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AU1 ▶

Characterization of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* CC398 and *mecC*-positive CC130 from Zoo Animals in the United Kingdom

AU2 ▶

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Little is known about the characteristics and diseases associated with methicillin-resistant *Staphylococcus aureus* (MRSA) in nondomestic animals. Four presumptive MRSA isolates, obtained from clinical ($n=3$) and surveillance specimens ($n=1$) from dwarf (*Helogale parvula*) and yellow mongooses (*Cynictis penicillata)* from a United Kingdom zoo, were analyzed by PCR for detection of *mecA* and *mecC*-mediated methicillin resistance, and virulence genes. Isolates were genotyped by multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) and *spa* sequence typing. Three isolates, obtained from the dwarf mongooses, carried *mecA*, *tetK*, and *fexA* resistance and virulence genes (*icaA*, *icaD*, and *sec*) and were typed to SCC*mec* IVa, *spa* type t899, and clonal complex (CC) 398. The fourth MRSA isolate, obtained from the femoral bone marrow of a yellow mongoose showing postmortem findings consistent with septicemia, carried *mecC* and was oxacillin/cefoxitin susceptible, when tested at 37°C but showed a characteristic MRSA susceptibility profile at 25°C ± 2°C. Furthermore, this isolate exhibited a different genetic background (SCC*mec*XI/t843/CC130) and had biofilm-associated genes (*bap*, *icaA*, and *icaD*) and *tetK* tetracycline resistance genes. This work describes the first isolation of livestock-associated MRSA CC398 from two zoo mongoose species where it was associated with both clinical disease and colonization, and the first isolation of *mecC* MRSA from a zoo species in the United Kingdom. Both reports highlight the potential for zoo species to act as reservoirs for these zoonotic agents.

AU4 ▶

Keywords: MRSA, *mecA*, *mecC*, zoo animals, *spa* typing, multilocus sequence typing

Introduction

METHICILLIN-RESISTANT *STAPHYLOCOCCUS aureus* (MRSA) is a leading cause of infections in humans and animals worldwide. Colonization and infections of domestic animals are of particular interest due to the risk of dissemination and as reservoirs of human infection.[1] Since the first report of MRSA in animals,[2] there have been several studies of MRSA infections in a variety of domestic animal species such as horses, chickens, dogs, and cats.[3,4]

Among European livestock, sequence type (ST) belonging to the clonal complex (CC) 398, also called livestock-associated MRSA (LA-MRSA), is prevalent.[5–7] Although this lineage is generally regarded as animal adapted and rarely associated with clinical manifestations in livestock (among poultry and pigs), recently it has been shown to be associated with colonization and occasional involvement in human disease.[8]

Methicillin resistance in *Staphylococcus* spp. is linked to the acquisition of *mecA* gene but a novel homolog, *mecC* located in a new staphylococcal cassette chromosome *mec* (SCC*mec*) cassette (SCC*mec* XI), has been identified and shown to have a 69% DNA sequence similar to *mecA* allotypes.[9] Isolates harboring *mecC* typically yield negative results for conventional *mecA* PCR, which can translate in detection errors in a subset of MRSA isolates[10] and subsequent therapeutic and public health implications.

While there are extensive MRSA surveillance studies in humans, companion animals, and livestock, there is less information on the occurrence of MRSA, especially CC398

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and *mecC*-positive MRSA in less common potential reservoirs such as wildlife and zoo animal species. Here, we describe the occurrence and characterization of MRSA CC398 in a colony of dwarf mongooses (*Helogale parvula*) and of *mecC*-positive MRSA infection in a yellow mongoose (*Cynictis penicillata*) from a zoo in the United Kingdom.

