High prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the *mecC* gene in a semi-extensive red deer (*Cervus elaphus hispanicus*) farm in Southern Spain

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Abstract

The objective was to determine the prevalence of *Staphylococcus aureus* nasal carriage in red deer of a semi-extensive farm and in humans in contact with the estate animals, and to characterize obtained isolates. Nasal swabs of 65 deer and 15 humans were seeded on mannitol-salt-agar and oxacillin-resistance-screening-agar-base. Isolates were identified by microbiological and molecular methods. Antimicrobial susceptibility profile was determined for 16 antibiotics by disk-diffusion and the presence of eight antibiotic resistance genes, seven virulence genes and genes of immune-evasion-cluster (IEC) was analyzed by PCR. *S. aureus* was typed by PFGE-SmaI, *spa*, *agr*, SCC*mec* and MLST. Isolates were detected in 16 deer (24.6%). Eleven *S. aureus* isolates were methicillin-resistant (MRSA), and five were methicillin-susceptible (MSSA). All MRSA harbored *mecC* gene and were *agr*-III/SCC*mec*XI/ST1945 (four *spa*-t843 and seven *spa*-t1535). All *mecC*-MRSA carried *bla*Z-SCC*mec*XI and *etd*2, were IEC-type-E, and belonged to the same PFGE pattern. The five MSSA were typed as *spa*-t2420/*agr*-I/ST133. Regarding humans, *S. aureus* was recovered from six samples (40%). The isolates were MSSA and were typed as *spa*-t002/*agr*-II, *spa*-t012/*agr*-III or *spa*-t822/*agr*-III and showed different IEC types (A, B, D and F). *bla*Z and *erm*(A) genes were detected, as well as *cna* and *tst* genes. As conclusion, red deer analyzed in this study are frequent carriers of *mecC*-MRSA CC130 (16.9%), they are characterized by few resistance and virulence determinants, and by the presence of IEC type-E. Deer could be a source of *mecC*-MRSA which could potentially be transmitted to other animals, or even to humans.

Introduction

Staphylococcus (*S.*) *aureus* can be found as part of the normal microbiota of humans and animals without causing disease, however, it is also an opportunistic pathogen that may cause minor and severe infections. Methicillin-resistant *S. aureus* (MRSA) infections represent a major therapeutic problem; this resistance is mainly due to the acquisition of the *mecA* gene that encodes a modified penicillin-binding protein (PBP2a) with low affinity for beta-lactam antibiotics. Recently, a new *mecA* gene homologue was described in *S. aureus*, *mecC* (García-Álvarez et al., 2011), which codifies a PBP that shows more affinity for oxacillin than for cefoxitin (Kim et al., 2012). MRSA carrying *mecC* gene has been found among humans, livestock, companion, and wild animals, in several European countries (Becker et al., 2014; Paterson et al., 2014). *mecC*-MRSA strains belong primarily to CC130 and ST425. These lineages have been regarded as animal-adapted lineages of *S. aureus*, and therefore *mecC*-MRSA could be considered as a new example of livestock (LA)-MRSA, with a zoonotic potential (Paterson et al., 2014). The study of *S. aureus* in animals is gaining relevance as it allows to follow the evolution of emerging methicillin-resistant strains, taking into consideration that animals can act as MRSA reservoirs and transmit them to humans and to other animals. The mechanism of adaptation of *S. aureus* to different hosts is not completely understood; nevertheless, there are immunologically relevant genes such as the immune evasion cluster (IEC) genes, whose presence suggests a possible human origin. It is worth noting that, occasional colonization and infections may however not require adaptation to a specific host, as shown for CC398 MRSA (Price et al., 2012). Studies about the presence of *S. aureus* in red deer (*Cervus elaphus*) are limited, and a low or absent prevalence of MRSA has been found (Meyer et al., 2014; Porrero et al., 2014a).

The objectives of this study were to determine the prevalence of *S. aureus* nasal carriage in red deer of a semi-extensive farm in Southern Spain where two wild small mammals harboring *mecC*-MRSA had been recently captured (Gómez et al., 2014), and to characterize obtained isolates. Humans in contact with the estate animals were also analyzed.

