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

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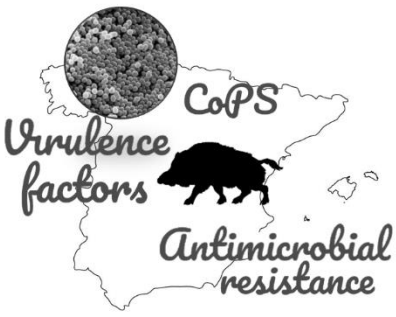
GRAPHICAL ABSTRACT

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



Abstract

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

1. Introduction

Coagulase positive *Staphylococcus* (CoPS) are part of the natural microbiota of skin and mucous membranes of humans and animals (Seinige *et al.* 2017). The *S. aureus*, *S. pseudintermedius* and *S. hyicus* (coagulase variable) species are included in the CoPS group, among others (Casanova *et al.* 2011).

S. aureus frequently colonizes humans and livestock (specially pigs), and can cause opportunistic infections and foodborne bacterial intoxications (Normanno *et al.* 2007; Porrero *et al.* 2014). Most of the *S. aureus* molecular characterizations have been focused on human and farm/domestic animal isolates (Seinige *et al*. 2017), but few data do exist about *S. aureus* isolates of wild animals. Since 2005, methicillin resistant *S. aureus* (MRSA) belonging to the lineage ST398, mostly of *spa*-type t011, was described as colonizer of pigs, humans exposed to pig farming, and other farm species, and was therefore designed as livestock associated (LA) MRSA, being implicated in human farm-

related infections (Pantosti 2012); this MRSA lineage shows in most cases tetracycline resistance. According to few reports, LA-MRSA of lineage CC398 might have evolved from MSSA-CC398 of human origin; the jump to livestock would have made it loose its human immune system adaptation genes (IEC), and acquire resistance to methicillin and tetracycline (Price *et al.* 2012). It has been reported in New York, that most of the CC398 strains involved in human infections were typed as t571, mostly sensitive to methicillin and tetracycline, properties that are uncommon in pig isolates (Smith and Wardyn 2015). Furthermore, other *S. aureus* clonal lineages such as CC130, CC599, CC59, CC1943 and CC425 are animal-adapted lineages, causing infections in animals and zoonoses in humans (Becker *et al.* 2014).

S. pseudintermedius (SP), is usual colonizer of dogs and to a lesser extend of cats (Perreten *et al.* 2010; Paul *et al.* 2012; Gómez-Sanz *et al.* 2013b, 2014; Stull *et al.* 2014). Besides causing skin infections and post-operative infections in pets, *S. pseudintermedius* is also responsible for some human infections (Börjesson *et al.* 2015; Grönthal *et al.* 2015; Somayaji *et al.* 2016; Lozano *et al.* 2017). The presence of *S. pseudintermedius* in humans suggests a transmission from dogs to people generally in contact with the mentioned animals; a low frequency of *S. pseudintermedius* carriage is generally observed in humans with no-pet contact, while higher rates are seen in people with dog-contact (Gómez-Sanz *et al.* 2013a). Although its detection in wildlife animals is scarce, it has been found in few cases. In fact, according to a report in 2012 of the Dutch Wildlife Health Centre (DWHC 2012), *S. pseudintermedius* was isolated from the meninges of a male fox found dead in woodlands in Germany. It has also been detected in healthy foxes in Denmark and Poland (Guardabassi *et al.* 2012; Nowakiewicz *et al.* 2016). Otherwise, *S. pseudintermedius* resistant to methicillin (MRSP) has emerged since the last decade, and its specific genotypes are mostly recovered from infections sites in dogs and cats (Perreten *et al.* 2010), but also in equines (Gómez-Sanz *et al.* 2014). In addition, in Canada, five MRSP isolates were detected from wild Norway rats trapped in an impoverished neighbourhood of Vancouver (Himsworth *et al.* 2013).

S. hyicus is a coagulase-variable species which has been found in animals with septic polyarthritis and bovine mastitis, and can cause exudative epidermitis in pigs and sepsis in humans (Taponen and Pyörälä 2009; Casanova *et al.* 2011). Data of *S. hyicus* from wildlife are really scarce; a single case has been reported in Spain, where *S. hyicus* was

isolated from both lung and skin of a young wild boar affected by epidermitis (Pérez *et al.* 2013).

