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

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ABSTRACT

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

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

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

1. Introduction

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

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

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Table 1
Phenotypes and genotypes of antimicrobial resistance in 161 CoNS isolates of wild boar.

Species	Number of isolates (total = 161)	Antimicrobial resistance phenotype[a] (nº of strains)		Genes detected (nº of strains)
S. sciuri	64	CLI	(6)	*lnu*(A) (3)
		TET	(4)	*tet*(K) (4)
		ERY-CLI (inducible)	(3)	*erm*(C)-*erm*(43)-*lnu*(A) (1)
				erm(F)-*erm*(43)-*mph*(C) (1)
				erm(A)-*erm*(43) (1)
		CLO	(1)	*fexA* (1)
		GEN	(1)	–
		TET-CLI	(1)	*tet*(K)-*tet*(L)-*lnu*(A) (1)
		Susceptible	(48)	
S. xylosus	21	TET	(2)	*tet*(K) (2)-*tet*(L) (1)
		Susceptible	(19)	
S. chromogenes	17	Susceptible	(17)	
S. equorum	13	TET	(2)	*tet*(K) (2)
		ERY	(2)	*msr*(A)/*msr*(B) (2)
		Susceptible	(9)	
S. vitulinus	13	TET	(1)	*tet*(K) (1)
		FOX	(1)	*mecA* (1)
		Susceptible	(11)	
S. fleurettii	8	CLI	(1)	–
		FOX	(2)	*mecA* (2)
		Susceptible	(5)	
S. succinus	4	Susceptible	(4)	
S. hyicus	4	Susceptible	(4)	
S. saprophyticus	3	TET-CLI	(1)	*tet*(K)-*lnu*(A) (1)
		TET-ERY-CLO	(1)	*tet*(K)-*mrs*(A)/*msr*(B)-cat_{pC221} (1)
		Susceptible	(1)	
S. simulans	3	TET	(1)	*tet*(K) (1)
		Susceptible	(2)	
S. lentus	3	TET-CLI	(1)	*tet*(K) (1)
		ERY-CLI (inducible)	(1)	*erm*(43)-*lnu*(A) (1)
		Susceptible	(1)	
S. cohnii	2	TET-ERY-CLI	(1)	*tet*(K)-*erm*(B) (1)
		TET	(1)	*tet*(K) (1)
S. haemolyticus	2	FOX-SXT	(1)	*mecA-dfrG* (1)
		Susceptible	(1)	
S. hominis	1	TET	(1)	*tet*(K) (1)
S. epidermidis	1	Susceptible	(1)	
S. simiae	1	Susceptible	(1)	
S. felis	1	Susceptible	(1)	

[a] CLI, clindamycin; CLO, chloramphenicol; ERY, erythromycin; FOX, cefoxitin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

2. Material and methods

2.1. Coagulase-negative staphylococci isolation and identification

Nasal samples of 371 wild boars were collected in a previous study during a hunting period (2016) from six Spanish regions [15]. In the present study, these samples were tested for CoNS recovery. The samples were pre-enriched in Brain Heart Infusion broth (BHI, Conda, Spain) broth supplemented with NaCl 6.5% and incubated at 37 °C for 24 h. Aliquots were later inoculated on Mannitol Salt Agar (MSA, Conda, Spain), and Oxacillin Resistant Screening Agar Base (ORSAB, Oxoid, England, supplemented with 2 μg/mL oxacillin) media, and incubated at 37 °C for 24 h.

Up to three colonies per plate with staphylococci morphology were chosen and streaked on BHI agar plates (Scharlau, Spain). After incubation at 37 °C for 24 h, the colonies were identified using the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker). Because *S. hyicus* is coagulase-variable, its capacity to produce coagulase was studied by tube-test using lyophilized rabbit plasma (BIOMÉRIEUX). CoNS isolates were maintained and further characterized in this study.

2.2. Antimicrobial susceptibility testing and antimicrobial resistance genes detection

The susceptibility to cefoxitin, gentamicin, tobramycin, tetracycline, chloramphenicol, erythromycin, clindamycin, and trimethoprim/sulfamethoxazole, was performed by the disc-diffusion method [16] for all the recovered isolates. The antibiotics considered in this work were among those included for disk diffusion method by EUCAST [16], and for which acquisition of resistance genes can occur by horizontal transfer mechanisms. All CoNS isolates recovered from each sample showing different species and/or different antibiotic resistance phenotype were maintained for further studies.