Materials and methods

Farm description

The study was performed in a semi-extensive red deer farm located in Cádiz (Southern Spain). The number of deer in the estate was 410 hinds and 72 stags. Deer were kept within large fenced (6-8 ha) enclosures in batches of approximately 60-80 females; males were kept in separate enclosures. Mention that, there are cattle and Iberian swine farms next to the deer farm, and the red deer also coexist with several wild species (such as *Oryctolagus cuniculus*, *Apodemus sylvaticus*, *Rattus rattus*, *Muss pretus, Crocidura russula*, *Mustela nivalis*, *Alectoris rufa*, *Anas platyrhynchos*, several passerine species as well as different species of reptiles). The farm is located in one of the main European pathways for bird migration between Africa and Europe, therefore several migrating bird species may come in contact directly or indirectly with farmed deer. The most representative migrating bird species are: *Pernis apivorus*, *Milvus migrans*, *Neophron percnopterus*, *Ciconia ciconia*, *Ciconia nigra*, *Circaetus gallicus*, *Circus pygargus*, *Circus aeruginosus*, *Accipiter nisus*, and *Pandion haliaetus*. The two wild mice (*A. sylvaticus*) in which *mecC*-MRSA was recently reported were also captured in this estate (Gómez et al., 2014).

Sample collection

Nasal swabs were collected randomly from 65 healthy deer (64 females and one male) of different ages (from 2 to 13 years old) in January 2014.

In addition, 15 nasal swabs were collected from human volunteers; 10 were from people in direct contact with the deer farm and other wild animals which sits alongside the farm; the remaining five samples were collected from staff of the Spanish Wildlife Research Institute (IREC) in contact with wild animals of the Iberian Peninsula, other than farmed deer. Before sampling, all volunteers gave their informed consent to participate in this study. All swabs were introduced in vials containing Amies agar gel medium (Copan, Murrieta, USA) and preserved refrigerated (4ºC) until analyzed.

Statistical analysis

Associated Clopper-Pearson 95% exact confidence intervals (95% CI) were estimated to assess for the statistical uncertainty of each of the proportions calculated. IBM SPSS v20.0 software was employed for statistical analyses.

S. aureus isolation and identification

Nasal swabs were inoculated into brain heart infusion broth (BD, France) with 6.5% NaCl and incubated at 37ºC for 24-48 h. Afterwards, 100 µL were seeded on mannitol salt agar (BD, France) plates and oxacillin resistance screening agar base (Oxoid, England) plates supplemented with oxacillin (2 mg/L). One to four colonies per plate with *S. aureus* morphology were recovered and initially identified by conventional methods (Gram staining, coagulase and DNase test). Identification of *S. aureus* was carried out by amplification of the *nuc* gene (Gómez et al., 2014). Only *S. aureus* strains showing different phenotypes of antimicrobial resistance of each sample and *spa*-type were further characterized.

Antimicrobial susceptibility testing and detection of antimicrobial resistance genes

Antimicrobial susceptibility testing to 16 antimicrobial agents (penicillin, oxacillin, cefoxitin, kanamycin, gentamicin, tobramycin, streptomycin, tetracycline, chloramphenicol, trimethoprim/sulfamethoxazole, erythromycin, clindamycin, ciprofloxacin, linezolid, mupirocin, and fusidic acid) was performed by disk-diffusion method (CASFM, 2010; EUCAST, 2014). Inducible or constitutive clindamycin resistance was determined by the double-disk diffusion test (D-test).

Presence of eight antimicrobial resistance genes [*mecA*, *mecC*, *blaZ*, *blaZ*-SCC*mec*XI (new *blaZ* allotype), *erm*(A), *erm*(B), *erm*(C), and *erm*(T)] was investigated by PCR (Cuny et al., 2011; García-Álvarez et al., 2011; Lozano et al., 2011).