Staphylococcus species have acquired resistance to antibiotics for decades. According to the World Health Organization (WHO) reports on Antibiotic Resistance (2014) and Antibiotic Consumption Surveillance (2018), the emergence of antimicrobial-resistant bacteria is a worldwide public health concern, including MRSA (Dickmann *et al.* 2017), due to limited options for treatment, although also implicate economic constraints (Antonanzas *et al.*, 2015). Nevertheless, since little is known about molecular epidemiology of CoPS in free-living wild animals, this study aimed to determine the frequency and diversity of CoPS in wild boar nasal samples, to analyse their resistance phenotypes and genotypes and to check the possible dissemination of the LA clonal lineage CC398 to wild animals.

2. Material and methods

2.1. Sample collection

Nasal samples of 371 wild boars were collected with aseptic swabs in commercial hunting events (known as "monterías") during a hunting period (october-december 2016) from six Spanish regions [Toledo (n=123), Ciudad Real (n= 62), Huelva (n=72), Sevilla (n=71), Cádiz (n=21), and Cáceres (n=22)] by the SaBio group of the Hunting Resources Research Institute (IREC /CSIC-UCLM-JCCM), Ciudad Real (Spain). Animals were not particularly hunted for this study; SaBio group assisted to "monterías" for sample collection. Swabs included in Amies (Copan, Murrieta, USA) transport medium were immediately transported in ice to the University of La Rioja for processing.

2.2. CoPS isolation and identification

The samples were pre-enriched in tubes with 5ml of Brain Heart Infusion (BHI) broth (Conda, Spain) supplemented with NaCl 6.5%, and incubated at 37ºC for 24h. Aliquots were later inoculated on Mannitol Salt Agar (MSA) (Conda, Madrid, Spain), and Oxacillin Resistant Screening Agar Base (ORSAB) (Oxoid, Hampshire, England), supplemented with 2 µg/ml oxacillin) media, and incubated at 37ºC for 24h.

Up to two colonies per plate with staphylococci morphology were chosen and streaked on BHI Agar plates (Scharlab, Barcelona, Spain). After incubation at 37ºC for 24h, the colonies were identified using the matrix-assisted laser desorption/ionization time of

flight (MALDI-TOF) mass spectrometry (Bruker, Massachusetts, USA). To confirm the identification of *S. pseudintermedius*, digestion of *pta* gene amplicon with *Mbo*I endonuclease was performed by PCR-restriction fragment length polymorphism (RFLP) (Bannoehr *et al.* 2009). The capacity to produce coagulase was studied in *S. hyicus* isolates by tube-test using lyophilized rabbit plasma (BIOMÉRIEUX, Lyon, France). CoPS isolates were maintained and further characterized.

2.3. Molecular Typing and virulence study

All the isolates belonging to *S. aureus* and *S. pseudintermedius* species were typed (*spa* and *agr*) as previously described (Lozano *et al.* 2012; Moodley *et al.* 2009; Perreten *et al.* 2010). Multilocus sequence typing (MLST) was performed for 28 representative strains (one of each *spa* types detected, and all strains with new *spa* types). For this purpose, PCR and partial sequencing of seven housekeeping genes for *S. aureus* and *S. pseudintermedius* were performed (www.pubmlst.org), to determine the sequence type (ST) and the clonal complex (CC), as previously described (Lozano *et al.* 2012). The virulence genes studied were *lukF/lukS-PV, tst, eta,* and *etb* for *S. aureus* (Gómez *et al.* 2016a), and *lukS/F-I, siet, expA, expB and* sec_{canine} for *S. pseudintermedius* isolates (Gómez-Sanz *et al.* 2011; Iyori *et al.* 2011). Furthermore, the gene *scn* belonging to the human Immune evasion cluster (IEC) was studied for *S. aureus* isolates (Wamel *et al.* 2006; Benito *et al.* 2014).

2.4. *Antibiotic susceptibility testing and antibiotic resistance gene detection*

The susceptibility to penicillin, cefoxitin (oxacillin in the case of *S. pseudintermedius*), gentamicin, tobramycin, tetracycline, chloramphenicol, erythromycin, clindamycin, ciprofloxacin, linezolid, and trimethoprim/sulfamethoxazole was analysed by disk-diffusion method (EUCAST, 2018). In addition, streptomycin susceptibility was also tested (CASFM, 2018).

The detection of the following antimicrobial resistance genes was performed by PCR, according to the phenotype of resistance: beta-lactams (*blaZ, mecA,* and *mecC*), tetracycline [*tet*(K), *tet*(M), and *tet*(L)], and streptomycin [*str*, and *ant*(6)-Ia] (Benito *et al.* 2014).