The detection of the following antimicrobial resistance genes was performed by PCR, according to the phenotype of resistance: beta-lactams (*mecA,* and *mecC),* tetracycline [*tet*(K)*, tet*(L), and *tet*(M)], aminoglycosides [*aac*(6′)-Ie–*aph*(2″)-Ia], macrolides- lincosamides [*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(Y)*, erm*(T), *erm*(43), *mph*(C), *msr*(A)/*msr*(B)*, lnu*(A)*, lnu*(B)*, vga*(A)], trimethoprim (*dfrA*, *dfrD*, *dfrG*, *dfrK*), and phenicols (cat_{pC194}*,* cat_{pC221}*,* cat_{pC223}*, cfr, fexA* and *fexB*) [17–23]. Besides, for the isolates resistant to chloramphenicol, two additional novel genes were tested: the gene *optrA* because it has been detected in enterococci and *S. aureus* and confers combined resistance to oxazolidinones and phenicols [24,25], and the gene *poxtA* whose expression in *S. aureus* and *E. faecalis* was able to decrease susceptibility to phenicols, oxazolidinones and tetracyclines [26]. Positive and negative control strains of the Universidad de La Rioja have been included in all PCR reactions.

3. Results

3.1. Diversity of CoNS species recovered from wild boars

Among 371 animals tested, 206 were positive to *Staphylococcus* spp. (55.5%), both coagulase-positive *Staphylococcus* (CoPS) (17.8%) and

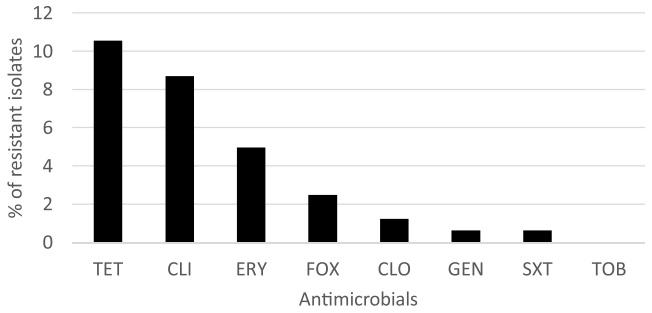

Fig. 1. Frequency of antimicrobial resistance among 161 CoNS isolates of wild boars. CLI, clindamycin; CLO, chloramphenicol; ERY, erythromycin; FOX, cefoxitin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TOB, tobramycin.

CoNS (37.7%). From 140 CoNS-carrier animals found, 161 CoNS isolates (one to three isolates of distinct species per animal, except for two animals where two isolates of the same species but with different antimicrobial resistance phenotype were recovered) were obtained and included in this study. The following patterns of coexistence of species were found among the positive animals (number of animals): *S. sciuri/S. equorum* (2); *S. sciuri/S. xylosus* (2); *S. sciuri/S. xylosus/S. simulans* (1); *S. sciuri/S. fleurettii* (1); *S. sciuri/S. hominis* (1); *S. sciuri/S. succinus* (1); *S. sciuri/ S. succinus/ S. fleurettii* (1); *S. sciuri/S. vitulinus* (1); *S. vitulinus/ S. xylosus* (2); *S. equorum/S. xylosus* (2); *S. equorum/S. cohnii* (1); *S. saprophyticus/ S. chromogenes/ S. cohnii* (1). Seventeen distinct species were identified in total, with predominance of *S. sciuri* (n = 64), *S. xylosus* (n = 21) and *S. chromogenes* (n = 17), followed by *S. equorum* (n = 13), *S. vitulinus* (n = 13) and *S. fleurettii* (n = 8). Other 11 CoNS species were detected in less frequency, as it is shown in Table 1.

3.2. Antimicrobial susceptibility of CoNS isolates

The resistance phenotype and genotype of the isolates are summarized in Table 1. Up to 125 of the 161 CoNS isolates were susceptible to all the tested antimicrobial agents (77.6%), with a 100% of susceptibility for the following species: *S. chromogenes*, *S. succinus*, *S. epidermidis*, *S. simiae, S. hyicus* (coagulase negative), and *S. felis*. The remaining 36 isolates showed resistance to at least one of the tested agents (22.4%), including one isolate presenting a multidrug resistance phenotype (*S. saprophyticus*) being resistant to three antimicrobial families: tetracycline, macrolides and phenicols. The following resistance rates were observed: tetracycline (10.5%), clindamycin (8.6%), erythromycin (4.9%), cefoxitin (2.5%), chloramphenicol (1.2%), gentamicin (0.6%), and trimethoprim/sulfamethoxazole (0.6%) (Fig. 1). Tetracycline-resistance was mediated by *tet*(K) gene in all cases and associated with *tet*(L) gene in two isolates, and erythromycin-resistance by either *msr*(A)/*msr*(B), *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(43) or *mph* (C) genes. All the isolates with phenotype of resistance to erythromycin and inducible resistance to clindamycin harbored the gene *erm*(43). None isolates carried the *erm*(T) gene. The *lnu*(A) gene was identified in six clindamycin-resistant *S. sciuri*, *S. saprophyticus*, and *S. lentus* isolates, and the *dfrG* gene in one trimethoprim-sulfamethoxazole (SXT) resistant *S. haemolyticus* isolate. Four *S. fleurettii*, *S. haemolyticus* or *S. vitulinus* isolates were methicillin-resistant and carried the *mecA* gene. Besides, the *fexA* and cat_{pC221} genes were detected respectively in two chloramphenicol-resistant *S. sciuri* and *S. saprophyticus* isolates, both susceptible to linezolid and negative by PCR for *optrA*, *poxtA*, *fexB* and *cfr* genes.