Virulence genotype and detection of immune evasion cluster genes

The presence of the virulence genes *lukF/S*, *tst*, *cna*, *eta*, *etb*, *etd* and *etd2* was investigated by PCR (Lozano et al., 2011; Monecke et al., 2013a). Isolates were additionally tested by PCR for the presence of the IEC system genes (*scn*, *chp*, *sak*, *sea* or *sep*) (Gómez et al., 2014).

Molecular typing of S. aureus isolates

All *S. aureus* isolates were characterized by *spa* typing (www.ridom.com) and *agr* typing as previously described (Gómez et al., 2014). One deer isolate of each *spa*-type was selected as representative strain for molecular characterization by multilocus sequence typing (MLST) (www.mlst.net), and their Clonal Complexes (CCs) were achieved using eBURST analyses. The type of staphylococcal cassette chromosome *mec* (SCC*mec*) was studied by PCR in all MRSA isolates (Shore et al., 2011).

Analysis of the clonal relatedness of isolates

The genetic relationship among obtained MRSA isolates was performed by pulsed-field-gel-electrophoresis (PFGE) of total DNA restricted with *Sma*I enzyme, as previously described (Murchan et al., 2003). PFGE patterns were visually analyzed.

Isolates which showed indistinguishable band patterns were regarded as belonging to the same PFGE-type. Patterns with 1-3 different bands were considered as closely related and classified as PFGE-subtypes.

Results

S. aureus recovered from samples of red deer

S. aureus was isolated in nasal samples of 16 of 65 tested deer (24.6%, 95% CI 14.8-36.9). Isolates recovered from the same samples presented identical phenotypic characteristics. For this reason, only one *S. aureus* isolate per sample was maintained and further characterized. Eleven of the 16 *S. aureus* isolates obtained were MRSA, representing 16.9% (95% CI 8.8-28.3) of tested animals. The remaining five isolates were MSSA. Table 1 shows the characteristics of these isolates.

Characterization of MRSA isolates from red deer

All the 11 MRSA isolates showed resistance to beta-lactams, but susceptibility to the remaining tested antimicrobials. None were positive for the *mecA* PCR, but the *mecC* gene was detected in all of them. These *mecC*-positive isolates carried the SCC*mec* type XI, the resistance gene *blaZ*-SCC*mec*XI, the virulence gene *etd2*, and presented IEC type E. Four of the MRSA isolates were typed as *spa*-t843/*agr*-III and the remaining seven MRSA as *spa*-t1535/*agr*-III. The MLST was performed in two representative strains, one with *spa*-t843 (C7697) and the other with *spa*-t1535 (C7705). Both strains were ascribed to the sequence type ST1945, and assigned to CC130. The PFGE technique was applied to MRSA strains and all of them corresponded to the same PFGE pattern A, though two subtypes were identified. All t843 isolates corresponded to the same subtype A1 but in the isolates with *spa*-type t1535 two subtypes were identified (A1 and A2).

Characterization of MSSA isolates from red deer

The five MSSA isolates recovered from deer showed susceptibility to all 16 tested antimicrobials, and none carried the tested virulence genes or the genes of the IEC system. All of them were typed as *spa*-t2420/*agr*-I, and they were ascribed to CC133 based on *spa* type and MLST, performed in one representative strain (C7696) that showed ST133.

S. aureus recovered from samples of humans and characterization of isolates

Regarding human nasal swabs, *S. aureus* was recovered from 6 out of 15 tested (40%, 95% CI 16.33-67.72). Three carrier people had direct contact with the deer farm; the remaining three positive people were staff of IREC in contact with wild animals, other than farmed deer. All *S. aureus* from humans were MSSA. Table 2 shows the characteristics of these isolates. Three different *spa*-types (t002/CC5, t012/CC30 and t822/CC30) were detected among them, and all but one of the *S. aureus* isolates were ascribed to some IEC type (A, B, D or F).

