes (CC8, CC9, CC22, CC25, CC97, CC130, CC133 and CC581) and two singletons (ST522 and ST1758), as well as 18 different *spa*-types. The most frequent *spa* types detected in goats were t337 (9 isolates), t759 (6 isolates) and t1534 (5 isolates). Among the three CCs covering 62% of the goat strains (CC133, ST522, and CC9), the *spa* types more frequently detected were t337 ($N = 9$) and t1534 ($N = 5$). The 21% belonged to ST522 ($N = 10$), with five isolates of *spa* type t1534 and CC133 ($N = 10$), with three of the five new *spa* types. No obvious change in *spa*- or MLST type distribution over time could be observed.

The ovine isolates ($N = 220$) clustered into four clonal complexes (CC130, CC133, CC1742 and CC9) and three singletons (ST522, ST1743 and ST1758), as well as 37 different *spa*-types. Three isolates were non-typeable by MLST. The most frequent *spa* types detected in sheep were t1534 (72 isolates), t2678 (29 isolates) and t3576 (20 isolates). Almost a half of the ovine strains belonged to the singleton ST522 (49%), followed by CC133 (39%) and CC130 (5%). The *spa* types most frequently associated to those MLST were t1534 and t3576 for ST522 ($N = 71$ and $N = 20$, respectively), t2678 for CC133 ($N = 29$) and t1773 for CC130 ($N = 5$). No obvious change in *spa*- or MLST type distribution over time could be observed.

Low frequencies of resistance were found for most antimicrobials, except for tetracycline (19%), penicillin (13.5%), streptomycin (6.7%) and erythromycin (6%). The largest difference between isolates from goat and sheep regarding antimicrobial susceptibility was the higher resistance of goat isolates to penicillin (46.8%), sulfamethoxazole (12.8%) and kanamycin (10.6%). The equivalent figures for the sheep isolates were 6.4%, 3.2% and 1.4% ($p < 0.05$, Fisher exact test) (Table 2).

An association between penicillin resistance and clonal complex was observed. Among the ovine isolates all three non-typeable isolates, all ST1743, the single identified CC9 and five of 11 CC130 isolates were penicillin resistant, whereas only two of 86 CC133 and none of the 108 St522 isolates were resistant. Among the caprine isolates all CC9, CC22 and CC25 were penicillin resistant, as were two of three CC8, but only one of ST522 and none of the CC133 isolates.

4. Discussion

In the current study we analyzed the clonal diversity of a collection of 267 strains of *S. aureus* from small ruminant mastitis in the centre of Spain. Our results show that all the isolates are *mec*A negative (MSSA) and that the strains have a considerable clonal diversity. A larger diversity of both MLST and *spa*-types were observed among the caprine isolates compared to the ovine isolates. This cannot be explained by differences in number of farms examined.

Table 2
Antimicrobial susceptibility of 267 *Staphylococcus aureus* isolates from mastitis in sheep and goats.

Antimicrobial agents	Number of isolates with a MIC (mg/L) of:																	Cut-off[a]	Percent resistance
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		
Cefoxitin							3	34	230									4	0
Chloramphenicol									1	134	128	2	2					16	1.5
Ciprofloxacin					115	138	13		1									1	0.4
Clindamycin				96	167	1	1	1		1								0.25	1.1
Erythromycin						98	153	2			14							1	6.0
Fusidic acid						262	4	1										0.5	1.5
Gentamicin							257	5	2			3						2	1.9
Kanamycin									245	14	2	1		5				8	3.0
Linezolid							2	90	173	2								4	0.7
Muropicin						266	1											0.5	0.4
Penicillin				231	4		3	8	20	1								0.125	13.5
Rifampicin	259	6	1															0.032	0
Streptomycin									56	130	63	7	11					16	6.7
Sulphamethozaxole													209	45	10	1	2	128	4.9
Synercid						263	3	1										1	0
Tetracycline						193	24	1			2	47						1	19.1
Tiamulin						3	187	75	2									2	0.7
Trimethoprim								252	12				3					2	5.6
Vancomycin							264	3										2	0

[a] EUCAST: *Staphylococcus aureus* data from the EUCAST MIC distribution website last accessed 5 April 2011 (http://www.eucast.org/); grey area, antimicrobial concentration tested.

ST522 was the most common MLST type observed among the ovine isolates (49%) and caprine isolates (21%). ST522 have previously been observed in two out of 32 caprine isolates examined from an European collection (Smyth et al., 2009), but to our knowledge not previously among isolates from sheep or documented as a predominant type. The second most common types was CC133. Previous studies of a limited number of isolates from small ruminants have found CC133 to be the predominant type (Jørgensen et al., 2005; Aires-de-Sousa et al., 2007; Ben Zakour et al., 2008; Smyth et al., 2009).

CC130 was in our study detected among the ovine and caprine isolates. This type has not previously been reported from small ruminants, but was identified among five percent of bovine isolates (Sung et al., 2008).

CC9 and CC25 which were common among our caprine isolates have previously mainly been found associated to pigs and humans, respectively (Rijnders et al., 2009; de Vries et al., 2009; Battisti et al., 2010; Hasman et al., 2010). All isolates belong to these CC-types were penicillin resistant. Three MSSA isolates belonging to CC8, which is a commonly observed MRSA clone in humans (Rijnders et al., 2009; de Vries et al., 2009), were also observed. CC25 is typically a human MSSA clone, while CC9 is common as a MSSA isolate in pigs, but MRSA isolates of this clonal complex have recently emerged in pigs. None of our CC8 or CC9 isolates were MRSA, but it is noteworthy that *Staphylococcus fleurettii* which was recently identified as the most likely origin of the *mec*A gene (Tsubakishita et al., 2010), was originally identified in goat milk cheese (Vernozy-Rozand et al., 2000). Further studies into the clonal diversity of these isolates and comparison to human isolates, could reveal whether they share a common origin and perhaps whether some MRSA isolates today found in humans could have a caprine origin.

We found 18 new *spa* types not previously described ($N = 65$ isolates). Three of these were detected in both goats and sheep (t7295, t7302 and t7304). Apart of the common ones, there were two and 13 new *spa* types only detected in caprine and ovine strains correspondingly, but some of them from non-related origins, which support the idea of diversity within the population. Moreover, we described nine new MLST profiles, three of them in both species; two only in goats and the other four only in sheep. The distribution of the new profiles indicates the genetic diversity of *S. aureus* in this population.

The distribution of *S. aureus* types among both goats and sheep in Spain differs considerably from that observed among humans in Spain. Thus, isolates belonging to CC30 seem to be the most common among healthy carriers (Lozano et al., 2011). Methicillin-susceptible isolates belonging to CC5 and CC30 are commonly found as causes of infections, whereas CC5 have been identified as the most common MRSA clone (Argudín et al., 2009; Vindel et al., 2009).

In general, a low frequency of antimicrobial resistance was observed. This is consistent with a limited use of antimicrobial agents for treatment of infections in small ruminants in Spain. It also suggests that most of the isolates examined were in fact of animal origin and not a result of human contamination of the samples obtained. More resistance was observed among the caprine compared to

the ovine isolates. This could suggest a higher selective pressure due to use of antimicrobial agents and/or an emergence of antimicrobial resistant types among the caprine isolates. Interestingly a strong association was observed between some types and penicillin resistance. This has previously been observed for bovine *S. aureus* (Vintov et al., 2003) and could suggest that isolates of these types have a human origin and that a continued use of penicillin for sheep and goats in Spain may lead to a change in predominating clonal types.

5. Conclusion

We found ST522 to be the predominant MLST type among *S. aureus* from sheep and a common type among isolates from goats. CC130 and CC133 were also common among isolates from sheep, while CC9, CC25 and CC133 were common types among goats. This indicates a host specificity of ST522, CC130 and CC133 to small ruminants. A strong association between penicillin resistance and isolates of CC9, CC22 and CC25 was observed. The finding of typical human associated CC25 clone and the pig associated CC9 among isolates from goats require further studies.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.10.015.

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5.3. Detección y caracterización de MRSA en animales salvajes de vida libre

La presencia de LA-MRSA en animales de abasto implica la existencia de un reservorio potencial de MRSA para la población (83, 107, 108). El hecho de que se encontraran MRSA en animales criados en extensivo (126), apuntaba a una posible exposición a LA-MRSA por parte de los animales salvajes de vida libre.

Por este motivo se decidió incluir la detección de MRSA en un proyecto de investigación sobre la interacción sanitaria entre fauna silvestre y ganadería extensiva. Se incluyeron en el estudio el buitre leonado, la cabra montés, el ciervo y el jabalí, animales que fueron muestreados mediante captura o tras la caza, tanto en fincas de caza como en parques naturales. De todos los animales se tomaron muestras nasales y de piel, exceptuando los buitres que se muestrearon sólo en fosa nasal. Aunque la mayoría de las muestras analizadas fueron negativas, 12 animales fueron portadores asintomáticos de MRSA (0,9%), no detectándose diferencias estadísticamente significativas entre los hospedadores analizados. No pudo encontrarse ninguna evidencia de relación entre el origen de los animales y la presencia de MRSA, aunque el número de animales positivos detectados limitó dicha comparación. En total se obtuvieron 13 aislados de 12 animales (un animal fue positivo a las dos muestras procesadas) de un total de 1.342 animales chequeados. La mayoría de los aislados pertenecieron al ST398 (n=11) aunque también se detectó ST1 (n=2). Ambos genotipos habían sido definidos previamente como *S. aureus* colonizadores de más de un hospedador (101, 127), hecho que ratifican nuestros resultados. El análisis conjunto de las líneas genéticas de MRSA encontradas y el perfil de resistencia a antimicrobianos, sugirieron un posible origen animal para los aislados ST398 (resistencia a tetraciclina) y humano para los aislados ST1 (sensibles a ciprofloxacina y resistentes a tetraciclina, clindamicina y eritromicina).

La presencia de MRSA en animales salvajes de vida libre, implica que los animales salvajes pueden constituir un reservorio de MRSA y demuestra la importancia de incluir estos animales en el estudio de las enfermedades que se transmiten entre el hombre y los animales (128).

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Methicillin resistant *Staphylococcus aureus* (MRSA) carriage in different free-living wild animal species in Spain

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a life-threatening pathogen in humans and its presence in animals is a public health concern. The aim of this study was to measure the prevalence of MRSA in free-living wild animals. Samples from red deer (n = 273), Iberian ibex (n = 212), Eurasian Griffon vulture (n = 40) and wild boar (n = 817) taken from different areas in Spain between June 2008 and November 2011 were analyzed. Characterization of the isolates was performed by *spa* typing, multi-locus sequence typing (MLST) and antimicrobial susceptibility testing.

A low prevalence of MRSA was found with 13 isolates obtained from 12 animals (0.89%; 95% CI: 0.46–1.56). All MRSA sequence types belonged to ST398 (t011 and t1451) and ST1 (t127). Genotypes and antimicrobial susceptibility patterns (tetracycline resistance in ST398 and clindamycin–erythromycin–tetracycline resistance in ST1) suggest that the MRSA found probably originated in livestock (ST398) or humans (ST1). This is the first report of MRSA carriers in free-living wild animals in Europe. Although our data showed that MRSA prevalence is currently low, free-living wild animals might act as reservoir and represent a potential risk for human health.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was initially found as an opportunistic pathogen in hospitals and was named hospital-associated (HA)-MRSA. Later, MRSA was also found to infect immune-competent individuals, principally causing skin and soft tissues infections (community-associated (CA)-MRSA; Pantosti, 2012). In 2004, surveillance and control programs established for humans in some European countries led to the finding of a new MRSA associated with swine production (Voss et al., 2005; Huijsdens et al., 2006), known as livestock-associated (LA)-MRSA.

This potential reservoir of LA-MRSA in food animals has resulted in several studies, which showed that ST398 (the most widespread sequence type or ST of LA-MRSA) was broadly disseminated across Europe and the rest of the world, particularly in swine (European Food Safety Authority, 2009; Smith and Pearson, 2010). Spanish studies revealed that the prevalence of MRSA in Iberian pigs raised outdoors was lower than in standard white pigs, but the presence of MRSA in outdoor pigs has highlighted the risk of exposure of wild animals to MRSA (Porrero et al., 2012b). Although studies have demonstrated the transmission of microbial agents between domestic animals and wildlife (see, for example, Gortazar et al., 2007), to our knowledge, wildlife carriage of MRSA has not been specifically evaluated. The aim of this study was to examine the prevalence of MRSA in free-living wild animals in Spain in order to identify whether MRSA has yet reached wildlife.

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Materials and methods

Study population

A total of 1342 apparently healthy animals including 273 red deer (*Cervus elaphus*), 212 Iberian ibex (*Capra pyrenaica*), 817 wild boar (*Sus scrofa*) and 40 Eurasian Griffon vultures (*Gyps fulvus*) were sampled between June 2008 and November 2011 (Table 1). Sampling areas included 10 different Spanish provinces where these animal species are located (Fig. 1).

Sample collection

Samples were obtained after hunting or through box-trapping. A total of 2546 samples from nares and/or skin swabs were analyzed for the presence of MRSA (Table 1). Only the nares were sampled in the Eurasian Griffon vultures and other species had samples taken from both the nares and the skin. Skin swabs were taken from the retro-auricular area in red deer and wild boar and from the inguinal-mammal area in Iberian ibex. These areas were selected because they were appropriate for *S. aureus* detection (Roberson et al., 1998; Smith et al., 2005; Porrero et al., 2012b). Nasal swabs were taken by introducing a sterile swab through the nostrils. Skin swabs were also taken with sterile swabs, sampling approximately 1 cm^2 of skin at the base of the ear or at the inguinal-mammal area. Swabs were held in Amies transport medium (Deltalab) to protect them from drying.

All samples were taken with particular care to avoid cross contamination both between samples and by operators. Technicians were instructed in order to ensure the quality of the samples and they were also tested to confirm that they were negative for MRSA (nares and auricular skin).

Samples were kept at a temperature between 2 °C and 25 °C (room temperature) and transported to the laboratory for processing within 13 days of sampling according to the relevant European Regulation (European Commission, 2008).

Bacterial isolation, identification and characterization

Samples were processed following a double enrichment procedure (European Commission, 2008). Swabs were cultured in salty Mueller–Hinton (6.5% NaCl, Oxoid). After incubation at 37 °C for 16–20 h, 1 mL was transferred to 9 mL tryptone

Table 1
Samples tested and positive for methicillin resistant *S. aureus* (MRSA) distributed by animal species.

	Red deer	Iberian ibex	Wild boar	Eurasian Griffon vulture	Total
Samples	543	369	1594	40	2546
Nasal swabs	269	211	795	40	1315
Skin swabs[a]	274	154	797		1225
Other[b]		4			4
ND			2		2
Tested animals	273	212	817	40	1342
Positive animals	1	2	7	2	12
% (CI)	0.37 (0.01, 2.02)	0.94 (0.11, 3.37)	0.86 (0.35, 1.76)	5.00 (0.61, 16.92)	0.89 (0.46, 1.56)
Isolates	1	2	8	2	13
Nasal swabs		1	3	2	6
Skin swabs[a]	1	1	5		7
spa type (*n*)	t011 (1)	t011 (1) t1451 (1)	t011 (6) t127 (2)	t011 (2)	

[a] Skin swabs were taken from the retro-auricular area in red deer and wild boar and from inguinal-mammal area in Iberian ibex;
[b] In these cases, Iberian ibex were sampled on ear (*n* = 2) and retro-auricular skin (*n* = 2); ND, not determined; CI: Exact 95% Fisher's confidence interval.