4. Discussion

CoNS are considered in general, less pathogenic than CoPS and thus, they have received less attention in the past. Nevertheless, they are being increasingly recognized as opportunistic pathogens in humans and animals, and they are receiving more consideration in the last years [9,27], mostly in isolates of humans [8,28,29] but also in those of livestock and food [6,7,9,30]. Nonetheless, still scarce information does exist about CoNS in wild animals [11], including wild boars.

Wild boars are known to be a reservoir for *S. aureus* [14,31] and other CoPS as *S. pseudintermedius* [15,32]. Besides, CoNS have been isolated during the fermentation of wild boars sausages in Croatia, although they were not identified to the species level [33]. In the present study, it has been detected a high diversity of species (n = 17) among CoNS recovered from wild boars, with predominance of *S. sciuri* and *S. xylosus* (53%), followed by *S. chromogenes, S. equorum* and *S. vitulinus,* representing all of them almost 80% of total isolates. *S. sciuri* and *S. xylosus* were also the most frequent CoNS species (93%) in wild tree-frogs fecal samples [13]. Moreover, *S. sciuri* was also the predominant CoNS species found among pig and dog isolates in Nigeria [34,35]. The species *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus,* recognized as opportunistic pathogens in human infections [11,29], represented 4% of the total CoNS recovered from wild boars in the present study. *S. epidermidis*, *S. saprophyticus*, and *S. sciuri*, among others, were isolated from birds of prey in Portugal [11].

Previous studies have shown the frequent detection of antimicrobial resistant isolates among CoNS recovered from human infections [36,37] or in the hospital environment [38], and some authors postulate that CoNS could be a reservoir of important resistance genes that could be transferred to *S. aureus* isolates [39,40]. According with data of 2015 of the Prevalence study of nosocomial infections in Spain (EPINE), CoNS were responsible of 6.2% of total infections [41]; three multicentric studies showed a decrease of the antibiotic resistance rates of CoNS from 2006 to 2014, although the percentages in 2014 were still relatively high to beta-lactams (74%), macrolides-lincosamides (40%), and aminoglycosides (40%) [41]. In addition, in a study on staphylococci from fecal samples of healthy children in Spain, CoNS isolates showed high level of resistance to beta-lactams (83.6%), macrolides-lincosamides (69.4%), and aminoglycosides (52.7%), although resistance to tetracycline and chloramphenicol was observed in few isolates [42]. In the present study, a high proportion of CoNS isolates showed susceptibility for all tested antimicrobials (77.6%), being our antimicrobial resistance rates very low compared to those detected in clinical or healthy human isolates. The less antibiotic pressure that probably occurs in natural environments than in hospitals and human habitats could account for this situation.

Nevertheless, relevant antimicrobial resistance genes were detected among the remaining 36 isolates that showed resistance for at least one of the antimicrobials tested. The most frequent resistance genes detected were those related to tetracycline [*tet*(K)], lincosamides [*lnu*(A)] and cefoxitin/methicillin resistance (*mecA*), although resistance to other agents were also found, as those for chloramphenicol (*fexA* and *cat*), trimethoprim (*dfrG*) or macrolides-lincosamides [*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(43), *mphC* and *msr*(A)/*msr*(B)]. All the isolates with resistance to erythromycin and inducible resistance to clindamycin harboured the gene *erm*(43) associated to another gene implicated in that resistance phenotype except the *S. lentus* isolate which only harbored *erm*(43). In fact, that gene was first described in a *S. lentus* isolate of dog origin which was negative for the previously known *erm* genes; it was later found in chicken and human *S. lentus* isolates also lacking other *erm* genes [23]. CoNS strains isolated from wild boar meat fermented sausages were all susceptible to penicillin, oxacillin, gentamicin and streptomycin while resistance to tetracycline, erythromycin and lincomycin was observed [33]. Beta-lactams, tetracyclines, lincosamides and sulphonamides are antimicrobial families very frequently used in the animal field (food-producing animals and pets) [10], whereby residues of antimicrobials or resistant bacteria could arrive to the natural environments [43].

