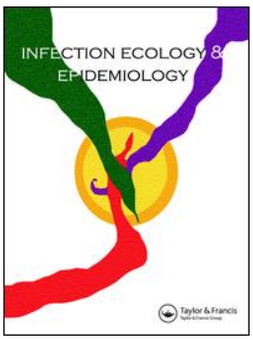

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ORIGINAL RESEARCH ARTICLE

Methicillin resistance gene diversity in staphylococci isolated from captive and free-ranging wallabies

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Background: Infection with methicillin-resistant staphylococci (MRS) can be life-threatening in humans and its presence in animals is a cause for public health concern. The aim of this study was to measure the prevalence of MRS in captive and free-ranging wallabies over a 16-month period in South Australia, Australia.

Materials and methods: Eighty-nine purified staphylococcal isolates recovered from 98 captive and free-ranging wallabies' anterior nasal swabs were used in this study. All isolates were tested for the presence of the *mecA*, *mecA1*, and *mecC* genes. Multiplex PCR-directed SCC*mec*-typing, *ccrB*-typing, and determination of the minimal inhibitory concentration of oxacillin were performed on *mec*-positive isolates.

Results and discussion: In total, 11 non-*Staphylococcus aureus* MRS were isolated from 7 out of 98 animals, corresponding to a 7.1% carriage rate. The SCC*mec* types I, III, and V were identified by multiplex PCR and sequencing of the *ccrB* gene. This is the first report of MRS carriage in both captive and free-ranging wallabies in Australia. These data demonstrate a low prevalence of MRS and no association between wallaby captivity status and MRS carriage could be assigned. These animals may act as a reservoir for the exchange of genetic elements between staphylococci. Furthermore, the *mecA* genes of animal isolates were identical to that found in human MRS strains and thus the possibility of zoonotic transfer must be considered.

Keywords: *macropods*; *wildlife*; Staphylococcus; *methicillin-resistance*; *SCC*mec

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a frequent pathogen of humans and many animal species. In addition, methicillin-resistant coagulase-negative staphylococci (MRCNS) has long been recognised as important human and animal pathogens. Both MRSA and MRCNS are of interest to human and animal medicine and are collectively known as methicillin-resistant staphylococci (MRS). The *mec* genes encoding resistance to methicillin and almost all β-lactam antibiotics are carried by a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), which is able to integrate into the staphylococcal chromosome at a specific site within the 3′ end of the ribosomal methyltransferase (1, 2). The SCC*mec* element is highly variable in various staphylococcal species. To date, 11 SCC*mec* types (I–XI) and numerous subtypes have been identified in MRSA (2–4). In 2011, a new divergent *mecA* homologue, designated *mecC*, located in a new SCC*mec* cassette–designated SCC*mec* type XI, was described in *S. aureus* (5, 6). This homologue is not detectable using routine *mecA*-specific PCR approaches, and various studies have searched for this new element in different animal hosts (7–10).

The origins of the SCC*mec* elements remain unknown, but it is believed that the *mecA* gene began with a single common ancestor. Homologues of the *mecA* gene have been found in *Staphylococcus sciuri*, *S. vitulinus*, and *S. fleurettii*. Furthermore, the *mecA* gene of *S. fleurettii* has 99 to 100% sequence homology to MRSA strain N315 thus indicating that a direct precursor to the methicillin resistance determinant for MRSA is present in *S. fleurettii* (11–14).

Although studies on MRSA in humans, companion animals, and livestock have been widely documented, there is still a scarcity of information on infections, carriage,

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and the role of this particular pathogen in wildlife. Here, we report the assignation of *mec* types to staphylococcal isolates and from captive and free-ranging wallabies from South Australia, Australia.

Materials and methods

Bacterial isolates

A collection of 89 staphylococcal isolates obtained from captive and free-ranging wallabies in a surveillance study undertaken in 2009–2010 was used in this study (15). Free-ranging wallaby samples were obtained from three colonies living in the Anangu Pitjantjatjara Yankunytjatjara (APY) Lands (102,650 km^2). The APY Lands, classified as a 'managed conservation and natural environment resource' by the Australian Department of Agriculture, are located in the far north-west corner of South Australia, Australia, and are home to approximately 2,230 people across 33 communities and outstations (15, 16). Anterior nasal swabs were collected from 68 captive and 30 free-ranging wallabies during routine health examinations. All animals were assessed as apparently healthy at the time of sampling. Staphylococcal species identification, antibiotic susceptibility profiles, and the presence of the β-lactamase resistance operon from the 89 isolates have been reported previously (15, 17).