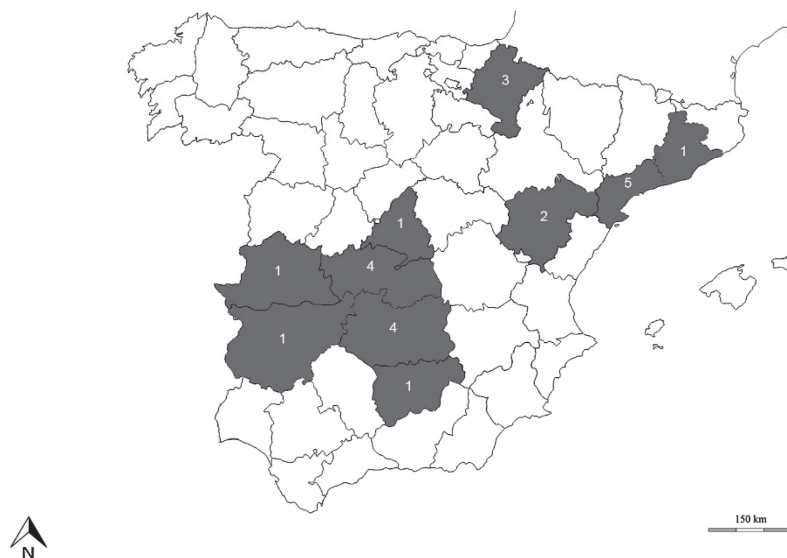

Fig. 1. Sampling areas of Spain from which animals were examined. Numbers indicate the species sampled in each province: Wild boar, regions 1, 4 and 5; Iberian ibex, region 2 and 5; Eurasian Griffon vulture, region 3; red deer, region 4.

soy broth (Oxoid) containing cefoxitin (3.5 mg/L, Sigma–Aldrich) and aztreonam (75 mg/L, Sigma–Aldrich) and incubated at 37 °C for 16–20 h. Finally, 25 µL were streaked onto Brilliance MRSA plates (Oxoid), which were incubated for 24–48 h at 37 °C. Denim blue colonies, presumptive positive for MRSA, were confirmed by PCR as methicillin-resistant (Stegger et al., 2012). The *spa* typing, multi locus sequence typing (MLST) and antimicrobial susceptibility testing were determined as previously described (Porrero et al., 2012a) with the exception of the following primers for MLST (*arc*C: dn 5′-CGATTTGTTGTTGATTAGGTTC-3′; *tpi* (up 5′-CAT-TAGCAGATTTAGGCGTTA-3′), which were specifically designed for the present study.

Statistical analysis

The proportion of MRSA positive samples in red deer, Iberian ibex, Eurasian Griffon vulture and wild boar were compared using Fisher's exact test. Exact Fisher's confidence intervals (CI) at 95% were calculated. Analyses were carried out using the software SPSS 19.0 and WinPepi 11.24.

Results

Most of the samples were negative for MRSA (Table 1). The proportion of positive animals was 0.37% (95% CI: 0.01–2.02) in red deer (1 positive/273 tested animals), 0.94% (95% CI: 0.11–3.37) in Iberian ibex (2/212), 5% (95% CI: 0.61–16.92) in Griffon vulture (2/40) and 0.86% (95% CI: 0.35–1.76) in wild boar (7/817). No significant differences between species ($P = 0.083$) were detected. Thirteen MRSA isolates were recovered from 12 animals. Six (46.2%) and seven (53.8%) isolates were recovered from nasal and skin swabs, respectively. Only one animal (wild boar) was MRSA positive for both skin and nasal swabs.

Two MLST and three *spa* types were detected. Most MRSA isolates (84.6%) belonged to ST398, 76.9% were ST398-t011 (10/13) and 7.7% were ST398-t1451 (1/13). Two isolates (15.4%) belonged to the ST1-t127 genotype.

Antimicrobial susceptibility testing confirmed all isolates as MRSA. Moreover, all isolates showed tetracycline resistance. Eight of the isolates (61.5%) were resistant to ciprofloxacin (all t011), and five (38.5%) were resistant to erythromycin and clindamycin (three t011 and two t127). No resistance was detected to chloramphenicol, fusidic acid, linezolid, mupirocin, quinupristin–dalfopristin, rifampin, sulfamethoxazole and vancomycin.

Discussion

There are only a limited number of studies on the prevalence of MRSA in wild animals and most of these have focused on captive animals which have had regular contact with humans (Janssen et al., 2009; Schaumburg et al., 2012; Vercammen et al., 2012). Recently, Wardyn et al. (2012) detected MRSA in wild patients at a care clinic that were sampled upon arrival. However, the present study is the first specifically to evaluate the presence of MRSA in free-living wild animals. Three species of artiodactyls (wild boar, red deer and Iberian ibex) were selected due to their ecological relationship with livestock and the possibility of exchanging pathogens (Wacheck et al., 2009; Martin et al., 2011). Vultures were examined because their feeding habits could increase their potential for contamination (Martin et al., 2011). The overall presence of MRSA in the wild species analyzed was low (0.89%), ranging between 0.37% (red deer) and 5.0% (Eurasian Griffon vulture) (Table 1). The higher MRSA detection in vultures might be related to its scavenging practices (Martin et al., 2011).

Our results are similar to those found in free-ranging bottlenose dolphins in the course of a study to characterize the microbiota of these animals, where MRSA was detected 3.9% of animals (Morris et al., 2010). The MRSA frequency isolation rates in free-living wild animals differ from those reported in carrier food animals, mainly pigs, which generally have higher levels of MRSA (EFSA, 2009).

About half of the MRSA-positive animals would not have been detected if only one sample per animal had been taken (Table 1). Therefore, despite the limited number of positive animals registered in our study, double sampling notably improved the detection of positives, and this finding should be considered when a low frequency of MRSA isolation is expected.

The three genotypes ST398-t011, ST398-t1451 and ST1-t127 found in our study have been previously reported in livestock and humans (Pantosti, 2012). ST398 is the most frequent ST detected in livestock (Smith and Pearson, 2010; Fluit, 2012). It has been reported mainly in pigs but also detected in cattle, horses, poultry, and even in food and human beings (Smith and Pearson, 2010). Price et al. (2012) studied an ST398 collection from different hosts, and reported that tetracycline resistance was related to animal origin. All of our ST398 isolates showed tetracycline resistance; this suggests that these isolates originated from livestock. So although the wild animals sampled came from locations with limited agricultural activity, there was still potential contact between wildlife and cattle reared outdoors, as has been shown for other pathogens (Mentaberre et al., 2012).

ST1 is considered a CA-MRSA (David and Daum, 2010). However, it has also been associated with mastitis in ruminants (Juhasz-Kaszanyitzky et al., 2007) and it is part of the MRSA population carried by healthy pigs (EFSA, 2009). Franco et al. (2011) characterized a collection of ST1-t127 from animal origin and humans and defined a porcine cluster (PC) and a human cluster (HC). The PC presented resistance to tetracycline, macrolide–lincosamide and fluoroquinolone. Our ST1-t127 isolates were susceptible to ciprofloxacin and resistant to tetracycline, clindamycin and erythromycin. This resistance pattern is more similar to that displayed by a porcine isolate grouped in the HC, which was resistant to clindamycin but susceptible to ciprofloxacin (Franco et al., 2011). Those authors hypothesized that this strain could have been introduced into the porcine holding by a human carrier. Thus, our t127 isolates may well have originated from a human source too.

Both ST1 and ST398 have been defined as MRSA lineages without pronounced host specificity for colonization and infection (Cuny et al., 2010) and even as multiple host colonizers (Fitzgerald, 2012). This would explain the detection of these genotypes in different wild animal species as seen in our study.

Difference in prevalence between wild animals and livestock carriage of MRSA together with the genotypes described and antimicrobial susceptibility data suggest that MRSA in wild animals could originate from livestock (ST398) or human (ST1) carriers.

Conclusions

To the best of our knowledge, this is the first report linking MRSA carriers to free-living wild animals in Europe. The *spa* type, MLST and antimicrobial susceptibility patterns suggest that the MRSA found are probably related to humans (ST1) and livestock (ST398). Although our data show that MRSA prevalence is low, free-living wild animals might act as a reservoir and represent a potential risk for human health.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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5.4. Detección y caracterización de MSSA en animales salvajes de vida libre

Conocer la estructura genética de la población MSSA permite comparar las líneas genéticas entre las poblaciones de MSSA y MRSA, ya que la captación del SCC*mec* por aislados MSSA constituye el origen de los MRSA (21, 62, 83). Aunque hay líneas genéticas que presentan la capacidad de colonizar gran número de hospedadores (107, 129), *S. aureus* presenta en general especificidad de hospedador (130, 131). En el caso de los animales salvajes de vida libre, el desconocimiento de los genotipos más prevalentes de MSSA junto con la detección de MRSA aparentemente vinculados al entorno humano y animal (LA-MRSA) hacían necesario el análisis de la población MSSA. El estudio permitiría también conocer la proporción de animales salvajes de vida libre que eran portadores de *S. aureus* y evaluar el tipo de muestra/s más idónea (piel y/o fosa nasal) para la detección de animales portadores.

Se tomaron muestras nasales y de piel de cabra montés, ciervo y jabalí así como muestras nasales de buitre leonado, y los aislados obtenidos fueron confirmados como MSSA y caracterizados mediante *spa typing*, MLST y resistencia a antimicrobianos.

Los resultados mostraron una detección de portadores de MSSA de entre el 5,0%-22,9%, no habiendo diferencias estadísticamente significativas entre hospedadores. Los aislados procedían mayoritariamente de muestras nasales, aunque en el caso de los ciervos y especialmente del jabalí, el doble muestreo aumentó la capacidad de detección de portadores. Al comparar las líneas genéticas mayoritarias detectadas en cabra montés, ciervo y jabalí, los tipos *spa* y los STs estaban asociados al hospedador de origen ($p < 0.05$).

En general, los aislados fueron sensibles a los antimicrobianos analizados y no se identificaron diferencias en los patrones de resistencia a antimicrobianos entre las especies animales estudiadas. Los porcentajes de resistencia más altos fueron detectados frente a penicilina (15,2%), resistencia ampliamente diseminada en animales de abasto y personas (10, 22, 32, 132-134). Además, se detectó una asociación entre la resistencia a penicilina y ST5, línea genética habitualmente relacionada con infecciones nosocomiales en personas (80) y causante de cojeras en aves (27).

Los valores de diversidad genética obtenidos de acuerdo al índice de Simpson (SID) fueron superiores en aislados MSSA que en aislados MRSA, observándose que las líneas genéticas de los aislados MRSA detectados en el mismo entorno (135) se detectaron sólo esporádicamente entre los aislados MSSA.

Title

Carriage of *Staphylococcus aureus* in free-living wild animals

Running title

S. aureus in wildlife

Byline

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Abstract

The presence of methicillin susceptible *Staphylococcus aureus* (MSSA) have been analysed in different free-living wild animals to assess the genetic diversity and predominant genotypes on each animal species. Samples were taken from the skin and/or nares and isolates were characterized by *spa* typing, MLST and antimicrobial susceptibility testing. The proportion of MSSA carriers was 5.00%, 22.93%, 19.78% and 17.67% in Eurasian griffon vulture, Iberian ibex, red deer and wild boar, respectively (P = 0.057). A higher proportion of isolates (P = 0.000) were recovered from nasal samples (78.51%) than skin samples (21.49%), but the 9.26% of red deer and 18.25% of wild boar would have been undetected if only nasal samples had been tested. Sixty-three different *spa* types were identified, including 25 new *spa* types. The most frequent were t528 (43.59%) in Iberian ibex, t548 and t11212 (15.79% and 14.04%) in red deer and t3750 (36.11%) in wild boar. By MLST, 27 STs were detected of which 12 had not been described previously. The most frequent were ST581 for Iberian ibex (48.72%), ST425 for red deer (29.82%) and ST2328 for wild boar (42.36%). Isolates from Eurasian griffon vulture belong to ST133. Host specificity has been observed for the most frequent *spa* types and STs (P = 0.000). The highest resistance percentage was found against benzylpenicillin (average 22.2%) although most of the *S. aureus* isolates were susceptible to all antimicrobial tested. Basically, MSSA isolates were different from those previously detected for MRSA in the same animal species.

Introduction

Staphylococcus aureus is a commensal microorganism in animals and humans [1] that colonize mainly the nares but also the throat and skin [2-4]. *S. aureus* is also causative agent of several diseases as pneumonia, wound and bloodstream infections [3], and colonization has been associated to clinical infection [5, 6].

Methicillin resistant *S. aureus* (MRSA) emerged by the integration of resistance mechanisms in methicillin susceptible *S. aureus* (MSSA) [7, 8]. The acquisition of *mecA* [9] or *mecC* [10] is a public health concern due to limited options for treatment. Moreover, MRSA infections are related to longer hospitalization stays and higher mortality [11, 12].

MRSA have been detected in domestic animals [13, 14]. Genetic background and antimicrobial resistance of *S. aureus* have been associated with host specificity in livestock [8, 15, 16]. Companion animals are normally colonized by human-related genotypes although some studies have described colonization factors that determine host specificity [8, 13]. MRSA detection in free-living wild animals in Spain revealed a very low prevalence

but genotypes related to livestock and humans [17, 18]. Genetic diversity of MSSA has been studied in domestic animals, also revealing predominant genotypes in different hosts [19, 20]. However, little is known about the molecular epidemiology of the susceptible *S. aureus* population in free-living wild animals. In this study, we investigated the presence of MSSA in free-living wild animals (Eurasian griffon vulture, Iberian ibex, red deer and wild boar) to assess the genetic diversity and predominant genotypes of MSSA on each animal species.

Materials and methods

Sampling

Apparently healthy animals were captured (box-trapping) or hunted between March 2009 and November 2011 in 10 different Spanish provinces. Animal were sampled with sterile swabs through the nares and/or swabbing on approximately 1 cm^2 of skin (ears or inguinal-mammal area) as previously described [17]. In total, 2,230 samples from 1,183 animals were tested including Eurasian griffon vulture (*Gyps fulvus*), Iberian ibex (*Capra pyrenaica*), red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*). The number of individuals per animal species and the number of samples tested are shown in Table 1. Sampling details were previously described [17].

Isolation and identification

Recovery of *S. aureus* (MSSA) was achieved by direct plating on Baird Parker agar with rabbit plasma fibrinogen (bioMerieux) to obtain black colonies with an opaque halo around them as presumptive *S. aureus*. One colony per sample was confirmed as MSSA (*S. aureus mecA* and *mecC* negative) by PCR as previously described [21].