The *lnu*(A) gene was detected in six isolates of three different CoNS species (*S. sciuri*, *S. saprophyticus*, and *S. lentus*). This gene has been

previously detected in *S. sciuri* [44], *S. saprophyticus*, *S. chromogenes*, *S. haemolyticus*, *S. simulans*, and *S. epidermidis* [45]. The *lnu*(A) gene seems to be located in small mobilizable plasmids which are likely transferred easily between different staphylococcal species [44,45]. So far it is an unusual gene that codifies a lincosamide nucleotidyltransferase conferring resistance to this important family of antimicrobials for human and animal medicine [46]. It is mostly detected in animal staphylococci (both CoNS and CoPS), as was the case for nine CoNS isolates from bovine mastitis in Germany [45], and methicillin-resistant *S. aureus* (MRSA) isolates of CC398 lineage from pigs in Spain [47].

Among the tested antimicrobials, tetracycline presented the highest resistance percentage (10.5%). Tetracycline resistance was always mediated by the *tet*(K) gene, alone or associated with *tet*(L) gene. The *tet*(K) was also the responsible gene of tetracycline-resistance in CoNS recovered from birds of prey [11], healthy dogs [35] and mastitis buffaloes' milk in Egypt [48]. These results suggest that *tet*(K) might be the most common gene in animal-derived-CoNS resistant to tetracycline.

One strain of *S. saprophyticus* presented a multidrug resistance phenotype that included tetracycline [*tet*(K)], erythromycin [*msr*(A)/*msr*(B)] and chloramphenicol (cat_{pC221}). *S. saprophyticus* is one of the CoNS species mostly involved in human pathologies, especially in urinary tract infections [5]. Also, two *S. saprophyticus* isolates from birds of prey presented a multidrug resistance phenotype with the following genotypes: *tet*(K), cat_{pC223} for one strain and *tet*(K), *tet*(L), cat_{pC223}, *dfrG* for the other one [11]. According to these data, the capacity of this opportunist pathogen to acquire different antimicrobial resistance determinants must be taken into consideration.

In this study, one *S. haemolyticus* isolate was resistant to SXT and harbored the *dfrG* gene. The *dfrG* gene is known to play an important role in the mediation of that resistance in certain clones of *S. pseudintermedius* and *S. aureus* of animal source, but also in *S. aureus* of human origin [49]. Moreover, four CoNS isolates were resistant to cefoxitin [*S. vitulinus* (n = 1), *S. fleurettii* (n = 2), *S. haemolyticus* (n = 1)] and carried the *mecA* gene. In fact, *S. vitulinus* and *S. fleurettii* among animal-derived isolates were known to harbor this important resistance gene [3,50]. Also, *S. haemolyticus* is a prevalent hospital pathogen highly resistant to beta-lactams, and it has been suggested as an important reservoir for the *mecA* gene [38,51].

Few strains were resistant to macrolide-lincosamide and phenicols, showing the presence of either *msr*(A)/*msr*(B) or *erm*(B) gene, and either *fexA* or cat_{pC221} gene, respectively whether resistant to erythromycin or chloramphenicol. Bacterial resistance to chloramphenicol is most commonly mediated by chloramphenicol acetyltransferase (CAT) genes [52]. But in this study, in addition to the *cat* gene detected in one *S. saprophyticus* isolate, *fexA* has been found out in one chloramphenicol resistant *S. sciuri* strain. As mentioned in previous papers, *fexA* was first identified in 2004 in a bovine *S. lentus* isolate [53], and later on found in other staphylococcal species isolated from livestock and Equidae (pigs, cattle, horses) [52]. Recently, it has been detected the *fexA* gene associated in the same plasmid with the *cfr* and *optrA* linezolid resistance genes [24]; however, our *fexA*-positive *S. sciuri* isolate lacked both genes. Furthermore, a novel phenicol-oxazolidinone-tetracycline resistance gene named *poxtA* has been described and identified in the genome of an MRSA of hospital origin [26]; but it was absent in our chloramphenicol-resistant strains.

5. Conclusion

CoNS were detected in approximately one-third of the wild boars tested, with a high diversity of species, some of them predominant as *S. sciuri* and *S. xylosus*. Although a high proportion of isolates showed antimicrobial susceptibility, the prevalence of resistance for tetracycline was relatively high (10.5%), mostly mediated by the *tet*(K) gene. Of relevance is the detection of CoNS of different species carrying unusual genes of resistance to macrolides [*erm*(F), *erm*(43)], clindamycin [*lnu*(A)] or chloramphenicol (*fexA*). Considering the evolution of all these resistance genes in staphylococci in natural ecosystems, the analysis of the forces that drive this evolution and the risk of transference of resistance genes among pathogens and ecosystems is required.

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