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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 SCCmec 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 SCCmec 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.3,4 In Portugal, colonization and infection with MRSA has been described in pigs, horses, calves, dogs and cats.5–7 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.3,4,9

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).4,5,7,10 Infection (i) and colonization (c) isolates were obtained from pigs in 2008 (n = 17, i + c), environmental dust samples from breeding pig sheds in 2008 (n = 14), humans
<table>
<thead>
<tr>
<th>Clonal complex</th>
<th>Origin</th>
<th>SCCmec type</th>
<th>spa type</th>
<th>MLST</th>
<th>Resistance phenotype</th>
<th>Resistance genotype</th>
</tr>
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<tbody>
<tr>
<td>CC5</td>
<td>dog (i)</td>
<td>II</td>
<td>t002</td>
<td>ST105</td>
<td>ERY, FQ, CLI, EtBr</td>
<td>erm(A), CAAT insertion at -10 motif of norA promotor</td>
</tr>
<tr>
<td></td>
<td>cat (i)</td>
<td>NT</td>
<td>t311</td>
<td>ST5</td>
<td>FUS</td>
<td>fusC</td>
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<tr>
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<td>horse (c)</td>
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<td>t062</td>
<td>ST5</td>
<td>ERY, CLI, FUS</td>
<td>erm(C), fusC</td>
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<td>ND</td>
<td>ERY, KAN</td>
<td>msr(A), apha3, sat</td>
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<tr>
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<td>II</td>
<td>t002</td>
<td>ND</td>
<td>ERY, CLI, FQ, KAN, SXT, EtBr</td>
<td>erm(A), aadd, CAAT insertion at -10 motif of norA promotor</td>
</tr>
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<td>ST22</td>
<td>erm(C), GTTGTAATACAAT insertion at -10 motif of norA promotor</td>
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<td>ND</td>
<td>FQ</td>
<td>erm(C), mph(C), msr(A)</td>
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<td>t032</td>
<td>ND</td>
<td>FQ</td>
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<td>IV</td>
<td>t1865</td>
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<td>FQ</td>
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<td>t032</td>
<td>ND</td>
<td>FQ</td>
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<td>FQ</td>
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<td>tet(M), tet(K), vga(A), dfrK</td>
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<td>V</td>
<td>t011</td>
<td>ND</td>
<td>TET, CLI, TIA, SXT,</td>
<td>tet(M), tet(K), vga(A), dfrK</td>
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<tr>
<td></td>
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<td>V</td>
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<td>V</td>
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<td>tet(M), tet(K), vga(A)</td>
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<td>t011</td>
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<td>t011</td>
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<td>tet(M), tet(K), vga(A), dfrK</td>
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<tr>
<td></td>
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<td>V</td>
<td>t011</td>
<td>ND</td>
<td>TET, ERY, CLI,</td>
<td>tet(M), tet(K), vga(A), dfrK</td>
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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 anti-
microbial 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, fusi-
dic 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 pheno-
type.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 bio-
cide 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 sub-
jected to ST398-specific PCR and SCCmec typing using primers described
previously.8 CC5 and CC22 strains were compared by SmaI PFGE, while
CC398 strains were compared by Apal PFGE, using a previously described
protocol.8 Nine strains were subjected to MLST. Eleven strains were ran-
domly 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 associ-
ated 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 SCCmec type IV and the CC398
isolates type V (n=46) and type IV (n=1). Among the CC5 iso-
lates, three had SCCmec type II, one had type IV and two carried
non-typeable SCCmec elements, which are currently sequenced.
All CC398 strains were resistant to tetracycline due to the pres-
ence of the tet(M) gene alone or in combination with tet(K) or
tet(L) genes. All CC22 and bovine CC398 strains were fluoroquino-
lone resistant. One porcine, one canine and the six bovine CC398
were resistant to chloramphenicol and florfenicol due to the pres-
ence of the fexA gene. Genes dfrK and vga(A) were present in
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 GTTGAATACAA (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: isaA, isda, hsdSx, aur, sspa, sspB, sspP, sdc, hysA1, setC, ssl02, capS, icaA, icaC, icaD, vwb, bbp, cflA, cflB, ebpS, eno, fib, fnbA, map, spa and splB. None of the strains carried the ACME locus, the epidermal cell differentiation inhibitor (ednA, ednB or ednC), exfoliative toxins (eta, etB or etD), biofilm-associated protein (bap) or toxic shock syndrome toxin 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), dfk (P = 0.0001), tet(K) (P = 0.0001), tet(M) (P = 0.0001) and vga(A) (P = 0.0001)].