Molecular characterization

All confirmed *S. aureus* isolates were characterized by *spa* typing sequencing the variable fragment of protein A [22]. Multi Locus Sequence Typing (MLST) was performed according to the protocol of Enright *et al.* [23] with the exception of self-designed primers for *arc*, *tpi* and *yqi*L housekeeping genes [17, 18]. MLST was performed on representative isolates from all *spa* types found per host. At least one isolate per *spa* type and host together with *S. aureus* with *spa* types containing less than three repeats [24] were characterized by MLST (n=88). Based on these results, sequence types (STs) were assigned.

Phenotypic antimicrobial resistance

Isolates were also tested for antimicrobial susceptibility by broth microdilution [20]. Briefly, minimum inhibitory concentration (MIC) was determined by microdilution using Sensititre *Staphylococci* plate EUST (Treck Diagnostic Systems) and interpreted according to the epidemiological cut-offs established by the European Committee on Antibiotic Susceptibility Testing (http://www.eucast.org/). Antimicrobials tested are shown in Table 2. Only one isolate per animal with the same *spa* type was tested for antimicrobial susceptibility testing.

Statistical analysis

Comparison of the proportion of positive animals in Eurasian Griffon vulture, Iberian ibex, red deer and wild boar was performed; Pearson´s chi-squared test and Confidence Intervals (CI) at 95% were calculated by SPSS 20 software and the online tool developed by WinEpi (http://www.winepi.net/sp/disease/cprev1.asp) respectively. Detection of *S. aureus* in nasal samples and skin samples was compared by McNemar´s test (SPSS 20).

Simpson's Index of Diversity (SID) and Jackknife pseudo-values Confidence Intervals (CI) at 95% were used to estimate the genetic diversity of MSSA isolates based on *spa* types [25], except for Eurasian griffon vultures because of the limited number of *S. aureus* isolates.

Fisher’s exact test (SPSS 20) was calculated to analyze the relationship between hosts and *spa* types and STs. Comparison was performed for the most frequent ones in the collection (over 5% of the isolates).

Proportion of phenotypic resistance to antimicrobials was compared in Iberian ibex, red deer and wild boar using the Fisher’s exact test (SPSS 20).

Results

In total, 242 MSSA isolates were obtained (Table 1). The proportion of positive animals was 5.00% (95% CI: 0.00-11.75) in Eurasian griffon vulture (2 positive animals out of 40 tested), 22.93% (95% CI: 16.35-29.51) in Iberian ibex (36/157), 19.78% (95% CI: 15.05-24.51) in red deer (54/273) and 17.67% (95% CI: 14.87-20.47) in wild boar (126/473). No significant differences between species (P = 0.057) were detected.

A higher proportion of isolates (P = 0.000) were recovered from nasal samples (78.51%; 190/242) than from skin samples (21.49%; 52/242) in all animal species where comparison was possible (all except vultures). In red deer and especially in wild boar,

double sampling increased the proportion of positive animals detected. If only nasal samples had been tested, five positive red deer (5/54; 9.26%) and 23 positive wild boar (23/126; 18.25%) would have been overlooked. In Iberian ibex, the three positive animals in skin samples, were also positive in nasal samples.

Half of the animals (n=12) with both nasal and skin positive samples (3 Iberian ibex, 3 red deer and 18 wild boar) presented the same *spa* type in both samples. The other 12 (3 red deer and 9 wild boar) presented different *spa* types.

Sixty-three different *spa* types were identified, including 25 *spa* types not yet reported (Table 1). The most frequent *spa* types detected were t528 (17/39; 43.59%) in Iberian ibex, t548 (9/57; 15.79%) and t11212 (8/57; 14.04%) in red deer and t3750 (53/144; 36.11%) in wild boar. The two isolates from Eurasian griffon vulture belonged to *spa* type t7304 (Table 1). In general, *spa* types were mostly detected only in a single host. One exception would be t548, which was the most frequent *spa* type detected in red deer and the second most frequent *spa* type in wild boar (Table 1). However, differences between the most frequent *spa* types and hosts were statistically significant (P = 0.000).

Based on *spa* types, Simpson's Index of Diversity (SID) calculated was 0.928 (95% CI: 0.907-0.949). SID revealed that genetic diversity was significantly higher (P < 0.05) for red deer (0.913; 95% CI: 0.943-0.972) than for Iberian ibex (0.656; 95% CI: 0.775-0.894) and wild boar (0.794; 95% CI: 0.846-0.899) isolates (Figure).

MLST analysis yielded 27 different STs, 12 of which had not been described previously (Table 1). The most frequents STs were ST581 for Iberian ibex (19/39; 48.72%), ST425 for red deer (17/57; 29.82%) and ST2328 for wild boar (61/144; 42.36%). Isolates from Eurasian griffon vulture belonged to ST133 (Table 1). As described for *spa* types, STs were mostly found in a single host although some of them (ST1, ST5, ST133) were sporadically detected in more than one host (Table 1). One exception would be ST425 (Table 1), which was detected mostly in red deer (n=17) but also in wild boar (n=7) and in Iberian ibex (n=1). Nevertheless, differences between the most frequent STs and hosts were statistically significant (P = 0.000).

Antimicrobial susceptibly testing revealed that the most of the isolates were susceptible to all antimicrobial tested (Table 2). Non-significant differences were detected between the proportion of resistance in isolates from Iberian ibex, red deer and wild boar (P > 0.050) for any of the antimicrobial tested. The most noteworthy resistance percentage was found against benzylpenicillin, with 11.11% (95% CI: 0.84-21.38) of isolates from Iberian ibex, 19.30% (95% CI: 9.05-29.54) of isolates from red deer and 26.67% (95%

CI: 19.21-34.13) of isolates from wild boar being resistant to this antimicrobial (Table 2). Isolates with any resistance to antimicrobials in our panel (n=62) presented 11 phenotypic resistance patterns. The most frequent ones were resistance only to benzylpenicillin (35 isolates out of 230 isolates tested; 15.22%), resistance to benzylpenicillin-streptomycin (10/230; 4.35%) and resistance only to streptomycin (7/230; 3.04%). The remaining resistance patterns were represented only by one or two isolates with a maximum of resistance to three antimicrobials (benzylpenicillin-streptomycin-tetracycline). Most of the isolates with benzylpenicillin resistance (n=51) belonged to ST5 (30/51; 58.82%). Comparison between ST5 and proportion of benzylpenicillin resistance showed statistically significant differences (P = 0.000).

Discussion

In this study, the genetic background of MSSA isolates from wild animals was determined in order to identify predominant genetic lineages on different free-living wild animal species.

Carriage of *S. aureus* has been evaluated revealing that *S. aureus* colonization is frequent in free-living artiodactyls (Table 1). However, the carriage rates detected in this study are lower than those reported in different domestic animals such as pigs (36%) small ruminants (from 29 to 64%), donkeys (50%) and rabbits (56%) [26-31]. Unlike the results obtained for MRSA [17], detection rate of MSSA in Eurasian griffon vulture was lower than in Iberian ibex, red deer and wild boar, although the differences were non-significant (P = 0.057).

Most of the animals simultaneously sampled in nares and skin for isolating *S. aureus* were positive only in one sample (174/198; 87.88%), with nasal swabs being the better option for sampling (172/198; 86.87%). Despite the higher detection in nasal samples, some red deer (5/54; 9.26%) and even more wild boar (23/126; 18.25%) would have been undetected if only nasal samples had been tested. Therefore, double sampling (nares and skin) would be recommendable in studies dealing with detection of MSSA carriers. Similar results have been observed for MRSA [17, 32]. The *spa* types detected in nasal and skin samples were different in the 50% of the animals positive in both samples, thus indicating that double sampling increase the diversity of *spa* types found. This should be considered an additional benefit when studying genetic diversity of MSSA.

Some of the *spa* types and STs identified in MSSA isolates in our study have been previously isolated in other animal species, although usually in low frequencies. Thus, ST2328-t9857 (t3750 related *spa* type) was found in sheep in Denmark [28]; t3750 was

previously described in Spain in 2006 although the host was not recorded (http://spa.ridom.de/; last access October 1st 2013); ST5-t548 was found in human and in pigs in the United States and in the United Kingdom [33, 34]; ST1740 (single locus variant of ST581)-t528 was described in small ruminants in Spain [20]; and ST425-t6386 and t742 were found in humans in the United Kingdom [10]. Although these data indicate the capacity of *S. aureus* to colonize more than one host, the most frequent *spa* types and STs in our study were host-associated (P = 0.000), suggesting host specificity as previously observed by other authors [35, 36].

Most of the MSSA isolates from free-living wild animals presented very low proportion of phenotypic resistance, which is probably linked to the absence of selective pressure due to no antimicrobial use in these animals [37]. The highest resistance percentage found in our study was against benzylpenicillin (22.17%; 95% CI: 16.81-27.54%). Although the origin of these resistance is unknown, previous studies showed that it is broadly disseminated in food animals [20, 26, 27] and humans [38-40].

A previous study characterized MRSA isolates recovered from the same free-living animal species examined in this work [17]. In these study, MRSA isolates belonged to ST398 (t011 and t1451) and ST1 (t127). In order to compare these MRSA isolates with the MSSA isolates obtained in the present study, genetic diversity based on Simpson´s index (SID) was calculated for MRSA (0.410; 95% CI: 0.041-0.780), showing that SID for MRSA is much lower than that observed for MSSA (0.928; P = 0.0052). This higher genetic heterogeneity observed in MSSA isolates in free-living wild animals would likely reproduce the natural genetic diversity present in the *S. aureus* population as described in humans [7, 41]. This results agrees with the higher genetic diversity exhibited by MSSA in a multicentre study performed in Europe [7]. When comparing SID between the animal species included in the study (all except vultures), genetic diversity detected in red deer was higher (P < 0.05) than in wild boar and Iberian ibex, however, justification of such differences remains unclear. The *spa* types and STs detected among MRSA isolates in free-living wild animals [17] were identified only sporadically in MSSA isolates (Table 1). Thus, only 7 isolates belonging to genotype ST1-t127 (Table 1) represented the 2.89% of the MSSA isolates. Similarly, only 3 MSSA isolates (1.24%) belonged to ST398 (Table 1) and none of them belonged to the *spa* types t011 or 1451 detected in MRSA isolates [17]. The single t011 isolate (0.41%) was ST2729, a single locus variant of ST398. Antimicrobial susceptibility patterns of MRSA isolates (tetracycline resistance in ST398 and clindamycin-erythromycin-tetracycline resistance in ST1) suggested that they probably originated in livestock or humans, respectively [17]. Nevertheless, none of the ST1 and ST398 MSSA

isolates exhibited the antimicrobial susceptibility patterns observed in MRSA. Overall, MSSA population was different from that of MRSA in genetic diversity (SID), genotypes and antimicrobial resistance patterns.

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Figure

Genetic diversity of *S. aureus* isolates based on *spa* types: Simpson's Index of Diversity (Simpson's ID) and Jackknife pseudo-values confidence intervals (CI) at 95%. MSSA: methicillin susceptible *S. aureus*.

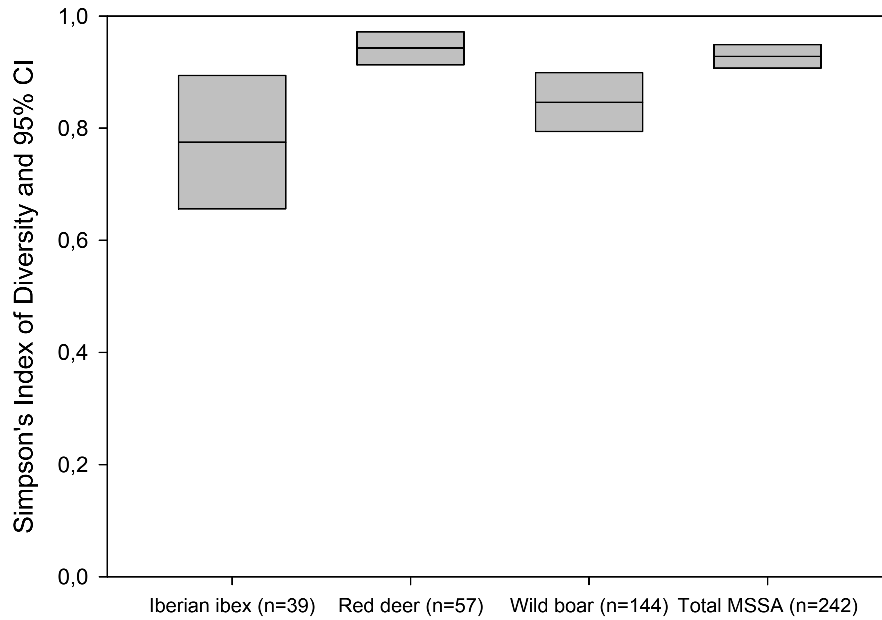

Table 1

Staphylococcus aureus detection and characterization by *spa* typing and Multi Locus Sequence Typing

Animal species	No. of animal tested	No. of positive animals	No. of samples / No. of isolates		MLST[a]	*spa* types[b] (number of isolates)
			Nasal	Skin		
Eurasian griffon vulture	40	2	40 / 2	0 / 0	ST133	t7304 (2)
Iberian ibex	157	36	157 / 36	103 / 3	ST5	t002 (4)
					ST130	t1736 (3)
					ST425	t3369 (1)
					ST2637	**t11221** (7)
					ST2639	t7229 (1), **t11216** (1)
					ST581	t528 (16), t843 (2), t1535 (1)
					ST2673	t528 (1)
					ST2328	t3750 (1), **t11501** (1)
Red deer	273	54	269 / 49	273 / 8	ST1	t098 (1), t127 (2), **t11223** (1)
					ST5	t548 (9), **t11210** (1)
					ST30	t342 (1)
					ST2681	t015 (1), **t11217** (1)
					ST133	t2678 (2)
					ST398	t571 (1)
					ST425	t1077 (1), t6386 (1), t6909 (1), **t11208** (3), t11212 (8), **t11228** (1), **t11231** (2)
					ST2640	t742 (2)
					ST350	**t11215** (5)
					ST522	t528 (1), t1534 (1), t3576 (3)
					ST2671	**t11211** (2), **t11226** (3), **t11233** (3)
Wild boar	713	126	694 / 103	694 / 41	ST1	t098 (2), t127 (5), t607 (2), t1407 (2), t2601 (1), **t11223** (1)
					ST5	t548 (18), t2516 (2), t7174 (2), **t11210** (5),

Animal species	No. of animal tested	No. of positive animals	No. of samples / No. of isolates		MLST[a]	*spa* types[b] (number of isolates)
			Nasal	Skin		
						t11214 (1), **t11219** (3)
					ST15	t084 (1)
					ST188	t189 (2)
					ST2672	t359 (1)
					ST2681	t015 (1)
					ST130	t6220 (1)
					ST133	t3583 (7), t10476 (2), **t11220** (1)
					ST2682	t6384 (1)
					ST2729	t011 (1)
					ST398	t034 (2)
					ST2675	**t11209** (2)
					ST425	t742 (3), t6909 (1), **t11222** (1), **t11225** (1), **t11232** (1)
					ST96	**t11218** (1)
					ST1643	t10712 (7)
					ST2328	t3750 (52), **t11227** (1), **t11230** (8)
					ST2641	**t11229** (1)
					ST2678	**t11502** (1)

MLST: Multi Locus Sequence Typing; ST: sequence type; a: at least one isolate per spa type per host-reservoir was selected to perform MLST (n=88), being the rest ones inferred; b: partially published in [42]; new spa types and STs identified in this study are in bold.