3. Results

Among the 371 samples of wild boar analysed, 66 of them carried CoPS isolates (17.8%). A total of 67 CoPS isolates were identified (one isolate per sample, except one sample for which one *S. aureus* and one *S. pseudintermedius* were obtained) and belonged to three distinct species: *S. aureus* (n=51), *S. pseudintermedius* (n=6), and *S. hyicus* (n=10). The identification of the *S. pseudintermedius* isolates was confirmed by the PCR-RFLP. *S. hyicus* was recovered from 4 additional samples, but they were excluded because they showed a coagulase-negative test.

Twenty-two different *spa* types were detected among the 51 *S. aureus* isolates, including three new *spa*-types registered as t16740, t16741 and t16809 (Table I). The MLST was performed for all the strains with new *spa* types (3 strains), and for one strain per each *spa* type detected (19 strains). Seventeen different STs were detected among the 22 tested strains, three of them presented one new allele sequence and were then registered as ST4368, ST4372 and ST4373. Other relevant STs were identified, as the following ones: ST398/CC398 (n=1), ST2328/CC133 (n=15), ST133/CC133 (n=5), ST425/CC425 (n=7), ST5/CC5 (n=5), ST1/CC1 (n=3), ST130/CC130 (n=2), and ST88/CC88 (n=1), among others. The clonal complex CC133 was the most frequently detected, including two sequence types and three *spa*-types (Table I).

The antimicrobial resistance phenotype and genotype and the *agr* results for *S. aureus* isolates are shown in Table I. Most of strains (n=38, 74.5%) were susceptible to all tested antimicrobials, and all but one strains, were methicillin-susceptible (MSSA). The unique MRSA strain was typed as ST398, t011, and *agr*-I, and showed resistance to penicillin and tetracycline, containing the *blaZ*, *mecA*, *tet*(M) and *tet*(K) genes. Ten *S. aureus* strains were resistant to penicillin (19.6%) and nine of them carried the *blaZ* gene. Five isolates showed resistance to streptomycin (9.8%), and all of them were *str*-positive. None of the *S. aureus* isolates harboured the *scn* gene of the IEC system, and two of them harboured either *luk-E* (strain of lineage ST1) or *luk-Dv* gene (strain of lineage ST88). These *luk*-E and *luk*-Dv genes were amplified when *lukF/lukS-PV* primers were used (while checking PVL genes) and were identified by sequencing.

S. pseudintermedius isolates were detected in six of the 371 wild boars tested (1.6%), and the results of the isolates are shown in Table II. These *S. pseudintermedius* isolates were submitted to MLST and *spa*-typing. In relation to MLST, one of the strains was ascribed to the sequence type ST455, and the other five strains presented new allele combinations, with three new STs registered as ST797 (n= 3), ST796 and ST798 (n=1, each one). Two

of the isolates were ascribed to *spa* types t02 and t15, and the remaining isolates could not be *spa*-typed. All the strains were typed as either *agr*II or *agr*III. Five of the strains showed streptomycin resistance (harbouring the *str* gene), one tetracycline resistance [carrying *tet*(M) and *tet*(L) genes] and one penicillin resistance. All *S. pseudintermedius* strains harboured the *lukS/F-I* and *siet* virulence genes.

Finally, *S. hyicus* isolates were detected in 14 of the 371 samples analyzed. However, four of them were negative to the coagulase test and the remaining 10 exhibited a positive coagulase test and were further characterized. All coagulase-positive *S. hyicus* isolates were susceptible to all tested antimicrobial agents.

4. Discussion

According with the results of this study, different CoPS species can colonize the nasopharynx of wild boar, being *S. aureus* the most frequent one (13.7% of tested animals), but *S. pseudintermedius* and *S. hyicus* species were also detected.

The *S. aureus* carriage rate detected in our study is in accordance with previous results obtained in this animal species in Spain (17.8%) (Porrero *et al.* 2014), but lower than data obtained in Germany (45.5%) (Seinige *et al*. 2017); nevertheless, a lower carriage rate was found in wild boar carcasses or lymph nodes (2%-3.2%) in Italy (Traversa *et al.* 2015). Differences in carriage rates could reflect different colonization level in the distinct countries, but it cannot be discarded the effect of different methodologies used for *S. aureus* recovery. *S. aureus* is frequently detected in other wild animals (Porrero *et al.* 2014; Mrochen *et al.* 2018).