Materials And Methods

Bacterial isolates

Clinical specimens obtained from live or dead mongooses were submitted for bacterial culture and yielded growths of presumptive MRSA isolates. The first isolate (Mo4) was obtained, in 2013, from the femoral bone marrow of a female yellow mongoose submitted for postmortem (PM) investigation that showed gross lesions consistent with septicemia. This mongoose was kept with a male that did not show any clinical signs of illness and was not screened for MRSA. The remaining three isolates were obtained in 2016 from a small colony of three dwarf mongooses: two isolates (Mo1 and Mo2) were from two animals that showed mild clinical signs of infection (conjunctival swelling in one animal and a nonhealing facial ulcer in the other). The third isolate (Mo3) was obtained from a clinically healthy mongoose from this group, which was screened for MRSA carriage by taking oropharyngeal and perianal swabs, following the previous findings of clinical MRSA cases.

Swabs taken from the lesion sites, from the bone marrow of the yellow mongoose and from the areas screened for carriage, were plated on 5% sheep blood agar (SBA), MRSA Brilliance-2 agar (all the media from Oxoid, Basingstoke, United Kingdom) and incubated aerobically at 37°C ± 2°C. Fresh colonies with morphology suggestive of *S. aureus* on SBA were used for DNA extraction by boiled preparation. Two multiplex PCRs, one targeting *nuc*, *femB*, and *mecA* genes[11] and a second one targeting *femB*, *mecA*, and *mecC*[10] were used for confirmation of MRSA status and the obtained amplicons were sequenced for confirmation.

Characterization of strain types, antimicrobial resistance, and virulence genes

A PCR specific for *S. aureus* CC398 was used to screen the confirmed MRSA isolates.[12] Molecular characterization of isolates was performed by multilocus sequence typing (MLST),[13] SCC*mec* typing (type I–IV, XI),[10,14] and *spa* sequence typing.[15] The *spa* types were determined using the *spa*Typer software available at http://spatyper.fortinbras.us.

All isolates were screened by PCR for the presence of virulence genes *lukS-PV* and *lukF-PV*[16] encoding for Panton-Valentine leukocidin (PVL), which are associated with many community-acquired lineages[17]; for the presence of the exfoliative toxin genes *eta* and *etb*,[18] commonly associated with skin diseases in animals; and for the entorotoxin gene *sec*, which has been associated with increased severity of skin diseases.[19] In addition, the presence of *bap*, *ica*A, and *ica*D[20] genes associated with biofilm production and which play a role in the development of persistent infections[21] was investigated. Furthermore, we sought to detect the presence of *sip*, an integrase carried by the Staphylococcal pathogenicity island (SaPI) that usually carries *bap*.[22] Finally, a panel of the most common antimicrobial resistance genes found in the United Kingdom MRSA CC398[23] was also included for screening (*ermA*, *ermB*, *ermC*, *tetK*, *tetM*, *aacA-aphD*, *fexA*, *cfr*, and *dfrK*).[24–28] Positive and negative controls for PCR reactions were included in each assay.

Antimicrobial susceptibility testing

Minimum inhibitory concentration (MIC) testing for 21 different antimicrobials (Table 2) was performed by broth microdilution (Sensititre COMPAN1F, Trek Diagnostics System, East Grinstead, UK) according to the manufacturer's instructions; *S. aureus* ATCC 29213 was included for quality control. Linezolid (10 μg) susceptibility testing was performed on all isolates by disc diffusion (DD) on Mueller–Hinton agar according to Clinical Laboratory Standards Institute (CLSI) recommendations.[29] In addition, oxacillin (1 μg) and cefoxitin (30 μg) susceptibility for the MRSA isolate Mo4 was performed by DD on Mueller–Hinton agar (+2% NaCl) according to CLSI guidelines[29] and MIC determined at both 37°C ± 2°C and 25°C ± 2°C. Antimicrobial susceptibility results were interpreted according to the CLSI breakpoits[29] except for chloramphenicol, linezolid, ticarcillin, and ticarcillin/clavulanic acid where EUCAST breakpoints were used.[30]

Results

All four *S. aureus* isolates presented typical growth on MRSA Brilliance-2 Agar at 24 hr with characteristic denim-blue colonies. Molecular testing showed that three MRSA isolates (Mo1, Mo2, and Mo3) had similar molecular characteristics, exhibiting phenotypic resistance to oxacillin and cefoxitin, which was encoded by the *mecA* gene. The fourth isolate (Mo4) produced typical colonies on the MRSA Brilliance-2 agar (although slightly smaller) but PCR testing failed to identify the *mecA* gene, thus, this isolate was reported as methicillin susceptible. Later discovery of the alternative *mecC* gene triggered retrospective investigation of this isolate with the second PCR, which identified the isolate to carry *mecC*. Further sequencing confirmed the identity of the *mecC* gene with 100% homology to the prototype sequence of *S. aureus* strain LGA251.