Table 2

Antimicrobial susceptibility and resistance of *S. aureus* isolates using the indicated cut-off value

Antimicrobial	Cut-off	Concentration* (μg/ml)	Nº of Resistant isolates / Nº of tested isolates			
			Eurasian griffon vulture (n=2)	Iberian ibex (n=36)	Red deer (n=57)	Wild boar (n=135)
Benzylpenicillin	0.125	0,12-2	0/2	**4/36**	**11/57**	**36/135**
Cefoxitin	4	0,5-16	0/2	0/36	0/57	0/135
Chloramphenicol	16	4-64	0/2	0/36	0/57	**1/135**
Ciprofloxacin	1	0,25-8	0/2	0/36	0/57	0/135
Clindamycin	0.25	0,12-4	0/2	0/36	0/57	0/135
Erythromycin	1	0,25-8	0/2	0/36	0/57	0/135
Fusidic acid	0.5	0,5-4	0/2	0/36	0/57	0/135
Gentamicin	2	1-16	0/2	0/36	0/57	0/135
Kanamycin	8	4-64	0/2	0/36	0/57	0/135
Linezolid	4	1-8	0/2	0/36	0/57	0/135
Mupirocin	0.5	0,5-2; 256	0/2	0/36	0/57	0/134
Quinupristin-dalfopristin	1	0,5-4	0/2	0/36	0/57	0/135
Rifampicin	0.032	0,016-05	0/2	0/36	0/57	0/135
Streptomycin	16	4-32	0/2	0/36	**5/56**	**15/133**
Sulfamethoxazole	128	64-512	0/2	**1/33**	**1/53**	0/135
Tetracycline	1	0,5-16	0/2	0/36	0/57	**4/135**
Tiamulin	2	0,5-4	0/2	0/36	0/57	0/135
Trimethoprim	2	2-32	0/2	0/36	**2/57**	**1/135**
Vancomycin	2	1-16	0/2	0/36	0/57	0/135

*range of studied concentration; Cut-off (last access 03/07/2013): http://www.eucast.org/mic_distributions/; bold letter: phenotypic resistance detected

5.5. Presencia de *mec*C en *S. aureus* de animales de abasto, animales salvajes y aguas residuales

La descripción de un nuevo mecanismo de resistencia a meticilina denominado *mec*C condujo al reconocimiento de nuevas líneas clonales de MRSA asociadas a rumiantes y personas (ST425, ST130) pero presentes también en otras especies animales (59, 129). Con el objeto de determinar la presencia en España de este nuevo mecanismo de resistencia a meticilina, analizamos nuestra colección de *S. aureus*. Los valores de concentración mínima inhibitoria (CMI) frente a cefoxitina detectados en los aislados MRSA-*mec*C previamente descritos, oscilaban entre 4-64 µg/ml (59, 129, 136) y el punto de corte epidemiológico establecido por el comité europeo para el análisis de sensibilidad a antimicrobianos (*European Commitee on Antimicrobial Susceptibility Testing* o EUCAST) para la cefoxitina es 4 µg/ml (CMI > 4 µg/ml se considera resistente). Así, aislados de MRSA-*mec*C presentan valores de CMI que pueden ser categorizados como sensibles o como resistentes. Estos datos nos llevaron a realizar un estudio de aislados procedentes de animales de abasto (cerdos y vacas), animales salvajes (buitre leonado, cabra montés, ciervo, gamo, jabalí y muflón) y efluentes urbanos *mec*A negativos, independientemente de los valores de CMI obtenidos frente a cefoxitina.

Tras analizar 361 aislados, se identificaron 4 aislados MRSA-*mec*C, 3 procedentes de animales salvajes (una de jabalí y dos de gamo) y 1 de agua residual. Las líneas genéticas detectadas fueron ST425 (aislados animales) y ST130 (aislados de agua residual), coincidentes con los aislados MRSA-*mec*C previamente detectados en otros países (59, 129, 136-138). Los valores de CMI frente a cefoxitina en nuestros aislados oscilaron entre 2-16µg/ml, valores incluso inferiores a los descritos previamente para MRSA-*mec*C (59, 129, 136). En relación al resto de antimicrobianos analizados, exceptuando la cepa de agua residual que era resistente a eritromicina, todos los aislados fueron sensibles a antimicrobianos no β-lactámicos, resultados que se corresponden con los datos previamente publicados (129, 137).

Este estudio constituye la primera detección de aislados de MRSA-*mec*C no clínicos en España, y, aunque la presencia de cepas de MRSA-*mec*C es baja (1,1%), pone de manifiesto la diseminación de este mecanismo de resistencia.

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Staphylococcus aureus Carrying *mecC* Gene in Animals and Urban Wastewater, Spain

To the Editor: A new methicillin resistance mechanism gene, a divergent *mec*A homologue named *mec*C (formerly *mec*A$_{LGA251}$), was recently described in *Staphylococcus aureus* (*1*). Methicillin-resistant *S. aureus* (MRSA) isolates carrying *mec*C have been recovered from humans, ruminants, pets, and other animals such as rats, seals, and guinea pigs (*1–3*). It has been suggested that *mec*C-carrying MRSA isolates might not be detected by using MRSA selective media (*4*). For *mec*C-carrying *S. aureus* isolates, cefoxitin MICs of 4–64 mg/L have been demonstrated (*1–2,4*), values that would normally include susceptible isolates, according to the epidemiologic cutoff value established by the European Committee on Antibiotic Susceptibility Testing (EUCAST; www.eucast.org). *mec*C-carrying *S. aureus* isolates have been classified as heteroresistant (*5*), and MICs can be affected by the drug-susceptibility testing method used (*1,5*).

These observations led us to retrospectively investigate the presence of *mec*C gene in a set of 361 *mec*A-negative *S. aureus* isolates collected during 2009–2012 (Table), independently of their susceptibility to cefoxitin. Isolates were recovered from healthy carriers in livestock (n = 39), from wild animals (n = 254), and from wastewater (effluents) from an urban sewage plant (n = 68). Specific amplification of the *mec*C gene was performed as described (*6*). The *mec*C-carrying *S. aureus* isolates were tested by broth microdilution using Microtiter EUST plates (Trek Diagnostic Systems, East Grinstead, UK) for susceptibility to benzylpenicillin, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, florfenicol, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, rifampin, sulfamethoxazole, streptomycin, quinupristin-dalfopristin, tetracycline, thiamulin, trimethoprim, and vancomycin. Additionally, susceptibility to oxacillin was determined by using microScan Gram Positive Combo panel 37 (Siemens, Erlangen, Germany). MICs were interpreted according to EUCAST epidemiologic cutoff values.

*mec*C was detected in a total of 4 isolates from wild boar (n = 1), fallow deer (n = 2), and urban wastewater (n = 1); these isolates represent 1% of the 361 tested isolates. The 3 isolates recovered from animals were susceptible to all antimicrobial drugs tested other than β-lactams and to oxacillin (MICs 0.5–1 mg/L) but were resistant to penicillin (MICs 0.5–2 mg/L). Two of the isolates were resistant to cefoxitin (MICs 8 and 16 mg/L) and the third was susceptible (MIC 4 mg/L). The wastewater isolate was resistant to penicillin (MIC 2 mg/L) and erythromycin (MIC 16 mg/L) and susceptible to all other antimicrobial drugs tested, including cefoxitin (MIC 4 mg/L) and oxacillin (MIC ≤0.25 mg/L).

Table. Testing of *Staphylococcus aureus* isolates for presence of methicillin resistance mechanism gene *mec*C, Spain

Isolate source	Year(s) of isolation	No. *mec*C-positive isolates	*spa* type	MLST	CC	Antimicrobial resistance profile
Livestock, n = 39						
Cattle, n = 5	2011	0				
Fattening pigs, n = 34	2009, 2011	0				
Wild animals, n = 254						
Eurasian griffon vulture, n = 2	2011	0				
Fallow deer, n = 2	2012	2	t11212	ST425	CC425	PEN, FOX
			t11212	ST425	CC425	PEN
Iberian ibex, n = 39	2009–2010	0				
Mouflon, n = 2	2009	0				
Red deer, n = 61	2009–2011	0				
Wild boar, n = 148	2009–2011	1	t11212	ST425	CC425	PEN, FOX
Urban wastewater, n = 68	2011	1	t843	ST2676	CC130	PEN, ERY

*MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; PEN, benzylpenicillin; FOX, cefoxitin; ERY, erythromycin.

Previous studies have described *mec*C-positive isolates as susceptible to all antimicrobial drugs tested except β-lactams (*2,3*), although sporadic resistance to fluoroquinolones has been found (*4,7*). We additionally found erythromycin resistance in 1 *mec*C-carrying *S. aureus* isolate. For the 4 *mec*C-carrying *S. aureus* isolates we detected, MICs of oxacillin were interpreted as susceptible, and 2 isolates were susceptible to cefoxitin according to EUCAST guidelines, findings that agree with previous reports (*1–2,4*). Thus, *mec*C presence is not always linked to resistance phenotypes for cefoxitin or oxacillin; such unclear findings could hinder the detection of *mec*C-carrying isolates.

We further characterized the 4 *mec*C-carrying *S. aureus* isolates by *spa* typing and detection of Panton-Valentin leukocidin (PVL) toxin genes (*6,8*). Multilocus sequence typing (MLST) was performed according to Enright et al. (*9*) by using self-designed primers *arc* (down 5′-CGATTTGTTGTTGATTAGGTTC-3′), *tpi* (up 5′-CATTAGCAGATTTAGGCGTTA-3′), and *yqi*L (down 5′-GATTGGYTCACCTTTRCGTTG-3′). All 4 isolates were PVL negative. The 3 animal isolates were assigned to a new *spa* type (t11212) and to clonal complex (CC) 425 and sequence type (ST) 425 (Table). ST425 has been previously associated with *mec*C-carrying *S. aureus* isolates in cattle and humans (*1–2*); the animals we sampled were from a game estate and may have had contact with cattle and with urban wastewater. The wastewater isolate was assigned to *spa* type t843 and to a new allelic profile, ST2676, in CC130 (Table). ST2676 represents a single-locus variant of ST130 carrying a different allele for the gene *aro*E. MRSA isolates of CC130 have been associated with humans and animals (*1–4,6*). This result indicates that *mec*C-carrying *S. aureus* isolates can be found in urban wastewater, which may act as an environmental reservoir, as has been demonstrated for *mec*A-carrying *S. aureus* (*10*).

In conclusion, we detected the methicillin resistance mechanism gene *mec*C in nonclinical *S. aureus* isolates from animals and urban wastewater in Spain. Although our data indicate that the frequency of this resistance mechanism is low, this gene appears to be expanding to new areas. Prospective studies should be performed to evaluate epidemiologic changes and to analyze the genetic lineages that carry this resistance mechanism.

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Schmallenberg Virus Antibodies in Adult Cows and Maternal Antibodies in Calves

To the Editor: Schmallenberg virus (SBV), a novel orthobunyavirus that is transmitted by *Culicoides* spp. biting midges, spread through herds of ruminants across Europe during 2011–2013. The virus reached as far as Finland in the north, the Republic of Ireland in the west, Turkey in the east (*1*), and Spain in the south. The clinical effect of SBV infection in ruminant livestock appears to be limited (*2*), and a vaccine to prevent the infection has been developed (*3*). There are no data to refute the assumption that natural SBV infection results in long-term immunity, as was seen earlier with natural infection of cattle with bluetongue virus serotype 8 (*4*). Newborn calves acquire passive immunity by ingestion and absorption of antibodies present in colostrum. Passive immunity can, however, block the production of serum antibodies when vaccine is administered to calves that have maternally derived antibodies (*5*). To determine the titers and persistence of SBV antibodies in adult cows and the decay of maternal antibodies in calves over time, we studied a herd of cattle from a dairy farm in the eastern Netherlands during April 2012–April 2013.

The dairy farm is the only location in the Netherlands where monitoring for biting midges was continuously conducted during the 2011–2013 SBV epidemic and where SBV RNA was detected in biting midges caught during 2011–2012 (*6,7*). The dairy herd comprised 110 animals: 60 milking cows (average age 4.0 years) and 50 heifers (average age 1.5 years) and calves (<1.0 year of age). No clinical signs or symptoms of SBV infection were observed in any of the cattle at the end of 2011 or during 2012. However, during the study period, 3 calves were stillborn, none of which had the characteristic malformations observed after SBV infection. Gross pathology confirmed that the calves did not have SBV infection, and all tissue samples were negative for SBV by reverse transcription PCR.

During the 12-month study, we obtained 4 blood samples from all animals in the herd. A virus neutralization test (VNT) was used to test the samples for antibodies (*8*). For optimal specificity and sensitivity, the VNT cutoff dilution was set at 1:8. Test dilutions ranged from 1:4–1:512. All samples were tested in duplicate; titers were determined using the Reed-Münch method and expressed on a $\log_2$ scale.

Blood samples were first obtained from the herd on April 19, 2012, after retrospective detection of SBV RNA in biting midges that had been collected from the farm on September 14, 2011 (*6*). The remaining 3 blood samples for each animal were collected on September 17, 2012; December 9, 2012; and April 23, 2013 (5, 8, and 12 months, respectively, after the first collection). SBV VNT results for the initial blood samples were positive for all cows ≥1 year of age and for all but four 6-month-old calves. One year later, blood samples for 98% of the cows ≥1 year of age and 50% of the cows <1 year of age were SBV seropositive. During the year, the mean $\log_2$ VNT titer of the adult cows dropped from 8.3 to 6.7.

It can be assumed that cows ≥1 year of age became infected with SBV around the time SBV-infected *Culicoides* biting midges were detected on the farm in September 2011 (*6*). Thus, at least 19 months after natural infection, these cows were probably protected against SBV when re-exposed to the virus. Of all cattle tested, 11 heifers seroconverted between April 2012 and September 2012, and 1 cow seroconverted between the September and December 2012 samplings. The low rate of seroconversion was matched by a 6× lower

5.6. Presencia de MRSA-*mec*C en una finca de caza

MRSA-*mec*C se ha considerado un nuevo LA-MRSA (139), habiéndose estudiado recientemente la posible transmisión entre animales y personas (115, 140). Aunque el contacto directo con animales de abasto parece el mecanismo más plausible de diseminación de MRSA (109), no en todos los casos de personas infectadas por MRSA-*mec*C se han podido identificar vínculos con la ganadería (95, 96, 141).