A high genetic diversity was detected among our *S. aureus* isolates (17 STs and 22 *spa* types), as also found in other studies of wild boar (Porrero *et al*., 2014; Seinige *et al*. 2017). The lineage ST2328/CC133, mostly associated to *spa*-type t3750, was the most frequently detected among *S. aureus* (all MSSA), not only in the present study (29.4%), but also in others (42.4%) (Porrero *et al*., 2014). That *spa*-type t3750 has been observed in 23% of *S. aureus* recovered from wild boar in Portugal (also MSSA), linked to the sequence types ST2328 and ST3220 (Sousa *et al.* 2017); however, this lineage was not found in another study performed in Germany (Seinige *et al.* 2017), in which ST1/t127 clone was predominant. On the other hand, other lineages identified in the present study, specially ST130 and ST425, seem to be frequent colonizers of wild animals, including

wild boar (Meemken *et al.* 2013; Monecke *et al.* 2016; Seinige *et al*. 2017; Gómez *et al.* 2014, 2015, 2016b).

MRSA ST398/CC398-t011, emergent livestock-associated lineage frequently detected in pigs (Gómez-Sanz *et al.* 2010; Lozano *et al.* 2012; Luzzago *et al.* 2014; Heikinheimo *et al.* 2016), was identified in the course of this study in one sample of a wild boar (1.9%), although it is scarcely detected in wildlife (Lozano *et al.* 2012; Monecke *et al.* 2016; Tegegne *et al*. 2017). This wild boar was hunted in a region in the South of Spain in which many Iberian pig farms are located. According to a previous study on pigs from Spanish slaughterhouses, 28% of the Iberian pigs were colonized by MRSA (most of them CC398), and a higher prevalence was found in white pigs (83%) (Porrero *et al.* 2012). The prevalence detected in wild boars in our study, is lower than the one referred for Iberian and white pigs. The ST398 strain obtained in our study harboured the *mecA*, *blaZ*, *tet*(M) and *tet*(K) genes, but not *scn* gene. An MRSA ST398/t899 was detected in one wild boar of 45 tested (2.2%) in a previous study in Portugal (Sousa *et al*. 2017), and, contained the *scn* gene (IEC-type B system), which suggests a human origin, contrarily to our strain. In addition, 20 MRSA isolates CC398/ (t011, t034, t1456, t1250) were detected from wild boar fresh meat in Germany (Kraushaar and Fetsch 2014). The CC398 lineage was also found in an European brown hare (Monecke *et al.* 2016), and in urban wastewater (Gómez *et al.* 2016a). These results lead us to think that the ST398 lineage has been spreading from the farm to the wild area, and make us be aware of the global concern of antimicrobial-resistance dissemination to different ecosystems.

A high rate of susceptibility to antibiotics was observed among *S. aureus* isolates. While its resistance to methicillin is still rare in wild boars (Seinige *et al*. 2017), resistance to penicillin, streptomycin and tetracycline is common in isolates of free-living wild animals, including wild boars (Porrero *et al.* 2014). Two MSSA isolates, ST1/t127 and ST88/t1951, harboured the virulence genes *luk-E* and *luk-Dv* respectively, that have been previously detected in wild boar *S. aureus* isolates (CC97, CC133, ST425) (Monecke *et al.* 2016). The lack of the *scn* gene of the human IEC system, point to an animal origin of the strains.

To our knowledge, the species *S. pseudintermedius* and *S. hyicus* have not been previously identified in wild boar, except for a case of pyoderma caused by a *S. hyicus* strain in a wild boar (Perez *et al*. 2013). *S. pseudintermedius*, frequent colonizer of dogs and cats (Perreten *et al.* 2010), has been nonetheless detected in other wild animals, such

as foxes (Nowakiewicz *et al.* 2016; Guardabassi *et al.* 2012), confirming that this species colonizes Canidae and Felidae. Its characteristics, including coagulase and exfoliative toxin production, make it become a great cause of zoonosis, leading to skin and soft tissues infections in humans (Somayaji *et al.* 2016; Lozano *et al.* 2017; Börjesson *et al.* 2015). In the present work, *S. pseudintermedius* was identified in 1.6% of the analysed wild boars, what suggests that it might be spread out more in wildlife. The *spa* type t02 detected among our *S. pseudintermedius* isolates was frequent among MRSP from dogs, although with different STs (Perreten *et al.* 2010; Ventrella *et al.* 2017).