Discussion

A high prevalence of red deer carriers of *S. aureus* and *mecC*-positive MRSA strains (24.6% and 16.9%, respectively) was detected. There are few studies on the prevalence of *S. aureus* in populations of red deer, and all of them were in free-living. Nevertheless, values observed in those studies were highly variable with ranges between 49% (Meyer et al., 2014) and 19.2 % (Porrero et al., 2014a). Regarding MRSA prevalence, to the best of our knowledge, only a Spanish study reported the detection of one MRSA isolate (presumably *mecA* positive) among 273 tested red deer (Porrero et al., 2013).

The 11 *mecC*-positive strains detected in our study presented two different *spa*-types (t843 and t1535), and belonged to closely-related PFGE-subtypes (A1/A2). This is the first report of MRSA carrying the *mecC* gene in red deer although it has been previously detected in other free ranging animals including other cervid species. In a Spanish study performed among wild animals, *mecC*-MRSA was detected in two fallow deer (*Dama dama*) and no *mecC*-positive MRSA was detected among 61 red deer (Porrero et al., 2014b). In that study the *spa*-type and ST/CC of *mecC*-MRSA strains (t11212/ST425/CC425) were different to those found in our study. As far as we know, only one study about the presence of *mecC*-MRSA in deer in other countries has been reported; in that case, the variant detected corresponded to ST130/CC130 in a fallow deer in Germany (Monecke et al., 2013b).

It is important to remark that in an earlier study performed by our group, *mecC*-positive MRSA strains were detected in fecal samples of two small wild mammals (*A. sylvaticus*) collected in the same estate where the deer lived (Gómez et al., 2014). The *mecC*-positive MRSA strains of small mammals presented identical characteristics to the majority of the *mecC* positive strains found in the present study: *spa*-type t1535, IEC-type E, and susceptibility to all antimicrobials except beta-lactams. In addition, these two mice isolates presented the same PFGE subtype A2 as one of the deer isolates (data not shown). The possibility that wild rodents, small carnivores and insectivores could be reservoirs and transmission vectors of MRSA, has been raised (Becker et al., 2014).

The fact of the transmission between different animal species has been already observed thanks to the use of whole-genome sequencing (Harrison et al., 2013) and the isolation of indistinguishable *mecC*-positive MRSA from wildlife and livestock sharing the same habitat has been documented (Loncaric et al. 2014).

On the farm studied, deer shared common places for eating and drinking, which could facilitate the spread of MRSA CC130 clone harboring the *mecC* gene among these animals. In fact, *mecC* strains with similar PFGE patterns were found among different deer individuals, indicating that deer-to-deer transmission is strongly suggested. It should be noted that farmed deer may be translocated for deer restocking purposes to big game estates, which supposes a potential transmission and spread of these *mecC*-MRSA strains to other ecosystems.

MRSA with the *mecC* gene has been also detected in humans, but the clinical cases caused by *mecC* isolates are still scarce. Recently some infections have been reported in Spain (Romero-Gómez et al., 2013; Cano et al., 2014; García-Garrote et al., 2014), and in other European countries, (García- Alvarez, 2011; Shore et al, 2011; Becker et al, 2014; Paterson et al, 2014).

The detection of the IEC-type E (*scn* and *sak*) among our *mecC*-positive deer isolates is relevant and raises questions about the origin of these apparently zoonotic isolates. These IEC genes were also present in *mecC*-MRSA detected from the two small mammals captured in the same estate (Gómez et al., 2014). To our knowledge, other than our group's studies, few investigations have determined the presence of IEC genes in *mecC*-MRSA and when tested, isolates have been negative (Cuny et al., 2011; Sabat et al., 2012; Monecke et al., 2013a; Paterson et al., 2014). It seems that the presence of IEC reflects the capacity to evade the human immune system, so, lack of IEC genes could be associated with an animal origin of strains. Our finding could be unexpected taking into account the dissemination of *mecC* gene in the animal environment and that CC130 has been considered as animal-adapted lineage. So, initially some authors have suggested that *mecC*-MRSA appeared possibly in ruminants (García-Álvarez et al.,

2011), though more studies are necessary to elucidate the animal origin of this clonal lineage and of the gene *mecC*.