La presencia de *mec*C en dos especies animales distintas (gamo y jabalí) dentro de una misma finca de caza (142) nos llevó a analizar en mayor profundidad otras especies animales que compartían el entorno, incluyendo animales de abasto criados en extensivo. El hecho de haber detectado MRSA-*mec*C en agua residual así como genotipos detectados previamente en personas (59, 142), hacía conveniente analizar muestras de agua del rio que atraviesa la finca y muestras de su personal. Tras realizar los análisis, sólo se detectaron tres aislados de MRSA-*mec*C procedentes de agua del rio, siendo negativos el resto de animales salvajes (jabalí y ciervo), animales de abasto (vacuno de carne) y las personas muestreadas. Las cepas detectadas presentaban los mismos tipos *spa* y MLST y similares perfiles de resistencia a antimicrobianos que los de las cepas obtenidas previamente de gamo y jabalí (142). El análisis de secuenciación masiva reveló que los aislados estaban muy relacionados entre sí, obteniéndose en total 132 polimorfismos de nucleótido único (*Single Nucleotide Polymorphisms* o SNPs) entre los 6 aislados comparados. Además, se describió una nueva estructura de SCC*mec* tipo XI en el aislado de jabalí, al presentar este un fragmento adicional de ADN de aproximadamente 14,4 kb cuya secuencia incluye genes relacionados con un transposón conjugativo.

La presencia de MRSA-*mec*C en el agua del rio y en diferentes especies animales en el mismo entorno indica un posible papel del agua del rio en la diseminación de MRSA.

Title

Detection of *mecC*-MRSA isolates in river water: a potential role for water in the environmental dissemination.

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Running title

Presence of *mecC*-MRSA in river water.

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a public health concern due to limited treatment options. The recent description of a *mecA* homologue, *mecC* in human and cattle, led to studies to detect this new variant in human and other animal species. Detection of *mecC* in wild boar and fallow dear in a Spanish game estate led us to further investigate the presence of *mecC*-MRSA at this location. Samples from cattle, wild animals, workers and river water were tested. A further three *mecC*-MRSA isolates were obtained from river water. Molecular characterization (MLST and *spa* typing) and antimicrobial susceptibility testing (broth microdilution) showed that isolates were similar to those detected in wild animals. Whole genome sequencing confirmed that the isolates from the river water and wild animals in the same geographic area were all closely related isolates of ST425 *mecC*-MRSA. The presence of *mecC*-MRSA in the river water highlights the potential role of water in the dissemination of *mecC*-MRSA.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent causes of invasive bacterial disease in Europe (Grundmann *et al.*, 2010; Gagliotti *et al.*, 2011). Resistance to β-lactam antibiotics in MRSA is mediated by the expression of an alternative penicillin-binding protein (PBP2a) which is encoded by the *mecA* gene. In 2011, a divergent form of the *mecA* gene named *mecC*, was described in humans and cattle (García-Alvarez *et al.*, 2011), and more recently has been detected in a range of animal species, including wild animals (García-Alvarez *et al.*, 2011; Paterson *et al.*, 2012; Walther *et al.*, 2012; Porrero *et al.*, 2014). More recent studies in Denmark using traditional epidemiology and whole genome sequencing have shown that *mecC*-MRSA can be transmitted from livestock to humans (Harrison *et al.*, 2013; Petersen *et al.*, 2013). Although previous studies have identified direct contact as the most plausible mechanism of MRSA transmission between animals and humans (Verkade and Kluytmans, 2014), the potential role of other mechanisms of dissemination remains unknown.

The presence of antibiotic resistant bacteria in water have been previously recognized and is likely due to selective pressure linked to antimicrobial residues in water and high concentration of microorganisms, which promote exchange of genetic material (Baquero *et al.*; 2008, Lupo *et al.*, 2012). Specifically, MRSA has been reported to survive in river and water environments (Tolba *et al.*, 2008). In municipal wastewater and recreational marine water, detection of MRSA has been associated with shedding by colonized people (Borjesson *et al.*, 2009; Plano *et al.*, 2011; Plano *et al.*, 2013). However, although

wastewater treatment plants diminish the MRSA proportion, wastewater discharge might act as a reservoir for MRSA (Borjesson *et al.*, 2009; Rosenberg Goldstein *et al.*, 2012). Recently, *mecC*-MRSA have been detected in an urban effluent in Spain (Porrero *et al.*, 2014).

A number of human *mecC*-MRSA cases have been identified in Spain (Romero-Gómez *et al.*, 2013; García-Garrote *et al.*, 2014), with no obvious epidemiological links to livestock or wild animals. We previously identified three *mecC*-MRSA isolates from wild boar (n=1) and fallow deer (n=2) in the same game estate (Porrero *et al.*, 2014). This, combined with the low prevalence of MRSA in wild animals in Spain (Porrero *et al.*, 2013), led us to further investigate the presence of *mecC*-MRSA in the game estate, so we returned to more comprehensively sample wildlife, domestic animals, workers and river water. *mecC*-MRSA isolates were detected and subjected to whole genome sequencing to understand the phylogenetic relationship between isolates. Analysis identified that closely related isolates were circulating in both wildlife and river water, highlighting a possible mechanism for spreading between animals and potentially even transmission on to humans.

Results and discussion

The presence of *mecC*-MRSA in wild animals led us to perform additional studies in the same game estate where *mecC*-MRSA was isolated (Porrero *et al.*, 2014). None of animals (n=88) examined were positive for *mecC*-MRSA (or *mecA*-MRSA) corroborating the low frequency of *mecC*-MRSA isolates in wild animals (Porrero *et al.*, 2014). None of the persons working within the game estate (n=14) were positive for MRSA. However, additional *mecC*-MRSA were isolated from two water subsamples of the river water taken inside the game state, this being the first report of isolation of *mecC*-MRSA from an environmental source. The three *mecC*-MRSA isolates from the river´s water had identical genotype based on MLST and *spa* typing (ST425-t11212) to those isolated from wild animals (Porrero *et al.*, 2014). Antimicrobial resistance profiles were also similar, with isolates from the water being resistant to benzylpenicillin and cefoxitin but susceptible to all other antimicrobials tested as previously described (Porrero *et al.*, 2014). Those antimicrobial susceptibility profiles fit with those previously described for *mecC*-MRSA isolates (Paterson *et al.*, 2014)

Whole genome sequencing was carried out to further elucidate the genetic relationship between the wild animals and river water isolates. Phylogenetic analysis indicated that all the isolates were closely related, differing by a total of 132 SNPs (Fig. 1). Wild animal and river water isolates clustered into three separate clades which were differentiated by ~100

SNPs (Figure 1) One of the clades contained the two isolates from fallow deer (ZTA12/02038STA and ZTA12/02038STB) differing from each other by 1 SNP. These isolates differed from the isolate from the wild boar (ZTA09/03698-9ST) by ~70 SNPs. The three river water isolates (ZTA13/00933–98ST, ZTA13/00933–98SA and ZTA13/00933–60SA) clustered in a different clade (Fig. 1); isolates ZTA13/00933–98SA and ZTA13/00933–98ST were identical and differed from isolate ZTA13/00933–60SA by 33 SNPs. The three isolates from the river water differed from the isolate from the wild boar (ZTA09/03698-9ST) by ~50 SNPs and from isolates of fallow deer by ~100 SNPs. Recently, up to 40 SNPs have been identified between isolates from a single colonised human (Golubchik *et al.*, 2013). Overall, phylogenetic analysis indicated that all the isolates were highly related which suggests the existence of a closely related *mecC*-MRSA ST425-t11212 population circulating between the different animal species and the water environment in the investigated game state. Water represents a major pathway for the dissemination of bacteria (Vaz-Moreira *et al.,* 2014) and contaminated river water has been suggested as potential reservoirs of MRSA, if such water sources become contaminated with these organisms (Tolba *et al.*, 2008). Therefore, the presence of *mecC*-MRSA in the river water highlight the potential role of water in the dissemination of *mecC*-MRSA. The environmental dissemination of *mecC*-MRSA via water could be a possible explanation for cases in humans with no obvious livestock interaction identified (Basset *et al.*, 2013; Romero-Gómez *et al.*, 2013; García-Garrote *et al.*, 2014). Both wild animals and livestock sharing a common water source might also facilitate transmission between different species of animal. Further studies will be necessary to investigate the role that water could play in the maintenance and spread of MRSA between different environmental compartments.

Further analysis of the genome sequence of the *mecC*-MRSA isolates from the river water and wild animals (n=6) only identified the resistance genes *mecC* and *blaZ*$_{LGA251}$, which fit with the observed antimicrobial resistance phenotypes.

Comparison of the SCC*mec* type XI of the six *mecC*-MRSA isolates with that of the strain LGA251 revealed that it was highly conserved (99.94% identity over 29,422 bp) in all the isolates. The isolate from wild boar ZTA09/03698-9ST contained an extra ~14.4 kb insertion (Figure 2) that corresponds to a putative conjugative transposon inserted in one hypothetical protein (SARLGA251_00430; NCBI accession no: NC_017349). This transposon exhibited higher than 95% nucleotide identity with transposons present in a number of mobile genetic elements (plasmid pSK53; NCBI accession no: GQ915272; a SCC*mec* type II; NCBI accession no: AB435014) or the chromosome (NCBI accession no:

HE681097) of other *S. aureus* isolates (Han *et al*., 2009; Holden *et al*., 2013). It contains a number of genes involved in conjugative transposition (Table S1) including *tcpA*, *tcpE* and *tcpC*. The presence of a conjugative transposon inserted in the SCC*mec* type XI element in the isolate from the wild boar is of interest, as it could potentially mediate the transfer of the SCC*mec* type XI cassette between bacteria. Overall, our study demonstrates for the first time the presence of *mecC*-MRSA in an environmental water source and highlights the potential role of water in the dissemination of *mecC*-MRSA.

Experimental procedures

Samples

We tested wild boars (n=10), red deer (n=10), veal calves (n=20) and beef cattle (n=48) that share pastures in a game estate of >3,000 ha located in the centre of Spain in which *mecC*-MRSA had been detected (Porrero *et al*., 2014). Samples were collected between November 2012 and August 2013. Samples were also collected from workers of the game state (n=14) and from the water of a river (n=7) that runs through the game estate at two different locations; one approximately 0.5 km from its entry into the estate (n=6) and a second sample 4.4 km upstream, outside the game estate (n=1).

Isolation and identification

Nasal swabs were cultured in 9 mL of Mueller–Hinton broth (6.5% NaCl, Oxoid) and incubated at 37 ºC for 16–20 h. One mL was then transferred to 9 mL tryptone soy broth (Oxoid) with cefoxitin (3.5 mg/L, Sigma–Aldrich) and aztreonam (75 mg/L, Sigma–Aldrich) and incubated at 37 º C for 16–20 h. Finally, 25 µL was streaked onto Brilliance MRSA plates (Oxoid) and incubated for 24–48 h at 37 ºC (Porrero *et al*., 2013). Denim blue colonies were confirmed as MRSA (*mecA* or *mecC* positive) or methicillin susceptible *S. aureus* (MSSA; *mecA* and *mecC* negative) by PCR using primers *spa*-1113f-*spa*-1514r, *mecA* P4-*mecA* P7 and *mecA*$_{LGA251}$ MultiFP- *mecA*$_{LGA251}$ MultiRP (Stegger *et al*., 2012). All water samples consisted of 1 L of stream water and were divided into 100 x 1 mL subsamples that were processed separately. Subsamples were cultured in 9 mL Mueller–Hinton broth (6.5% NaCl, Oxoid) and incubated at 37ºC for 16–20 h. After this enrichment, direct culturing onto Baird Parker with rabbit plasma fibrinogen (bioMerieux) and selective enrichment for MRSA was performed as described for nasal swabs (Porrero *et al*., 2013).

Molecular and phenotypic characterization

All confirmed *S. aureus* were characterized by *spa* typing (Harmsen *et al*., 2003) and Multilocus Sequence Typing (MLST) (Enright *et al*., 2000; Porrero *et al*., 2013; Porrero *et*

al., 2014). Antimicrobial susceptibility testing was carried out by microdilution and Minimum Inhibitory Concentrations (MICs) were interpreted according to the European Committee on Antibiotic Susceptibility Testing (EUCAST) epidemiological cut-offs (Porrero *et al.*, 2012). Antimicrobials tested were benzylpenicillin, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, quinupristin-dalfopristin, rifampicin, streptomycin, sulfamethoxazole, tetracycline, tiamulin, trimethoprim and vancomycin (EUST plates, Trek Diagnostics).

Genome sequencing and bioinformatics

To perform epidemiological investigations (Harrison *et al.*, 2013), whole genome sequencing (WGS) was carried out in all *mecC* positive isolates (n=6). Overnight cultures were grown in tryptic soy broth (TSB) at 37º C with 200 rpm shaking. Genomic DNA was then extracted from 1 ml of the overnight cultures using a MasterPure Gram Positive DNA Purification Kit (Cambio, UK). Illumina library preparation was carried out as described by Quail and collegues (Quail *et al.*, 2008). Hi-seq sequencing was carried out following the manufacturer's standard protocols (Illumina, Inc, USA).

The whole genome based phylogeny was generated by mapping the fastq files against the LGA251 reference genome (EMBL accession no: FR821779) using SMALT (www.sanger.ac.uk/smalt). Single Nucleotide Polymorphisms (SNPs) located in mobile genetic elements were removed (Harrison *et al.*, 2013) and a maximum likelihood tree was generated using RAxML (Stamatakis *et al.*, 2005). Genomes were assembled *de novo* using Velvet (Zerbino and Birney, 2008) and contigs reordered against LGA251 using Mauve (Darling *et al.*, 2004).

Staphylococcal Cassette Chromosome *mec* (SCC*mec*) of those isolates carrying *mecC* were compared with SCC*mec* type XI of strain LGA251 using ACT (Carver *et al.*, 2005), Artemis (Rutherford *et al.*, 2000) and BLAST (Zhang *et al.*, 2000). Identification of acquired antimicrobial resistance genes in sequenced isolates was also done using Resfinder (http://cge.cbs.dtu.dk/services/ResFinder/) (Zankari *et al.*, 2012) and manually using BLAST.

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Figure 1

Phylogenetic relationships between the ST425 *mecC*-MRSA isolates in the game estate. Figure shows an unrooted maximum likelihood tree generated from SNPs in the core genome. The branch length for LGA251 has been trimmed. Bootstrap values for branches are shown in black. The number of differentiating SNPs for each branch is shown below each branch in bold.

