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Journal of Antimicrobial Chemotherapy

Clonal diversity, virulence patterns and antimicrobial and biocide susceptibility among human, animal and environmental MRSA in Portugal

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Objectives: The objective of this study was to identify the *Staphylococcus aureus* clonal types currently circulating in animals, humans in contact with animals and the environment in Portugal based on genetic relatedness, virulence potential and antimicrobial/biocide susceptibility.

Methods: Seventy-four *S. aureus* isolates from pets, livestock, the environment and humans in contact with animals were characterized by SCC*mec* typing, *spa* typing, PFGE and CC398-specific PCR, by antimicrobial and biocide susceptibility testing and by detection of resistance genes and genes for efflux pumps. Representative strains were analysed by DNA microarray and MLST.

Results: The *S. aureus* isolates represented 13 *spa* types and 3 SCC*mec* types and belonged to three clonal complexes (CC5, CC22 and CC398). Most of the isolates were multiresistant and harboured the resistance genes that explained the resistance phenotype. The *qacG* and *qacJ* genes for biocide resistance were detected in 14 isolates (all MRSA CC398), while 4 isolates (3 CC5 and 1 CC22) had insertions in the −10 motif of the *norA* promoter. Isolates of the clonal lineages associated with pets (CC5 and CC22) harboured specific sets of virulence genes and often a lower number of resistance genes than isolates of the clonal lineage associated with livestock animals (CC398).

Conclusions: We found, for the first time in animals in Portugal, four strains belonging to CC5, including ST105-II, a lineage that has been previously reported as vancomycin-resistant *S. aureus* in Portugal. Moreover, for the first time the *qacG* and *qacJ* genes were detected in MRSA CC398 strains. Active surveillance programmes detecting MRSA not only in livestock animals but also in companion animals are urgently needed.

Keywords: *mecA*, staphylococci, public health, CC5, CC398, CC22

Introduction

Staphylococcus aureus, especially MRSA, are a major problem in the healthcare system and are also disseminated into the community.[1] In Portugal, a country with a high prevalence of nosocomial MRSA, MRSA of the clonal complexes CC22 and CC5 are the main clones causing infections in people attending healthcare centres and EMRSA-15 (ST22-IVh) accounts for more than 50% of the total isolates in hospitals.[1] The first European vancomycin-resistant *S. aureus* (VRSA), a CC5 MRSA clone ST105-II, was recently described in Portugal.[2] Animals can also become colonized and infected by MRSA, and might act as a reservoir for human infections.[3] In Portugal, colonization and infection with MRSA has been described in pigs, horses, calves, dogs and cats.[4–7] However, little is known about the potential of these strains to colonize/infect humans, especially those in contact with animals. Furthermore, animal MRSA strains can harbour antimicrobial resistance genes and/or efflux pumps that could potentially be transmitted to human MRSA strains, limiting the efficacy of antimicrobial/biocide treatment.[3,8,9]

The objective of this study was to identify and characterize the MRSA clonal types currently circulating in animals, humans in contact with animals and the environment in Portugal.

Materials and methods

Bacterial isolates

This study included all of the MRSA isolated at the Antibiotic Resistance Laboratory (Faculty of Veterinary Medicine, University of Lisbon) from 2001 to 2014, from all over Portugal (from routine diagnostic and national monitoring and surveillance programmes).[4–7] Infection (i) and colonization (c) isolates were obtained from pigs in 2008 (n=17, 11 i + 6 c), environmental dust samples from breeding pig sheds in 2008 (n=14), humans

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Table 1. Characteristics of the 74 MRSA isolates