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

<table>
<thead>
<tr>
<th>Origin</th>
<th>Clone</th>
<th>agr group</th>
<th>Haemolysins and leukotoxins</th>
<th>Enterotoxins</th>
<th>IEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (i)</td>
<td>CC5-1002-II</td>
<td>II</td>
<td>hla, hlb, hld, lukF, lukS, lukD, lukE</td>
<td>seg, seg, sei, sej, sem, sen, seo, seu, ser, egc</td>
<td>chp, sak, scn</td>
</tr>
<tr>
<td>Human (c)</td>
<td>CC5-1002-II</td>
<td>II</td>
<td>hla, hlb, hld, lukF, lukS, lukD, lukE</td>
<td>seg, seg, sei, sej, sem, sen, seo, seu, ser, egc</td>
<td>chp, sak, scn</td>
</tr>
<tr>
<td>Human (c)</td>
<td>CC5-1002-IV</td>
<td>II</td>
<td>hla, hlb, hld, lukF, lukS, lukD, lukE</td>
<td>sej, seg, sei, sej, sem, sen, seo, seu, ser, egc</td>
<td>chp, sak, scn</td>
</tr>
<tr>
<td>Horse (c)</td>
<td>CC5-1062-nt</td>
<td>II</td>
<td>hla, hlb, hld, lukF, lukS, lukD, lukE</td>
<td>seg, sei, sem, sen, seo, seu, egc</td>
<td>chp, sak, scn</td>
</tr>
<tr>
<td>Dog (i)</td>
<td>CC22-t032-IV</td>
<td>I</td>
<td>hla, hlb, hld, lukF, lukS</td>
<td>sec, seg, sei, sel, sen, seo, seu, egc</td>
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<tr>
<td>Human (c)</td>
<td>CC22-t032-IV</td>
<td>I</td>
<td>hla, hlb, hld, lukF, lukS</td>
<td>sec, seg, sei, sel, sen, seo, seu, egc</td>
<td>chp, sak, scn</td>
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<td>Calf (c)</td>
<td>CC398-t108-V</td>
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<td>hla, hlb, lukF, lukS</td>
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<td>Environmental dust</td>
<td>CC398-t1255-V</td>
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<td>Pig (i)</td>
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<td>I</td>
<td>hla, hlb, lukF, lukS</td>
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<td>Pig (c)</td>
<td>CC398-t011-V</td>
<td>I</td>
<td>hla, hld, lukF, lukS</td>
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</tbody>
</table>

### Discussion

During recent years, we have observed a significant increase in the number of MRSA descriptions in animals in several countries, including Portugal, 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. 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. 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. 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. MRSA isolates from other countries also belonged to CC5. In addition to the possibility of pets being a reservoir and distributor of VRSA, companion animals can also carry vanA-carrying VRE and thereby raise the chances of acquisition of the vanA gene cluster.

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 of 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. Companion animals seem to carry S. aureus clones 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 and both genes have also been detected among staphylococci of bovine and caprine origin in Norway. Biocides are extensively used in animal husbandry, including quaternary ammonium compounds. 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.
**MRSA in Portugal**

**Acknowledgements**
We thank Kerstin Meyer (Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany) for excellent laboratory assistance.

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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/).

**References**