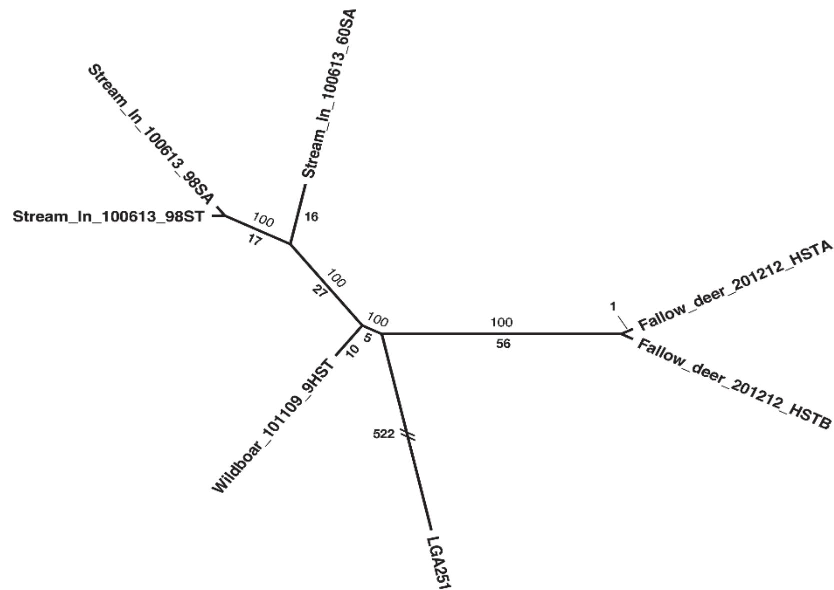

Figure references:

Wild boar_101109_9HST (ZTA09/03698-9HST); Fallow_deer_201212_HSTA (ZTA12/02838HSTA); Fallow_deer_201212_HSTB (ZTA12/02838HSTB); Stream_In_100613_60SA (ZTA13/00933-60SA); Stream_In_100613_98SA (ZTA13/00933-98SA); Stream_In_100613_98ST (ZTA13/00933-98ST).

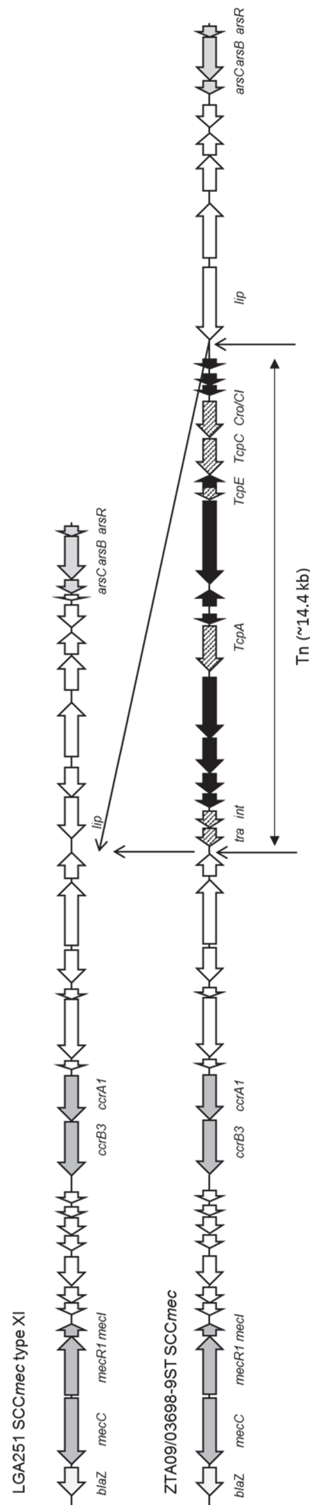

Figure 2

Comparison of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) type XI of strain LGA251 and strain ZTA09/03698-9ST (SCC*mec* element of strain ZTA09/03698-9ST was submitted to the EMBL database under accession number: pending).

Table S1

Putative conjugative transposon (~14.4 kb)

Product	Size (bp)	Orientation
Putative transposase (pseudogene)	1023	-
Hypotetical protein	411	-
Hypothetical protein	591	-
Putative traG membrane protein	1047	-
Hypothetical protein	1839	-
FtsK/SpoIIIE family protein	1359	-
Hypothetical protein [*Staphylococcus aureus*]	267	-
Hypothetical protein	477	+
Hypothetical protein	2496	-
Putative TcpE family protein	384	-
Putative conjugative transposon protein TcpC	1101	-
Replication initiation factor family protein	1092	-
Hypothetical protein	303	-
Hypothetical protein	321	-
Hypothetical protein	294	-

5.7. Diversidad genética y genotipos predominantes de *S. aureus* en aguas

Estudios previos sobre la presencia de MRSA en aguas residuales y aguas de uso recreativo determinaron que los aislados detectados en el agua son parte de la microbiota de los individuos en contacto con el agua (143-145). La comparación de los aislados existentes en agua residual urbana no tratada y agua de rio permitiría observar diferencias en las poblaciones de MRSA y MSSA circulantes en el agua en dos nichos ecológicos aparentemente diferenciados.

Se tomaron una muestra de efluentes urbanos pre-tratamiento en una depuradora de aguas residuales ubicada en un área con una población superior a los 3 millones de habitantes y una muestra de agua de rio dentro del término municipal de una población de casi 8.400 habitantes. Ambas muestras fueron subdivididas en 100 sub-muestras cada una, entendiendo que constituían alícuotas independientes que, de forma conjunta, ofrecen la imagen de la diversidad genética y los clones mayoritarios de MRSA y MSSA presentes en el agua.

La presencia de MRSA y MSSA en ambas muestras señala el posible papel del agua en la diseminación de *S. aureus*. Como era de esperar, la proporción de MRSA fue superior en el agua residual (42, 146), pero ambas muestras presentaron como genotipo mayoritario el ST125-t067, clon frecuentemente detectado en infecciones nosocomiales en España (21, 75, 91). Sin embargo y a pesar de los genotipos compartidos, cuando se analizan los tipos *spa* y STs mayoritarios en los aislados MSSA (ST5-t002, ST15-t084 y ST30-t012), existen diferencias estadísticamente significativas entre las líneas predominantes detectadas en agua del rio y aguas residuales.

En general, las líneas genéticas MRSA y MSSA más frecuentes detectadas en agua del río y efluentes urbanos son clones asociados a infecciones en personas (ST5, ST15, ST30, ST125), siendo un hallazgo puntual la detección de LA-MRSA en estas muestras.

Title

Staphylococcus aureus genetic lineages found in urban effluents and river water

Running title

Staphylococcus aureus in water

Byline

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Abstract

The presence of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) in river water and urban effluents was studied to analyse the snapshot of the *Staphylococus aureus* population and determine the genetic diversity and predominant genotypes on each ecological niche based on *spa* types and MLST. MRSA proportion in urban effluents was higher than in river water and differences were statistically significant (P<0.05). According to the Simpson's Index of Diversity based on *spa* types, MSSA isolates were more diverse than MRSA isolates (P<0.05). Predominant *spa* types and STs detected in MSSA river water isolates were different from those found in urban effluents. ST125-t067 was the predominant MRSA genotype detected in both urban effluents (67.6%) and river water (82.4%). Overall, the MSSA and MRA lineages most frequently found in river water and urban effluents were human associated clones (ST125-t067, ST5-t002; ST22-t032, ST30-t012 and ST15-t084). These results show the potential role of water in the *S. aureus* maintenance and dissemination.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) remains a public health concern as human pathogen [1, 2]. Different genetic lineages have been described as Hospital Associated-MRSA (HA-MRSA), Community-Associated-MRSA (CA-MRSA) and Livestock Associated-MRSA (LA-MRSA). Infections caused by HA-MRSA isolates are normally related to risk factors such as hospitalization, surgery or indwelling medical devices and multi-resistant isolates [3]. CA-MRSA affects to otherwise healthy people and infections have been linked to the presence of the toxin Panton-Valentine leukocidin or PVL [3]. Finally, LA-MRSA has been considered an occupational risk although its frequency of isolation is increasing in countries with low MRSA prevalence [4]. However, genetic differentiation between those groups is getting more complicated due to the incidence of HA-MRSA in the community and *vice versa* and due to the transmission of MRSA between humans and animals [5].

Direct contact was pointed out as the most feasible transmission route of *S. aureus* [4]. Nevertheless, colonized individuals might discharge bacteria into urban effluents and recreational water [6-8], which constitute a potential risk for dissemination. Moreover, the presence of MRSA in river water could indicate water is acting as a new reservoir for this pathogen (M. C. Porrero, E. M. Harrison, J. F. Fernández-Garayzabal, G. K. Paterson, A. Díez-Guerrier, M. A. Holmes, and L. Domínguez, submitted for publication).

These facts led us to study the presence of MSSA and MRSA in urban effluents and river water to assess the genetic diversity and predominant genotypes in each ecological niche.

Material and methods

The urban effluents sample was taken in July 2011 in a sewage plant that gathers wastewater from several urban collectors (untreated wastewater) in an urban nucleus with 3.3 million people and the river water sample was taken in September 2012 in the countryside, downstream the municipal term of a city with 8,392 people (http://www.ine.es/SID/Informe.do). Both samples were divided into 100 subsamples that were processed separately (n=100 subsamples per sample) to obtain the snapshot of the *S. aureus* population in the water.

Easch sub-sample (1 ml) was cultured on 9 ml of Muller-Hinton broth (6.5% NaCl, Oxoid) and incubated at 37ºC for 16-20h. One ml was then transferred to 9 ml tryptone soy broth (Oxoid) with cefoxitin (3.5 mg/l, Sigma–Aldrich) and aztreonam (75 mg/l, Sigma–Aldrich) and incubated at 37 º C for 16–20 h. Finally, 25 µl were streaked onto Brilliance MRSA plates (Oxoid) and incubated for 24–48 h at 37 ºC [9]. Denim blue colonies were confirmed as MRSA (*mecA* or *mecC* positive) or methicillin susceptible *S. aureus* (MSSA; *mecA* and *mecC* negative) by PCR using primers *spa*-1113f-*spa*-1514r, *mecA* P4-*mecA* P7 and $mecA_{LGA251}$ MultiFP- $mecA_{LGA251}$ MultiRP [10]. In parallel, 100 µl of incubated Muller-Hinton broth (6.5% NaCl) were cultured onto Baird Parker (BP) agar with Rabbit Plasma Fibrinogen (bioMerieux) and incubated at 37ºC during 24-48h. All black colonies coagulase-positive were selected as potential *S. aureus* and confirmed as MRSA or MSSA as described above.

All confirmed *S. aureus* were characterized by *spa* typing sequencing the variable fragment of protein A [10, 11]. Simpson's Index of Diversity (SID) and Jackknife pseudo-values Confidence Intervals (CI) at 95% were used to estimate the genetic diversity of *S. aureus* isolates based on *spa* types [12].

Multilocus Sequence Typing (MLST) was performed to at least one isolate per *spa* type and isolation route (n=103). Seven housekeeping genes were sequenced to obtain the alleles profile and sequence type (ST) according to protocols described before [13, 14]. Detection of Panton–Valentine leukocidin (PVL) was also carried out [10]. When more than one isolate MRSA with the same *spa* type was obtained from different isolation protocols in one subsample, only one of them was included in the data analysis as they were presumed as clonal.

Fisher's exact test (SPSS 20) was calculated to analyse the relationship between type of sample (urban effluents or river water) and the presence of MRSA and between the type of sample and the most frequent *spa* types and STs in the collection (n > 5 isolates).

Results

Both urban effluents and river water contained MRSA (102/169 and 34/115 respectively) and MSSA (67/169 and 81/115). Isolates detected are shown in the Table. A higher proportion of MRSA was detected in urban effluents (102/169; 60.4%) than in river water (34/115; 29.6%), being the differences statistically significant (P=0.000). All MRSA isolates were positive to *mec*A gene except one isolate that was positive to *mec*C (urban effluents). Only one isolate (MSSA) was positive to PVL (river water).

Isolates were grouped in 81 different *spa* types and 42 STs of which 12 *spa* types and 14 STs were common to both environments (Table). Per sample, 46 *spa* types and 23 STs were detected in urban effluents, and 47 *spa* types and 33 STs in river water. New *spa* types (n=10) and STs (n=7) have been firstly described in this study, with higher numbers from river water than from urban effluents (Table).

The number of different *spa* types detected for MSSA in river water (n=42) was higher than the number of *spa* types found in urban effluents (n=35) but lower for MRSA (n=7 and n=13 correspondingly). In MRSA, only one *spa* type covered the 82.4% of the MRSA isolates in river water (t067; 28/34), detecting for the rest of the *spa* types only one isolate per *spa* type. In urban effluents, 3 *spa* types represented the 84.3% of MRSA, being also t067 the *spa* type with higher values (69/102; 67.6%). In MSSA, 26 and 22 different *spa* types represented around 80% of isolates in river water and urban effluents respectively. The most frequent *spa* types detected per group were t002 (13/81; 16.0%), t012 (6/81; 7.4%), t078 (6/81; 7.4%) and t084 (5/81; 6.2%) in river water; and t012 (13/67; 19.4%), t084 (7/67; 10.4%) and t021 (5/67; 7.5%) in urban effluents. Comparison of the most frequent *spa* types in the collection (n > 5) revealed different predominant *spa* types for MSSA (P = 0.001) in urban effluents and river water but not for MRSA (P = 0.070).

Based on *spa* types, Simpson's Index of Diversity (SID) was calculated (Figure). SID for MRSA from river water was 0.326 (95% CI: 0.102-0.550) and 0.530 (95% CI: 0.412-0.648) in urban effluents. Regarding MSSA, values were 0.958 (95% CI: 0.934-0.981) in river water and 0.944 (95% CI: 0.910-0.978) in urban effluents, showing MSSA higher SID values than MRSA (P = 0.000).

Similarly, MLST results showed that MSSA population was more diverse than MRSA (Table). The most frequent STs detected for MRSA were ST125 for both urban effluents

(70/102; 68.6%) and river water (29/34; 85.3%), detecting in urban effluents also ST22 (9/102; 8.8%) and ST5 (9/102; 8.8%). Comparison of the most frequent STs in the collection (n > 5) in river water and urban effluents revealed that the differences between them were non-significant (P = 0.204). In MSSA, some of the most frequent STs were common to river water and urban effluents as ST5 (15/81; 18.5% in river water and 5/67; 7.5% in urban effluents), ST15 (8/81; 9.9% and11/67; 16.4%) and ST30 (11/81; 13.6% and 25/67; 37.3%). Moreover, ST25 (6/81; 7.4%), ST45 (5/81; 6.2%) and ST8 (5/81; 6.2%) were also frequent in MSSA isolated from river water and ST72 (7/67; 10.4%) in MSSA isolated from urban effluents. However, comparison of the most frequent STs in the collection (n > 5) revealed statistically significant differences between predominant STs isolated in river water and urban effluents (P = 0.004).

Discussion

The goal of this study was to analyse the presence of MSSA and MRSA in river water and urban effluents to assess the genetic diversity and predominant genotypes in each ecological niche. Due to the nature of the sample taken, each subsample of water was considered an individual sample by itself and the genetic diversity observed in the bacterial population obtained from all subsamples would reflect the snapshot of the *S. aureus* population found in the water.