SCC*mec*, *spa* typing, and MLST revealed that the three isolates from dwarf mongooses were typed to SCC*mec*IVa/t899/CC398, carried *tetK* encoding a tetracycline efflux pump and *fexA* encoding for a protein involved in the efflux of chloramphenicol and florfenicol. However, our isolates lacked *cfr* (often associated with *fexA* on a common mobile genetic element), which mediates resistance to linezolid and also resulted in phenotypic susceptibility to this antimicrobial agent. However, they had different virulence genes, where isolates Mo1 and Mo2 were positive for the biofilm-associated genes *icaA*, *icaD*, and the enterotoxin-encoding gene *sec* while Mo3 was positive only for *icaA*. *mecC*-positive isolate Mo4 (from the yellow mongoose) had a different genetic background (SCC*mec*XI/t843/CC130), and in addition to *tetK* also carried various biofilm production-associated genes (*bap*, *ica*A, and *ica*D).

All MRSA isolates lacked the virulence genes encoding for PVL (*lukS-PV* and *lukF-PV*), those encoding for exfoliative toxins associated with skin infection (*eta and etb*) and macrolides (*ermA*, *ermB*, and *ermC*), ribosomal-mediated tetracycline (*tetM*), gentamicin (*aacA-aphD*), and trimethoprim-sulfamethoxazole (*dfrK*) resistance genes. Molecular characteristics of the isolates investigated in this study are presented in Table 1. ◀T1

TABLE 1. DESCRIPTION AND CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* MONGOOSE ISOLATES

	Source	*Year*	*Site*	*CC398 PCR*	*MLST*	mecA	mecC	*SCC*mec *type*	spa-*typing*	icaA	icaD	bap	tetK	fexA	sec
Mo1	Dwarf mongoose	2016	Facial lesion	+ve	398	Pos	Neg	IVa	t899	Pos	Pos	Neg	Pos	Pos	Pos
Mo2	Dwarf mongoose	2016	Conjunctiva	+ve	398	Pos	Neg	IVa	t899	Pos	Pos	Neg	Pos	Pos	Pos
Mo3	Dwarf mongoose	2016	Pharyngeal swab	+ve	398	Pos	Neg	IVa	t899	Pos	Neg	Neg	Pos	Pos	Neg
Mo4	Yellow mongoose	2013	Bone marrow	−ve	130	Neg	Pos	XI	t843	Pos	Pos	Pos	Pos	Neg	Neg

CC, clonal complex; MLST, multilocus sequence typing; SCC*mec*, staphylococcal cassette chromosome *mec*.

Isolates Mo1, Mo2, and Mo3 exhibited resistance to all β-lactam antimicrobials, fluoroquinolones, chloramphenicol, tetracycline, and potentiated sulfonamides. Surprisingly, despite the growth on the selective MRSA Brilliance-2 Agar, *mecC*-positive MRSA isolate Mo4 revealed susceptibility to most β-lactams (except penicillin and ampicillin) including oxacillin and cefoxitin by both MIC and DD when tested at 37°C ± 2°C. Interestingly, repeating susceptibility testing at 25°C ± 2°C revealed resistance to all β-lactams antimicrobials (Table 2). ◀T2

Discussion

Unlike with farm or companion animals, data on prevalence and molecular characteristics of MRSA isolates in zoo animals are very scarce. In North American facilities, the MRSA USA300 strain was detected in an African elephant (*Loxodonta africana*) calf, which was shown to have become infected via an infected caretaker.[31] In Europe, *mecC*-positive MRSA isolates (CC130 and t528) were described in captive mara (*Dolichotis patagonum*) at Copenhagen Zoo[32] while none of the 93 different mammals (40 species and 19 families) investigated at the Royal Zoological Society of Antwerp were MRSA positive.[33]

Although isolation of MRSA CC398 or LA-MRSA is common among farmers (particularly pig farmers) in northern Europe, recent studies indicate a change in its epidemiology with an increasing number of colonizations in people without livestock contact,[34,35] which highlights the importance of identifying uncommon hosts that can act as potential reservoirs.