Clonal complex	Origin	SCC*mec* type	*spa* type	MLST	Resistance phenotype	Resistance genotype
CC5	dog (i)	II	t002	ST105	ERY, FQ, CLI, EtBr	*erm*(A), CAAT insertion at −10 motif of *norA* promotor
		II	t002*	ND	ERY, FQ, CLI, KAN, EtBr	*erm*(A), *erm*(B), *aadD*, CAAT insertion at −10 motif of *norA* promotor
	cat (i)	NT	t311	ST5	FUS	*fusC*
	horse (c)	NT	t062*	ST5	ERY, CLI, FUS	*erm*(C), *fusC*
	human (c)	IV	t002*	ND	ERY, KAN	*msr*(A), *aphA3*, *sat*
	human (c)	II	t002*	ND	ERY, CLI, FQ, KAN, SXT, EtBr	*erm*(A), *aadD*, CAAT insertion at −10 motif of *norA* promotor
CC22	dog (i)	IV	t032* (*n*=2)	ST22	FQ	
		IV	t025	ST22	ERY, CLI, FQ, EtBr	*erm*(C), GTTGTAATACAAT insertion at −10 motif of *norA* promotor
		IV	t2357 (*n*=2)	ST22, ND	ERY, CLI, FQ	*erm*(C)
		IV	t1865	ND	FQ	
	dog (c)	IV	t032	ND	FQ	
	cat (i)	IV	t032 (*n*=3)	ST22	FQ	
		IV	t032	ST22	ERY, CLI, FQ	*erm*(C), *mph*(C), *msr*(A)
	cat (c)	IV	t032 (*n*=2)	ND	FQ	
	human (c)	IV	t032* (*n*=4)	ND	FQ	
		IV	t1865 (*n*=2)	ND	FQ	
		IV	t020	ND	FQ	
		IV	t910	ND	FQ	
CC398	pig (i)	V	t011 (*n*=4)	ND	TET, CLI, TIA, SXT, TMP	*tet*(M), *tet*(K), *vga*(A), *dfrK*
		V	t011 (*n*=6)	ND	TET, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *vga*(A), *dfrK*, *qacG*
		V	t4571*	ND	TET, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *vga*(A), *dfrK*, *qacG*
	pig (c)	V	t011 (*n*=4)	ND	TET, CLI, TIA	*tet*(M), *tet*(K), *vga*(A)
		V	t011*	ND	TET, ERY, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*, *qacG*
		V	t011	ND	TET, CLI, TIA, TMP, EtBr	*tet*(M), *tet*(K), *vga*(A), *dfrK*, *qacG*
	pig shed	V	t011	ND	TET	*tet*(M)
		V	t011 (*n*=2)	ND	TET, CLI, TIA, SXT, TMP	*tet*(M), *tet*(K), *vga*(A), *dfrK*
		V	t011	ND	TET, KAN, GEN, SXT, TMP	*tet*(M), *tet*(K), *aadD*, *aacA-aphD*, *dfrK*
		V	t108 (*n*=2)	ND	TET, CLI, TIA	*tet*(M), *vga*(A)
		V	t108	ND	TET, CLI, TIA	*tet*(M), *tet*(K), *vga*(A)
		V	t108	ND	TET, CLI, TIA, SXT, TMP	*tet*(M), *vga*(A), *dfrK*
		V	t108	ND	TET, ERY, CLI, TIA, SXT, TMP	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*
		V	t108 (*n*=2)	ND	TET, SXT, TMP	*tet*(M), *tet*(L), *dfrK*
		V	t108	ND	TET, SXT, TMP, EtBr	*tet*(M), *tet*(L), *dfrK*, *qacJ*
		V	t108	ND	TET, CHL, FFC, FQ	*tet*(M), *fexA*
		V	t1255*	ND	TET, CLI, TIA, APR	*tet*(M), *vga*(C), *apmA*
	calf (c)	V	t108 (*n*=4)	ND	TET, CHL, FFC, FQ	*tet*(M), *tet*(K), *fexA*
		V	t108*	ND	TET, KAN, CHL, FFC, FQ	*tet*(M), *tet*(K), *aphA3*, *fexA*
		V	t108	ND	TET, ERY, CLI, CHL, FFC, FQ	*tet*(M), *tet*(K), *erm*(C), *fexA*
	dog (i)	V	t108	ST398	TET, CHL, FFC, FQ	*tet*(M), *fexA*
	horse (c)	IV	t011	ND	TET, KAN, GEN, SXT, TMP	*tet*(M), *aacA-aphD*, *dfrK*
	human (c)	V	t011 (*n*=2)	ND	TET, ERY, CLI	*tet*(M), *tet*(K), *erm*(C)

V	t011	ND	TET, CLI, TIA	*tet*(M), *tet*(K), *vga*(A)
V	t011	ND	TET, TMP	*tet*(M), *tet*(K), *dfrK*
V	t011*	ND	TET, ERY, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*, *qacG*
V	t011 (*n*=2)	ND	TET, ERY, CLI, TIA, TMP, EtBr	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*, *qacG*
V	t1255	ND	TET, TMP, EtBr	*tet*(M), *tet*(K), *dfrK*, *qacG*

APR, apramycin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; FFC, florfenicol; FQ, fluoroquinolones; FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin; ND, not determined; NT, non-typeable; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; TIA, tiamulin; TMP, trimethoprim.
Isolates with an asterisk were selected for microarray analysis.

in contact with animals in 2008–12 (*n*=18 c), calves in 2010 (*n*=6 c), dogs in 2008–14 (*n*=10, 1 i+9 c), cats in 2001–14 (*n*=7, 5 i+2 c) and horses in 2010 (*n*=2 c).