Both MSSA and MRSA have been detected in river water and urban effluents. As *S. aureus* is part of the human and animal microbiota [15], its presence in the water would be related to *S. aureus* shedding, as colonized individuals might discharge bacteria into urban effluents and recreational water [6-8]. Accordingly, previous studies have described the capacity of *S. aureus* to survive in wastewater and river water [16, 17]. The higher proportion of MRSA in urban effluents might be related to the higher concentration of antimicrobial resistant bacteria in wastewater [18, 19] and to the population density in the area of sampling [20].

Although *mec*C-MRSA have been found in our collection, its low prevalence has been previously recognised in other studies [21, 22].

Concerning PVL detection, only one MSSA isolate from river water was PVL positive in our collection, even though some studies described that PVL is increasing in the south of Europe and in some areas in Spain [23, 24].

Predominant MRSA detected in river water and urban effluents were common (differences between the most frequent *spa* types and STs found were non-significant). ST125-t067 was the most frequent MRSA genotype detected, and it has been frequently associated

with nosocomial infections in Spain [1, 25, 26]. The constant release of human and animal pathogens, like MRSA, into the environment through wastewater [19] would explain the relatively high proportion (29.6%) of MRSA in the river water. Other genotypes detected in MRSA from urban water isolates such as ST22-t032 and ST5-t002 have also been associated with human infections [5, 26-28]. The lower genetic diversity of MRSA isolates has been related with the widespread distribution of a limited number of MRSA genetic lineages [1, 29]. This is in agreement with the genetic diversity detected in our study applying the SID based on *spa* types (Figure), which showed a higher genetic diversity in MSSA than in MRSA for both river water and urban effluents ($P = 0.000$).

Regarding MSSA, some of the most frequent genotypes found in river water and urban effluents (as ST30-t012 and ST15-t084) have been previously identified in human healthy carriers and patients [27, 30]. In spite of the differences in the distribution of predominant *spa* types and STs ($n > 5$) between urban effluents and river water for MSSA ($P < 0.05$), the most frequent genetic lineages (ST5-t002 and ST15-t084 and ST30-t012) were found in both ecological niches.

Overall, MRSA and MSSA have been detected in river water and urban effluents, showing the potential role of water in the *S. aureus* dissemination. Genetic lineages detected in our study showed that predominant MRSA and MSSA in both river water and urban effluents have been human associated MRSA and MSSA [5, 27, 30]. LA-MRSA presence could be considered sporadic as ST398 MRSA and related clones (ST1094, which is single locus variant of ST398) were found in very low numbers in river water and in urban effluents. Other watercourses should be evaluated to assess the presence of these pathogens and their potential role in *S. aureus* dissemination.

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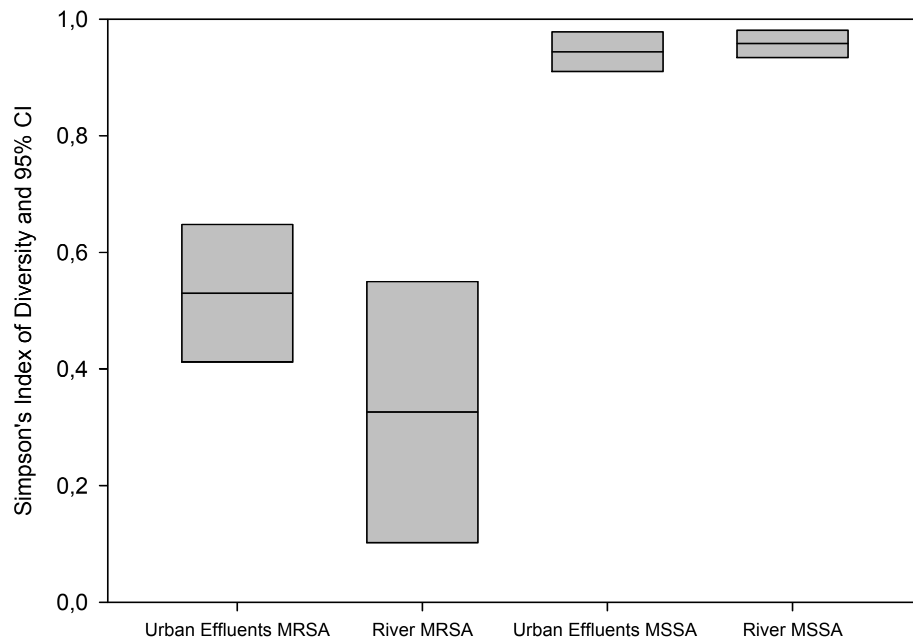
Figure

Genetic diversity of *S. aureus* isolates based on spa types: Simpson's Index of Diversity (Simpson's ID) and Jackknife pseudo-values confidence intervals (CI) at 95%. MRSA: methicillin resistant *S. aureus*; MSSA: methicillin susceptible *S. aureus*.



Table

spa types and sequence types (STs) of *S. aureus* found in river water and urban effluents

Sample	Methicillin resistance	Sequence Type (ST)	*spa* type (number of isolates)
River water	MRSA	ST1	t127 (1)
		ST5	t10422 (1)
		ST106	t056 (1)
		ST125	t067 (28), t837 (1)
		ST228	t3744 (1)
		ST398	t011 (1)
	MSSA	ST5	t002 (13), t311 (1), t586 (1)
		ST6	t701 (1)
		ST7	t091 (1)
		ST8	t008 (3), t024 (2)
		ST12	t160 (1)
		ST15	t084 (5), t335 (1), t803 (2)
		ST22	t223 (1)
		ST25	t078 (6)
		ST26	**t12664** (1)
		ST30	t012 (6), t399 (1), t5224 (1) t12633 (1), **t12667** (1), **t12668** (1)
		ST34	**t12773** (1)
		ST45	t015 (2), t065 (1), t728 (2)
		ST59	t216 (1)
		ST72	t148 (3)
		ST97	t267 (4)
		ST101	t056 (1)
		ST125	t067 (1)
		ST398	**t12666** (3)
		ST508	t050 (1)
		ST582	t605 (1)
		ST737	t4801 (1)*
		ST707	t4523 (1)
		ST919	**t12665** (1)
		ST2303	t1877 (1)
		ST2746	t073 (1)
		ST2747	t2724 (1)
		ST2748	t026 (1)
		ST2754	t5334 (1)
		ST2753	t748 (1)

Sample	Methicillin resistance	Sequence Type (ST)	*spa* type (number of isolates)
		ST2812	t021 (1)
Urban effluents	MRSA	ST1	t127 (5), t386 (2)
		ST5	t002 (8), t668 (1)
		ST22	t032 (9)
		ST72	t148 (1), t9350 (1)
		ST125	t067 (69), t3557 (1)
		ST398	t011 (1)
		ST1094	**t11245** (2)
		ST2674	t3200 (1)
		ST2676**	t843 (1)**
	MSSA	ST5	t002 (3), t010 (2)
		ST8	t008 (1)
		ST9	t8887 (1)
		ST15	t084 (7), t094 (2), t491 (1), t1877 (1)
		ST22	t005 (1)
		ST25	t2613 (1)
		ST30	t012 (13), t018 (1), t021 (5), t122 (1), t238 (1), t584 (1), t710 (1), t1306 (1), **t11235** (1)
		ST34	t089 (1), t166 (1)
		ST45	t065 (1), t620 (1), **t11236** (1)
		ST49	**t11246** (1)
		ST72	t1346 (4), t3092 (1), t3169 (1), t3682 (1)
		ST97	t5727 (1)
		ST106	t056 (3)
		ST121	t1114 (1)
		ST130	t843 (1)
		ST188	t189 (1)
		ST509	t375 (2)

MRSA: methicillin resistant *S. aureus*; MSSA: methicillin susceptible *S. aureus*; *PVL positive isolate; ***mec*C isolate [15]; bold letter: *spa* types and STs described in this study

Discusión

6. Discusión

S. aureus es uno de los agentes microbianos que con mayor frecuencia causa infecciones en personas (20, 147), situación que se agrava cuando se trata de MRSA debido a las limitaciones en el tratamiento (99). Los MRSA se diferenciaron inicialmente en HA-MRSA y CA-MRSA, y diversos estudios en individuos relacionados con el sector porcino dieron lugar a la descripción posterior de aislados LA-MRSA (32, 80). El hecho de que los animales de abasto pudieran actuar como reservorio de MRSA junto con la implicación de los animales salvajes y el medioambiente en la diseminación de microorganismos patógenos y resistencias a antimicrobianos (42, 128, 148, 149), nos condujo al estudio de *S. aureus* en animales de abasto, animales salvajes y agua (residual y fluvial) para analizar su posible implicación en la epidemiología de MRSA.

En el presente trabajo, la frecuencia de detección de MRSA fue muy diferente en los distintos animales analizados. La mayor proporción de MRSA se encontró en ganado porcino, con el 61% de animales positivos al considerar de forma conjunta el cerdo blanco y el cerdo ibérico (Capítulo 5.1, Tabla 2). El porcentaje de muestras positivas a MRSA fue especialmente elevado en el caso del cerdo blanco (83%), valor sensiblemente superior al obtenido en España en el estudio estandarizado de prevalencia realizado en la Unión Europea (108). En dicho estudio, España presentó los valores más altos de prevalencia de MRSA tanto en granjas de madres como en granjas de abuelas con porcentajes del 51,2% y 46,0% respectivamente. La sensibilidad del análisis disminuye cuando se utilizan muestras de polvo en comparación con las muestras de piel del pabellón auricular (150), y al emplear muestras agregadas en comparación con muestras individuales (151). El hecho de que en el estudio europeo (108) se analizaran muestras agregadas de polvo recogido en granjas comparado con la detección de MRSA basada en muestras individuales de piel de animales en el presente trabajo, podría explicar la mayor de prevalencia de MRSA detectada (Capítulo 5.1, Tabla 2). Otros aspectos que justificarían las diferencias entre ambos estudios podrían ser el lugar de muestreo (matadero en el presente trabajo o granja en el estudio europeo) o la fase productiva en la que se han tomado las muestras (cerdos de engorde en el presente estudio y granjas de madres en el estudio europeo). En este sentido se ha comprobado que la detección de MRSA en el matadero puede ser mayor como consecuencia de contaminaciones cruzadas durante el transporte de los animales al matadero (152) y que la proporción de MRSA aumenta al descender en la pirámide productiva (108, 153, 154).

El cerdo ibérico presentó una menor proporción de MRSA (28%; $P<0,05$) que el cerdo blanco (Capítulo 5.1, Tabla 2). Una mayor densidad productiva y una mayor importación de animales implican una prevalencia más alta de MRSA (102, 153). Así mismo, el uso de antimicrobianos ha sido reconocido como un factor que favorece la selección de MRSA en el ganado porcino (155, 156). En este sentido, la cría del cerdo ibérico se caracteriza por una menor densidad de animales en producción, una limitada interacción con animales de otras razas y en general un menor uso de antimicrobianos (157). Estas diferencias justificarían la menor proporción de MRSA detectada en el cerdo ibérico. Además, el hecho de que se detectara MRSA en animales criados en extensivo puso de manifiesto el riesgo de exposición a estos microorganismos para animales salvajes de vida libre que comparten el entorno.

A diferencia del ganado porcino, no se encontró MRSA entre los aislados de la colección de *S. aureus* de mastitis de pequeños rumiantes (Capítulo 5.2), resultados que concuerdan con la detección puntual de MRSA en casos de mastitis descrita por otros autores (41, 100). En el caso de los animales salvajes, estudios previos sobre la presencia de MRSA se habían basado en animales muestreados en cautividad y/o en contacto frecuente con personas (158-161). Sin embargo, en el presente estudio se analizaron animales salvajes de vida libre (buitre leonado, cabra montés, ciervo y jabalí) que actúan como reservorios de otros patógenos para el hombre y otras especies animales (162, 163). La detección de MRSA en animales salvajes de vida libre fue baja, oscilando entre el 0-5% dependiendo de la especie (Capítulo 5.3, Tabla 1). El hecho de haber tomado dos muestras por animal (muestra nasal y muestra de piel) mejoró notablemente la detección de animales positivos, ya que si sólo se hubiera tomado una muestra, se habrían considerado negativos prácticamente la mitad de los animales (sólo un animal fue positivo en las dos muestras). El buitre leonado presentó los mayores valores de detección de MRSA (5,0%), hecho que podría estar relacionado con sus hábitos alimenticios (163). En general, estas bajas prevalencias (0,9% de MRSA en el total de los animales analizados) concuerdan con la ausencia de presión selectiva debida a la nula o muy escasa exposición a antimicrobianos en animales salvajes de vida libre en comparación con los animales domésticos o el hombre (50, 164). Sin embargo, se han detectado anteriormente microorganismos resistentes en entornos sin conocida exposición a antimicrobianos (42). La presencia de microorganismos resistentes en el medioambiente debido a contaminaciones desde el entorno urbano y/o ganadero (146), podría explicar los aislamientos de MRSA en los animales salvajes estudiados, hipótesis que se corresponde con las características genéticas y fenotípicas de los aislados detectados en nuestro estudio (Capítulo 5.3). Este

trabajo representa la primera descripción en Europa de aislados MRSA en animales salvajes de vida libre y, aunque la frecuencia de detección es baja por el momento, pone de manifiesto el riesgo potencial que suponen los animales salvajes como reservorios de MRSA en el medioambiente.

Otro elemento a considerar en la diseminación de bacterias entre los diferentes compartimentos medioambientales es el agua (149). Las muestras de agua analizadas en nuestro estudio (Capítulo 5.7) presentaron diferentes proporciones de aislamiento de MRSA, siendo el porcentaje en aguas residuales (60,4%) mayor que en agua fluvial (29,6%). El hecho de que se aislara menos MRSA en agua fluvial podría deberse al entorno poblacional que rodeó el punto de muestreo. La muestra de agua de rio fue recogida en un municipio con 8.392 habitantes, mientras el agua residual pertenecía a un municipio con más de 3 millones de habitantes de acuerdo al Sistema de Información Demográfica (http://www.ine.es/SID/Informe.do). Se considera que a mayor población de influencia en el área, mayor es el nivel de contaminación (149). Por otro lado, se sabe que las plantas de tratamiento de agua residual son un punto de concentración de antimicrobianos y de microorganismos resistentes a antimicrobianos (146), con lo que la mayor proporción de MRSA podría estar relacionada también con el tipo de muestra, constituida en nuestro caso por el agua residual de diversos colectores urbanos y analizada tras la separación de solutos groseros.