MRSA CC398 is considered a nonhost specific lineage, which was isolated from different livestock species[1] and was also found in red deer (*Cervus elaphus*), Iberian ibex (*Capra pyrenaica*), wild boar (*Sus scrofa*), and Eurasian griffon vultures (*Gyps fulvus*) in Spain[36] and to the best of our knowledge, this is the first report of MRSA CC398 in zoo animals. Unlike in mainland Europe, this lineage has been less reported in the United Kingdom where it was first isolated in 2009 from horses[37] but, in the last few years, reports of isolation from United Kingdom dairy cattle,[38] poultry,[39] pigs,[40] and retail meat[41] have increased, suggesting that this lineage is becoming more common in the United Kingdom.

MRSA CC398 isolates usually lack several clinically important *S. aureus*-associated virulence factors, such as PVL, regardless of their origin.[42] However, in this study, 2/3 MRSA CC398 isolates (Mo1 and Mo2) obtained from clinical cases were positive for the *sec* gene, which encodes for the staphylococcal enterotoxin C, shown to be associated with increased severity of atopic dermatitis in humans and animals[43] while isolate Mo3 (obtained from a healthy animal) lacked this gene. Although the source of entry of these isolates into this zoo is still unclear, the molecular typing (SCC*mec*IVa/t899/CC398) suggests an animal origin.[44,45] However, the mongooses did not have any direct contact with other zoo species but had access to outdoor pens; so, contact with wild rodents or birds was possible.

Molecular typing also revealed that isolate Mo4 belonged to ST130 (CC130), a nonhost specific lineage already shown to be one of the predominant STs carrying *mecC* found both in humans and a range of animal species[9,10] This

Table 2. Minimum Inhibitory Concentrations of the Methicillin-Resistant *Staphylococcus aureus* Isolates Associated with Clinical Disease in Dwarf (*Helogale parvula*) and Yellow Mongooses (*Cynictis penicillata*)

MIC (μg/ml)	*Mo1*	*Mo2*	*Mo3*	*Mo4 (37°C)*	*Mo4 (25°C)*
Penicillin	**4**	**4**	**4**	**0.5**	**>8**
Ampicillin	**4**	**4**	**4**	**0.5**	**8**
Amoxicillin/clavulanic acid	**≤4**	**≤4**	**≤4**	≤4	**8**
Cefazolin	**≤4**	**≤4**	**≤4**	≤4	**>16**
Cefovecin	**4**	**4**	**4**	1	**>8**
Cefoxitin	**4**	**4**	**4**	≤2	**>16**
Oxacillin	**1**	**1**	**1**	0.5	**>4**
Cefpodoxime	**4**	**4**	**4**	≤2	**>16**
Ceftiofur	**4**	**4**	**4**	1	**>4**
Ticarcillin[a]	**16**	**16**	**16**	≤8	**64**
Ticarcillin/clavulanic acid[a]	**≤8**	**≤8**	**≤8**	≤8	**32**
Amikacin	≤4	≤4	≤4	≤4	≤4
Chloramphenicol[a]	**>16**	**>16**	**>16**	8	≤4
Clindamycin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Doxycycline	**>8**	**>8**	**>8**	≤2	≤2
Enrofloxacin	**>2**	**>2**	**>2**	≤0.25	≤0.25
Erythromycin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Gentamicin	≤1	≤1	≤1	≤1	≤1
Marbofloxacin	**>2**	**>2**	**>2**	0.5	≤0.25
Rifampin	≤1	≤1	≤1	≤1	≤1
Trimethoprim/sulfamethoxazole	**>2**	**>2**	**>2**	≤0.5	≤0.5

[a]For interpretation of test results EUCAST breakpoints (v. 6.0) were used. Values shown in bold = resistant.
MIC, minimum inhibitory concentration.