S. hyicus can be responsible for exudative epidermitis in pigs (Casanova *et al.* 2011) or bovine mastitis (Taponen and Pyörälä 2009). However, *S. hyicus* is rarely found in wildlife animals (Nowakiewicz *et al.* 2016). In our study, 2.7% of the tested wild boar carried coagulase positive *S. hyicus* isolates. *S. hyicus* was previously obtained of a wild boar affected by exudative epidermitis in a game estate in Spain (Pérez *et al.* 2013). That disease generally occurs in intensive pig farms, but since intensive wild boar game estates are being developed (Pérez *et al.* 2013), the prevalence of *S. hyicus* in this animal species might be higher than expected.

In conclusion, *S. aureus*, and in a lower level *S. pseudintermedius* and *S. hyicus*, are colonizers of wild boars. Wild boars seem to be a source for the lineage LA-SARM CC398 and is frequently colonized by MSSA isolates of the lineages CC130, CC133 and CC425. Furthermore, a high genetic diversity of *S. aureus* and *S. pseudintermedius* was found among recovered isolates.

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Table I: Phenotypic and genotypic characteristics of *S. aureus* isolates recovered from wild boar

Number of isolates	CC	ST (*Nº strains*)	*spa*-type (*Nº strains*)	*agr*	Antimicrobial resistance		Virulence genes (Nº strains)	*scn* gene
					Phenotype[c]	Genotype		
15	CC133	ST2328 (13)	t3750	III	Susceptible		-	-
		ST2328 (1)	t3750	III	PEN	-	-	-
		ST2328 (1)	t16741[b]	III	Susceptible		-	-
5	CC133	ST133 (1)	t3583	I	TET	-	-	-
		ST133 (4)	t3583	I	Susceptible		-	-
7	CC425	ST425	t742 (*1*), t11232 (*3*), t6292 (*1*), t11212 (*2*)	II	Susceptible		-	-
5	CC5	ST5 (2)	t002	II	PEN-STR	*bla*Z, *str*	-	-
		ST5 (1)	t002	II	PEN	*bla*Z	-	-
		ST5 (1)	t002	II	Susceptible		-	-
		ST5 (1)	t1094	II	PEN	*bla*Z	-	-
3	CC1	ST1 (1)	t127	III	PEN-STR	*bla*Z, *str*	-	-
		ST1 (2)	t127	III	Susceptible		*luk*-E (1)	-
2	CC130	ST130	t843	III	Susceptible		-	-
2	-	ST3224	t12923	IV	PEN	*bla*Z	-	-
1	**CC398**	**ST398**	**t011**	**I**	**PEN- FOX- TET**	***bla*Z, *mec*A, *tet*(M), *tet*(K)**	-	-
1	CC49	ST49	t208	II	Susceptible		-	-

1	CC88	ST88	t1951	III	Susceptible		*luk*-Dv	-
1	CC97	ST352	t1200	II	Susceptible		-	-
1	-	ST2681	t073	I	STR	*str*	-	-
1	-	ST2681	t073	I	Susceptible		-	-
1	-	ST4372[a]	t12827	III	PEN	*blaZ*	-	-
1	-	ST4372[a]	t12827	III	Susceptible		-	-
1	-	ST4368[a]	t16740[b]	III	STR	*str*	-	-
1	-	ST4373[a]	t16809[b]	III	Susceptible		-	-
1	-	ST1259	t548	II	Susceptible		-	-
1	-	ST2677	t3293	II	Susceptible		-	-

[a] new ST

[b] new *spa*-type

[c] PEN: penicillin; FOX: cefoxitin; TET: tetracycline, STR: streptomycin

Table II: Phenotypic and genotypic characteristics of *S. pseudintermedius* isolates recovered from wild boar

Strain	MLST	*agr*	*spa*-type	Antimicrobial resistance		Virulence genes
				Phenotype[b]	Genotype	
C9430	ST796[a]	III	NT	TET-STR	*tet*(M), *tet*(L), *str*	*luk S/F-I, siet*
C9614	ST797[a]	II	t02	STR	*str*	*luk S/F-I, siet*
C9431	ST797[a]	II	NT	STR	*str*	*luk S/F-I, siet*
C9432	ST797[a]	II	NT	STR	*str*	*luk S/F-I, siet*
C9711	ST798[a]	III	t15	PEN-STR	*str*	*luk S/F-I, siet*
C9712	ST455	III	NT	Susceptible	-	*luk S/F-I, siet*

[a] new ST

[b] PEN: penicillin; TET: tetracycline, STR: streptomycin

NT: non-typeable