Antimicrobial susceptibility testing and resistance genes

The 74 isolates were routinely tested by broth microdilution for their antimicrobial susceptibility to a panel of antimicrobials (ampicillin, amoxicillin/clavulanic acid, chloramphenicol, ciprofloxacin or enrofloxacin, erythromycin, florfenicol, fusidic acid, gentamicin, kanamycin, penicillin, trimethoprim, trimethoprim/sulfamethoxazole and tetracycline). Genes encoding resistance to β-lactams, aminoglycosides, macrolides, lincosamides, tetracyclines, fusidic acid, phenicols and trimethoprim were detected by PCR.[8,9]

Biocide susceptibility and efflux pump genes

Determination of ethidium bromide (EtBr) MICs was used as a simple screening procedure for identifying strains with an increased efflux phenotype.[9] MICs of chlorhexidine acetate, benzalkonium chloride and triclosan were determined to further characterize the efflux phenotype. *S. aureus* ATCC 29213 was used as a quality-control strain. The detection of the biocide efflux pump genes *norA* and its promoter region, *qacA/B*, *smr*, *qacG*, *qacH* and *qacJ* was performed by PCR and sequencing of strains with high EtBr MICs (>8 mg/L).[9]

Molecular typing

All strains were assigned a *spa* type through the *spa* server (http://www.ridom.de/spaserver/). The isolates were assigned to clonal complexes according to the database of the *spa* server. These strains were also subjected to ST398-specific PCR and SCC*mec* typing using primers described previously.[8] CC5 and CC22 strains were compared by SmaI PFGE, while CC398 strains were compared by ApaI PFGE, using a previously described protocol.[8] Nine strains were subjected to MLST. Eleven strains were randomly chosen for characterization using the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany).

Statistical analysis

The comparison of groups of categorical data was performed using the Fisher's exact test with a level of significance set at 0.05, using SPSS v20 (IBM, New York, USA).

Results

Overall, 13 *spa* types were identified (Table 1). According to the *spa* server, four of these *spa* types were associated with CC398 (*n*=47), six were linked to CC22 (*n*=21) and three were associated with CC5 (*n*=6). These assignments were confirmed by results from MLST and CC398-specific PCRs. The PFGE patterns of all isolates belonging to the same clonal complex showed >80% similarity (Figures S1 and S2, available as Supplementary data at *JAC* Online). The CC22 isolates had SCC*mec* type IV and the CC398 isolates type V (*n*=46) and type IV (*n*=1). Among the CC5 isolates, three had SCC*mec* type II, one had type IV and two carried non-typeable SCC*mec* elements, which are currently sequenced. All CC398 strains were resistant to tetracycline due to the presence of the *tet*(M) gene alone or in combination with *tet*(K) or *tet*(L) genes. All CC22 and bovine CC398 strains were fluoroquinolone resistant. One porcine, one canine and the six bovine CC398 were resistant to chloramphenicol and florfenicol due to the presence of the *fexA* gene. Genes *dfrK* and *vga*(A) were present in

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Table 2. Virulence characteristics of the 11 *S. aureus* characterized by the *S. aureus* Genotyping Kit 2.0 (Alere)

Origin	Clone	*agr* group	Haemolysins and leukotoxins	Enterotoxins	IEC
Dog (i)	CC5-t002-II	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*sed*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *seu*, *ser*, *egc*	*chp*, *sak*, *scn*
Human (c)	CC5-t002-II	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*sed*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *seu*, *ser*, *egc*	*chp*, *sak*, *scn*
Human (c)	CC5-t002-IV	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*sea*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *seu*, *ser*, *egc*	*sak*, *scn*
Horse (c)	CC5-t062-nt	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*, *egc*	*chp*, *sak*, *scn*
Dog (i)	CC22-t032-IV	I	*hla*, *hlb*, *hld*, *lukF*, *lukS*	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*, *egc*	—
Human (c)	CC22-t032-IV	I	*hla*, *hlb*, *hld*, *lukF*, *lukS*	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*, *egc*	*chp*, *sak*, *scn*
Calf (c)	CC398-t108-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Environmental dust	CC398-t1255-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Pig (i)	CC398-t4571-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Human (c)	CC398-t011-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Pig (c)	CC398-t011-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—

almost all porcine and environmental MRSA CC398 strains. All strains were susceptible to vancomycin.

Eighteen strains had high MICs of EtBr: 13 (all CC398) carried a *qacG* gene and had MICs of 16 mg/L, 1 strain (CC398) carried a *qacJ* gene and had an MIC of 32 mg/L and in 4 strains (3 CC5 and 1 CC22) the *norA* gene had an insertion of sequence CAAT ($n=3$) or GTTGTAATACAAT ($n=1$) in the −10 motif of the *norA* promoter and had MICs of 32 mg/L. MICs of benzalkonium chloride ranged from ≤0.125 to 4 mg/L and MICs of chlorhexidine acetate ranged from ≤0.125 to 1 mg/L. All strains had low MICs of triclosan (≤0.125 mg/L).