Aunque en los estudios realizados se detectaron aislados MRSA fundamentalmente *mec*A positivos (Capítulos 5.1, 5.3 y 5.7), también fue posible la detección de MRSA *mec*C positivos tanto en muestras de animales salvajes como en agua (Capítulos 5.5 y 5.6). En nuestro primer estudio de detección de MRSA-*mec*C, la proporción de aislados *mec*C positivos fue del 1,1% en el total de los aislados analizados (Capítulo 5.5, Tabla), constituyendo nuestro estudio la primera detección en España de este mecanismo de resistencia en aislados no clínicos de *S. aureus*. Con respecto a las características genéticas de los aislados *mec*C (Capítulo 5.5, Tabla), las cepas de animales salvajes fueron ST425-CC425 y la de agua residual ST2676-CC130. Ambos genotipos han sido relacionados previamente con la presencia de *mec*C tanto en animales como en el hombre (59), aunque MRSA-*mec*C ST130 se aísla con más frecuencia (165). De acuerdo con estos datos, los casos clínicos de MRSA-*mec*C positivos descritos en España se han debido a infecciones producidas por MRSA-*mec*C CC130 (95, 96). Cabe destacar que la detección de MRSA-*mec*C puede haberse subestimado puesto que el mecanismo inicial de detección de estos aislados se ha basado en la obtención en el laboratorio de fenotipos de resistencia a meticilina junto con la ausencia del gen *mec*A (59, 129, 137, 138, 140, 166, 167), y nuestro

estudio demuestra que hay aislados portadores de *mec*C con fenotipo sensible a cefoxitina (Capítulo 5.5, Tabla).

En relación a la detección de MSSA, su análisis pretendía conocer la estructura genética de la población de *S. aureus* en los diferentes reservorios en el estudio (12), dado que los MRSA surgen a partir de la captación de la resistencia a meticilina por aislados MSSA (21, 62, 83). Con ese propósito se ha estudiado la presencia de *S. aureus* en animales portadores y muestras de agua residual y fluvial.

Desafortunadamente, en los animales de abasto incluidos en el estudio, no pudimos analizar la frecuencia de portadores de MSSA, ya que el estudio de pequeños rumiantes se corresponde con aislados de una colección de *S. aureus* procedentes de mastitis y durante el estudio de prevalencia de MRSA en cerdo blanco y cerdo ibérico no se realizó el aislamiento de MSSA. Aun así, contamos con datos de la Red VAV del programa de vigilancia de resistencias en porcino del año 2009, donde el 38,2% de los animales en los que se aisló *S. aureus* portaban MSSA (Red VAV, datos no publicados). En el caso de los animales salvajes de vida libre, la frecuencia de portadores varió entre el 5,0%-22,9% (Capítulo 5.4, Tabla 1) para las diferentes especies animales analizadas (buitre leonado, cabra montés, ciervo y jabalí). Aunque las diferencias entre ellos no fueron estadísticamente significativas (P=0,057), es evidente que existen diferencias en el nivel de colonización de las distintas especies animales por *S. aureus*. Otros estudios, en su mayoría basados en muestras nasales para el aislamiento de MSSA, describen una mayor proporción de portadores de *S. aureus* en cerdos (36%), pequeños rumiantes (29-64%), burros (50%) y conejos (56%) (133, 134, 168-171). En nuestro trabajo, hemos analizado muestras nasales y muestras de piel de cabra montés, ciervo y jabalí (Capítulo 5.4). A pesar de que la frecuencia de portadores es inferior a la descrita para otras especies de animales de abasto, la proporción obtenida en animales salvajes podría haber sido incluso menor si se hubiera analizado únicamente un tipo de muestra, tal como se vio en la detección de portadores de MRSA (Capítulo 5.3, Tabla 1). Aunque la toma de muestras en fosas nasales fue más productiva en la detección de MSSA (el 78,5% de los aislados provienen de muestras nasales en comparación con el 21,5% de muestras de piel), el 9,3% de los ciervos y el 18,3% de los jabalíes se habrían considerado no portadores si no se hubiera muestreado también en piel (Capítulo 5.4, Tabla 1). Estos resultados destacan que en algunas especies animales como el ciervo y especialmente el jabalí, el doble muestreo mejora la detección de animales portadores de MSSA. Además, en la mitad de los animales que presentaron las dos muestras positivas a MSSA (12/24; Capítulo 5.4), se

obtuvieron diferentes tipos *spa*, lo que aumenta la diversidad genética en estudios epidemiológicos.

En el caso de las muestras de agua, la proporción de MSSA detectada fue del 39,6% en agua residual y el 70,4% en agua de rio. Puesto que *S. aureus* forma parte de la microbiota bacteriana de piel y mucosas en el hombre y los animales (6), su presencia en el agua estaría relacionada con la eliminación de este microorganismo por individuos colonizados tanto a los efluentes urbanos como a aguas de uso recreativo (143-145). Estudios previos han descrito la capacidad de *S. aureus* de sobrevivir en agua de rio y aguas residuales (145, 172, 173) y además, las aguas residuales se consideran una vía de entrada de microorganismos potencialmente patógenos desde el hombre y los animales al medioambiente (146).

El análisis de la diversidad genética de los aislados obtenidos mediante la aplicación del índice de Simpson (SID) basado en los tipos *spa* (21, 71, 174) indica que los aislados MSSA presentaron una mayor diversidad genética que los aislados MRSA (P=0,0000) en todos los reservorios en los que disponíamos de datos que permitían dicha comparación (Tabla 5).

Tabla 5. Índice de Simpson de diversidad genética (SID) basado en tipos *spa*

Resistencia a meticilina	Reservorio	SID (IC: 95%)*	n
MRSA	Animales salvajes	0,410 (0,041-0,780)	13
	Porcino 2009**	0,412 (0,322-0,502)	177
	Agua residual	0,530 (0,412-0,648)	102
	Agua fluvial	0,326 (0,102-0,550)	34
MSSA	Animales salvajes	0,928 (0,907-0,949)	242
	Porcino 2009**	0,921 (0,873-0,969)	29
	Agua residual	0,944 (0,910-0,978)	67
	Agua fluvial	0,958 (0,934-0,981)	81

MRSA: *methicillin resistant S. aureus*; MSSA: *methicillin susceptible S. aureus*; * Jackknife pseudo-values; ** Red VAV, datos no publicados.

Estos resultados coinciden con la mayor diversidad genética descrita en aislados MSSA de personas (21, 71, 175, 176) y con el hecho de que los MRSA hayan surgido a partir de la captación de la resistencia a meticilina por parte de un número limitado de líneas genéticas que posteriormente se han consolidado como los MRSA predominantes (62, 99, 177).

Así, en porcino hemos detectado MRSA ST398 como genotipo mayoritario, tanto en cerdo blanco como en cerdo ibérico (Capítulo 5.1, Tabla 2). El hecho de que ST398 se haya detectado también como genotipo mayoritario MRSA en cerdo ibérico (Capítulo 5.1, Tabla

2) implica que ambos comparten una misma población de MRSA. En el programa de vigilancia de resistencias de la Red VAV de porcino en 2009 (Tabla 6), ST398 resultó el genotipo mayoritario tanto entre los aislados MRSA (99,4%; 176/177) como entre los aislados MSSA (37,9%; 11/29). Estos resultados están en línea con lo señalado por otros autores (111, 112) y confirman el genotipo ST398 como clon adaptado al ganado porcino (83, 99).

Tabla 6. Aislados de *S. aureus* detectados en porcino en España

Reservorio	Resistencia a meticilina	ST	Tipo *spa* (n)
Cerdos de engorde en matadero (Porcino 2009*)	MRSA	ST1	t127 (1)
		ST398	t011 (134), t034 (3), t108 (21), t588 (1), t899 (1), t1197 (8), t1255 (1), t1451 (4), t4872 (2), **t11237** (1)
	MSSA	ST1	t127 (3), t607 (1)
		ST106	t056 (1)
		ST2680	t318 (1)
		ST398	t011 (6), t034 (2), t1197 (3)
		ST5	t548 (2)
		ST9	t337 (4), t1430 (1), t3270 (1), t3446 (3)
		ST97	**t11234** (1)
Granjas de reproductoras (EFSA 2008)	MRSA	ST1	t127 (2)
		ST398	t011 (132), t034 (1), t108 (23), t1197 (8), t1255 (2), t1344 (1), t1451 (1), t1456 (1), t2329 (3), t2330 (1), t4872 (1), t567 (1)

ST: *sequence type*; * Red VAV, datos no publicados; tipos *spa* y STs en negrita implica que son secuencias descritas por primera vez en este trabajo

Independientemente de la adaptación del genotipo ST398 al ganado porcino, su aislamiento no está limitado a esa especie animal como lo demuestra su detección en otras especies (83, 99, 107). En este sentido, en los programas de la Red VAV de ganado bovino de aptitud cárnica correspondientes con los años 2010 y 2011 (Red VAV, datos no publicados) todos los aislados MRSA detectados fueron ST398, si bien su frecuencia de aislamiento fue muy inferior (7/200; 3,5%) a la detectada en ganado porcino (Capítulo 5.1, Tabla 2). Un dato a tener en cuenta es que los mataderos de origen de estos animales son de sacrificio mixto (porcino y bovino), por lo que el origen de estos aislados podría deberse a contaminación cruzada en el matadero, como se ha descrito en otros estudios (169). Asimismo, ST398 fue el genotipo mayoritario (11/13; 84,6%) entre los aislados

MRSA de los animales salvajes. Todos estos datos demuestran la gran capacidad de colonización que tiene este genotipo de MRSA (101, 107, 127).

Estudios de secuenciación masiva han determinado que el CC398 tiene un origen ancestral humano. Una vez en el reservorio animal, el CC398 se ha adaptado, incluyendo la captación de resistencia a tetraciclina (*tet*M) y a meticilina (*mec*A), entre otros (113, 178). Así, la presencia de *tet*M se ha propuesto como marcador genético de aislados CC398 de origen animal (178). En nuestra colección, prácticamente todos los aislados MRSA CC398 analizados presentaron fenotipo de resistencia a tetraciclina (372/374; 99,5%).

A diferencia de la alta prevalencia de MRSA ST398 en España (108) y de la presencia de este clon como genotipo más frecuente en muestras de cerdo ibérico y animales salvajes (Capítulos 5.1 y 5.3), las muestras de agua analizadas presentan líneas genéticas mayoritarias distintas. Tanto en muestras de agua residual como en muestras de agua fluvial la línea genética más frecuente de MRSA fue ST125 (Capítulo 5.7, Tabla). Este clon es característico de infecciones nosocomiales por MRSA en España (21, 75, 89).

En el caso de los MSSA en animales, nuestro estudio en aislados de mastitis en pequeños rumiantes determinó la alta frecuencia de detección de ST133 y ST522 (31,1% y 44,3% respectivamente; Capítulo 5.2). Otros estudios habían detectado estos genotipos en rumiantes previamente, aunque el número de cepas en estudios anteriores era menor y ST522 no había sido descrito como predominante (120-123). ST133 se ha aislado también como genotipo mayoritario en un estudio realizado en portadores en pequeños rumiantes (169). Los animales salvajes presentaron MSSA de líneas genéticas mayoritarias diferenciadas para cada una de las especies animales analizadas (Capítulo 5.4, Tabla) tales como ST581 en cabra montés (48,7%), ST425 en ciervo (29,8%) y ST2328 en jabalí (42,4%). Algunos de estos ST o líneas genéticas relacionadas habían sido descritos previamente en rumiantes (132, 169), cerdos (179, 180) y personas (59, 179, 180), aunque en todos los casos con baja frecuencia de detección. Los genotipos mayoritarios de MSSA detectados en animales salvajes son en general diferentes de los genotipos MRSA (Capítulo 5.3, Tabla 1).

En relación a los aislados MSSA del agua residual y agua fluvial, las líneas genéticas predominantes fueron básicamente ST5, ST15 y ST30 (Capítulo 5.7, Tabla), si bien existieron diferencias en la proporción de cada una de ellas en los dos tipos de agua ($P<0,05$). Estos STs de MSSA aislados de aguas han sido previamente descritos en personas en España, tanto en portadores como en casos clínicos (79, 181). También en

el agua residual y fluvial los genotipos mayoritarios de MSSA detectados son diferentes de los genotipos MRSA (Capítulo 5.7, Tabla).

La presencia de *S. aureus* en el agua se ha relacionado con su eliminación por parte de individuos colonizados (143, 144, 173). En este sentido, el estudio que realizamos en una finca de caza donde se detectaron animales portadores de MRSA-*mec*C positivos ST425 (Capítulo 5.6), permitió la detección de aislados genéticamente relacionados en el agua del arroyo que cruza la finca. Un análisis de secuenciación masiva de los aislados de jabalí y gamo y del agua, evidenció la elevada relación genética entre ellos (Capítulo 5.6, Figura 1), diferenciándose únicamente en 132 SNPs.

La presencia de MRSA en agua podría justificar los casos de LA-MRSA en que los no se ha podido establecer un vínculo epidemiológico con la ganadería como en las infecciones descritas recientemente (95, 96, 141), ya que el agua puede contener microorganismos de individuos que comparten el entorno y así intervenir en su diseminación.

Conclusiones

7. Conclusiones

1. El cerdo ibérico y el cerdo blanco comparten una misma población de MRSA, siendo ST398 el genotipo mayoritario en ambos hospedadores.
2. La detección de aislados MRSA en animales salvajes de vida libre cuyo origen es atribuible a otros hospedadores, implica la existencia de interacciones entre reservorios, además de la participación de los animales salvajes en la epidemiología de MRSA.
3. En estudios epidemiológicos en los que se espera una baja prevalencia de *S. aureus*, la realización de doble muestreo mejora tanto la capacidad de detección de animales portadores como la diversidad genética obtenida.
4. Aunque se ha observado una gran diversidad en aislados MSSA de animales salvajes, las líneas genéticas mayoritarias detectadas en cabra montés, ciervo y jabalí se asocian al hospedador de origen.
5. La detección de MRSA *mec*C positivos en animales salvajes y agua representa la primera descripción en España de aislados no clínicos con dicho mecanismo de resistencia y evidencia su diseminación entre la población de *S. aureus*.
6. La detección de aislados de MRSA-*mec*C positivos sensibles a cefoxitina sugiere la necesidad de ampliar los criterios actuales de detección de *mec*C basados en el fenotipo de resistencia a cefoxitina y ausencia de *mec*A.
7. La presencia de aislados de *S. aureus* en el agua pone de manifiesto su papel potencial en la diseminación de este microorganismo, hecho que cobra especial relevancia en el caso de MRSA.

Conclusions

8. *Conclusions*

1. Iberian pigs and standard white pigs share the MRSA population, being ST398 the most frequent genotype in both hosts.
2. Detection of MRSA isolates in free-living wild animals, whose origin is attributable to other hosts, suggests the existence of interactions between reservoirs, besides the participation of wildlife in the epidemiology of MRSA.
3. Double sampling improves the detection of healthy animal carriers and the genetic diversity obtained in epidemiological studies when low prevalence of *S. aureus* is expected.
4. A wide diversity of MSSA isolates was observed in wild animals, however, the most frequent genetic lineages detected in Iberian ibex, red deer and wild boar are associated to host.
5. Detection of *mec*C-MRSA in animals and water represents the first description of nonclinical *mec*C-MRSA isolates in Spain and evidences the dissemination of this resistance mechanism among the *S. aureus* population.
6. Detection *mec*C-MRSA isolates susceptible to cefoxitin suggests the need for extending the current criteria for detecting *mec*C-MRSA based on cefoxitin resistance phenotype and absence of *mec*A gene.
7. The presence of *S. aureus* in water shows its potential role in the dissemination of this microorganism, which is particularly relevant in the case of MRSA.

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9. Bibliografía

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