Regarding the detection of MSSA isolates of the clonal complex CC133, this lineage is associated with ungulates, and was found in 5 of the 65 animals tested (7.7%, 95% CI 2.5- 17.1). All these isolates showed susceptibility to all antimicrobials, lacked the tested virulence genes and did not carry the genes of the IEC system, what could reflect an animal origin. ST133 was also detected in MSSA isolated from red deer and roe deer in other areas in Spain and Germany (Porrero et al., 2014a; Monecke et al., 2013b).

Six humans (being or not exposed to farmed deer) were carriers of *S. aureus* of different *spa*-types but all of them were MSSA. Among these strains we detected *spa*-types relating to CC5 and CC30, primarily associated with humans but both widely spread among humans and animals (McCarthy et al., 2012). However, we did not detect *mecC*-positive strains similar to those found in deer, and based on our results the animal-to-human transference could not be demonstrated.

In summary, red deer could be acting as *mecC*-MRSA hosts and could share these resistant microorganisms with other animals (such as small mammals) when they coexist in the same habitat. It is of interest the detection of the IEC-type E in these isolates when the IEC system seems to be more associated with adaptation to the human host. More studies should be performed in the future to analyze the evolution of *mecC*-MRSA in complex natural ecosystems.

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Table 1. Characteristics of the 16 *S. aureus* isolates recovered from deer [a].

Strain	Molecular typing						IEC genes (type)	Antimicrobial resistance phenotype[d]	Antimicrobial resistance genes	Virulence genes
	spa	*agr*	MLST	CC[b]	SCC*mec*	PFGE[c]				
C7697	t843	III	ST1945	**130**	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7702	t843	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7703	t843	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7704	t843	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7698	t1535	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7700	t1535	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7705	t1535	III	ST1945	**130**	XI	A2	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7707	t1535	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7708	t1535	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7709	t1535	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7711	t1535	III		130	XI	A1	*scn-sak* (type E)	PEN-OXA-FOX	*mecC-blaZ*-SCC*mec*XI	*etd2*
C7696	t2420	I	ST133	**133**		ND	-	Susceptible		-
C7701	t2420	I		133		ND	-	Susceptible		-
C7706	t2420	I		133		ND	-	Susceptible		-
C7710	t2420	I		133		ND	-	Susceptible		-
C7712	t2420	I		133		ND	-	Susceptible		-

[a] MLST, multilocus sequence typing; SCC*mec*, Staphylococcal cassette chromosome *mec*; CC, clonal complex; PFGE, pulsed field gel electrophoresis; IEC, immune evasion cluster.
[b] Bold letter: MLST was performed; Normal letter: CC presumptive according to *spa*-type.
[c] ND, not determined.
[d] PEN, penicillin; OXA, oxacillin; FOX, cefoxitin.

Table 2. Characteristics of the *S. aureus* isolates recovered from humans.

Strain	Molecular typing			IEC genes (type)[b]	Antimicrobial resistance phenotype[c]	Antimicrobial resistance genes	Virulence genes
	spa	*agr*	CC [a]				
C7690	t002	II	CC5	*scn-chp-sak-sep* (type F)	ERY-CLI (inducible)	*erm*(A)	-
C7692	t002	II	CC5	*sak*	Susceptible	-	-
C7693	t002	II	CC5	*scn-chp-sak* (type B)	PEN-ERY-CLI (inducible)	*blaZ-erm*(A)	-
C7691	t822	III	CC30	*scn-chp-sak-sea* (type A)	PEN-ERY-CLI (inducible)	*blaZ-erm*(A)	*tst-cna*
C7694	t012	III	CC30	*scn-sak-sea* (type D)	PEN	*blaZ*	*cna*
C7695	t012	III	CC30	*scn-sak-sea* (type D)	PEN	*blaZ*	*cna*

[a] CC: clonal complex presumptive according to *spa*-type
[b] IEC, Immune Evasion Cluster.
[c] PEN, penicillin; ERY, erythromycin; CLI, clindamycin.