The main virulence characteristics of the 11 *S. aureus* characterized by the *S. aureus* Genotyping Kit 2.0 are summarized in Table 2. MRSA CC5 strains belonged to *agr* type II and the others to *agr* type I. All strains carried the following virulence genes: *isaB*, *isdA*, *hsdSx*, *aur*, *sspA*, *sspB*, *sspP*, *sdrC*, *hysA1*, *setC*, *ssl02*, *cap5*, *icaA*, *icaC*, *icaD*, *vwb*, *bbp*, *cflA*, *cflB*, *ebpS*, *eno*, *fib*, *fnbA*, *map*, *splA* and *splB*. None of the strains carried the ACME locus, the epidermal cell differentiation inhibitor (*edinA*, *edinB* or *edinC*), exfoliative toxins (*etA*, *etB* or *etD*), biofilm-associated protein (*bap*) or toxic shock syndrome toxic 1 (*tsst-1*). The main differences in the carriage of virulence genes were detected in the enterotoxins, haemolysins, leukotoxins and immune evasion cluster (IEC) (Table 2). Interestingly, the human MRSA CC22-t032 strain carried the IEC, while the dog MRSA CC22-t032 strain did not.

Pet-associated MRSA (CC5 and CC22) were significantly more likely to carry enterotoxin genes [*seg* ($P=0.002$), *sei* ($P=0.002$), *sem* ($P=0.002$), *sen* ($P=0.002$), *seo* ($P=0.002$), *seu* ($P=0.002$) and enterotoxin gene cluster (*egc*; $P=0.002$)] and staphylokinase gene [*sak*; $P=0.015$)], while livestock-associated MRSA CC398 were significantly more likely to carry efflux pumps and particular antimicrobial resistance genes [*qacG* ($P=0.003$), *dfrK* ($P=0.0001$), *tet*(K) ($P=0.0001$), *tet*(M) ($P=0.0001$) and *vga*(A) ($P=0.0001$)].

Discussion

During recent years, we have observed a significant increase in the number of MRSA descriptions in animals in several countries, including Portugal,[4–7] despite only isolated studies describing MRSA in animals and a single surveillance study (European Union-wide baseline survey on MRSA conducted in 2008 in breeding pig holdings) conducted in Portugal. Studies on the role of animals, especially pets, in the transmission of MRSA into the community are still lacking. In Denmark MRSA CC398 constituted 31% of all new MRSA cases in 2013 and patients in contact with live pigs are screened for MRSA colonization when entering the hospital setting.[10,11] In contrast, livestock-associated MRSA constitutes a small percentage of the overall MRSA burden in Portugal and active screening does not include patients with animal contact.[12] This study showed that people in direct contact with animals (owners, handlers and veterinary personnel) carried similar MRSA clones as the animals they were in contact with. Especially worrying was the fact that humans in contact with companion animals carried clones (CC5 and CC22) circulating in hospitals and the community.[1] We found, for the first time in animals in Portugal, four strains belonging to CC5. One of these strains belonged to ST105-II, the same lineage as the recently described VRSA in Portugal.[2] VRSA isolates from other countries also belonged to CC5.[13] In addition to the possibility of pets being a reservoir and distributer of VRSA, companion animals can also carry *vanA*-carrying VRE and thereby raise the chances of acquisition of the *vanA* gene cluster.[14]

MRSA CC22 and CC5 strains carried significantly more enterotoxins than MRSA CC398, including *egc*, and at least one IEC gene. One dog MRSA CC22-t032 strain did not carry IEC genes, which might suggest host adaptation. Still a recent study found no significant difference in the presence or absence of the IEC between human and companion animal isolates when correcting for shared evolutionary history, suggesting that IEC conferred isolates with an extended host spectrum.[15] Companion animals seem to carry *S. aureus* clonal linages that are more virulent to humans than livestock animals, and so active surveillance of MRSA in companion animals seems to be urgently needed.

We found for the first time, to the best of our knowledge, the *qacG* and *qacJ* genes in MRSA CC398 strains. The *qacG* gene has been described in porcine MRSA isolates from clonal lineage ST9 in Hong Kong[16] and both genes have also been detected among staphylococci of bovine and caprine origin in Norway.[17] Biocides are extensively used in animal husbandry, including quaternary ammonium compounds.[14] The acquisition of *qacG* and *qacJ* by MRSA CC398, usually carried on plasmids, may aid to the persistence of MRSA in the environment, making the eradication of MRSA CC398 more difficult.

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Transparency declarations
None to declare.

Supplementary data
Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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