isolate was obtained from the PM tissues of a mongoose suspected of septicemia, carried biofilm-associated genes (*icaA* and *icaD*) and the *bap* gene, a surface protein first identified on biofilm-forming *S. aureus* strains from chronic bovine mastitis, strongly associated with the persistence of infection.[46] To the best of our knowledge, the presence of *bap* gene has not been previously reported in ST130 so far. Nevertheless, Prenafeta *et al.*[47] reported frequent biofilm production in *mecC*-positive MRSA isolates from the United Kingdom and although they have not investigated the presence of *bap* gene, all isolates were positive for *icaA* and *icaD* in that study. The *bap* gene in *S. aureus* is usually carried by the Staphylococcal pathogenicity island bovine 2 (SaPIbov2)[22] where *sip*, (which encodes for an integrase for SaPIbov2) can be used for its detection. However, *sip* was not detected in our isolates, which consequently did not allow us to confirm the localization of this gene on the mobile genetic element. Analysis of the flanking regions of the *bap* genes in other staphylococcal species has shown that it was not contained in SaPIbov2.[48] Nevertheless, variability at the primer binding position in our isolates could lead to the failure of *sip* gene detection.

Despite growing on Brilliance-2 MRSA media, the MIC results for this *mecC*-positive MRSA isolate revealed susceptibility to most β-lactams, when tested at 37°C, which reverted to resistant when tested at 25°C. This is in line with previous studies that showed that the optimal temperature for the activity and stability of the alternative penicillin-binding protein $PBP2A_{LGA}$ encoded by *mecC* is 25°C, which may explain this result.[49]

The recent discovery of MRSA carrying an alternative *mecA* gene indicates a group of MRSA isolates being underdetected by the current phenotypic and molecular diagnostic methods, due to the following factors: (1) the *mecC* and its product the LGA251 protein are not detected by the *mecA* PCR or the current commercial PBP2′ agglutination kits[50]; (2) some *mecC*-positive isolates grow poorly in primary culture on MRSA detection agars[51]; (3) some *mecC* isolates appear susceptible to cefoxitin despite being a better agent than oxacillin for detection of these isolates.[52] Although these scenarios can be overcome by the detection of *mecC* by PCR, most human and veterinary diagnostic laboratories do not have PCR facilities. Based on our results, to improve detection of *mecC* MRSA, we suggest that isolates yielding characteristic growth on the MRSA detection agars but which give negative results with the PBP2′ agglutination test and/or appear susceptible to oxacillin/cefoxitin when tested at 37°C should also be tested at 25°C. Further studies with a larger number of *mecC* isolates are necessary to confirm these findings, but this approach could increase *mecC* isolate detection by means affordable to most diagnostic laboratories.

The *mecC* isolate Mo4 was identified as *spa* type t843, which appears frequently among MRSA CC130 *mecC*-positive isolates from Europe, where it has been reported in the European hedgehog (*Erinaceus europaeus*), European brown hare (*Lepus europeaus*) and wood mice (*Apodemus sylvaticus*), humans, and livestock. It has also been linked to zoonotic transmission.[53–57] In the United Kingdom, 0.45% of human MRSA isolates carry *mecC* and most of these belonged to CC130.[58] In addition, *mecC* isolates with a similar genetic background (ST130, *spa* type843) have been identified in human isolates from Scotland and North-West England, where the zoo is located from which these specimens originated.[9]

Although the origin of MRSA CC398 carrying *mecA* and MRSA CC130 carrying *mecC* in the mongooses at this zoo remains unknown their detection highlights the potential for

both mongoose species to act as reservoirs for these zoonotic agents. Furthermore, given that both lineages have a broad host spectra and recognized zoonotic potential it underlines the possible transmission risk to other endangered species and to zoo personnel. In addition, the potential for inter-human transmission of both of these MRSA lineages has been already demonstrated,[59,60] therefore contact with staff working in zoos could to be a risk factor to assess when investigating the epidemiology of MRSA in people without livestock contact.

Disclosure Statement

No competing financial interests exist.

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