Detection of the mecA1, mecA, *and* mecC *elements*

All 89 staphylococcal strains were screened for the presence of *mecA* as previously described (18). Additional PCRs were performed for the detection of the *mecC* gene identified in all cefoxitin-resistant strains (5, 6). Furthermore, all *S. sciuri* isolates were screened for the presence of the ubiquitous *mecA1* homologue (19).

We tested the hypothesis that the carriage of *mecA* and *mecC* in staphylococci would be impacted by the captivity status of the wallaby host. The significance of these data was determined by using chi-square analysis. A p-value ≤ 0.05 was regarded as being statistically significant. The p-values are shown only for results that were statistically significant (20).

Phenotypic screening of cefoxitin resistance

In total, 22 oxacillin-resistant isolates from the 2009–2010 surveillance study and 11 *mecA*-positive isolates identified as described above were assessed for their ability to grow in the presence of cefoxitin by Kirby–Bauer disc diffusion test with a 30-μg cefoxitin disc (Oxoid, Basingstoke, UK). Inoculum preparation, inoculation, and incubation were performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (21). Instead of clinical breakpoints, for the present study, the results were evaluated according to the epidemiological cut-off values of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoints for *S. aureus* and coagulase-negative staphylococci. Data from the EUCAST disc diffusion distribution website were last accessed January 2016 (www.eucast.org). *S. aureus* strain ATCC 6538 and a clinical MRSA SCC*mec* II isolate were used as internal quality controls.

Determination of oxacillin minimum inhibitory concentration

Oxacillin minimum inhibitory concentration (MIC) analysis was carried out on 11 *mecA*-positive strains and two isolates that exhibited discordant results for *mecA*-specific PCR and cefoxitin resistance. Bacterial suspensions were adjusted to 0.5 McFarland standard in 0.85% saline. In total, 14 twofold dilutions of oxacillin were made to cover the concentrations 0.03125 to 256 μg/ml in Mueller–Hinton broth supplemented with 2% NaCl. The test was incubated aerobically at 37°C for 24 h and results were read with a MultiSkan® EX Type 355 (Thermo Fisher Scientific, SA, Australia) spectrophotometer at 595 nm. Interpretation of MIC breakpoints for all strains followed the guidelines provided by EUCAST; data from the EUCAST MIC distribution website were last accessed January 2016 (www.eucast.org). A clinical MRSA SCC*mec* II, *S. aureus* ATCC 6538, and *S. epidermidis* ATCC 12228 were used as quality control strains in addition to a sterility control.

*Typing of SCC*mec *and* ccr *elements*

All 11 *mecA*-positive isolates were typed using multiplex PCR methodologies 1 and 2 with 1.5 mM $MgCl_2$ to classify isolates into the main SCC*mec* types I, II, III, IV, and V. SCC*mec* IV subtyping was performed with multiplex PCR 3 (22). Supplementary SCC*mec*- and *ccr*-typing methodologies (23–25) were used in selected *mecA*-positive strains that could not be assigned a SCC*mec* type with the aforementioned multiplex PCR. Appropriate control strains that have been previously assigned to *mec* classes from Malik et al. were included for the SCC*mec*-typing protocols (26).

Nucleotide analysis of mec *and phylogenetic analysis*

DNA sequences were assembled manually using BioEdit v.7.1.11 (27) (available at www.mbio.ncsu.edu/bioedit/bioedit.html), subjected to homology analysis on National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genbank/) and multiple alignments performed with Clustal X2 with bootstrap 1000 (28) (available at www.clustal.org/clustal2/). The *mec* sequences from Ito et al. (29) were used as references. Approximate likelihood ratio test analysis was performed using PhyML 3.0 with bootstrap 100 analysis (www.phylogeny.fr/one_task.cgi?task_type=phyml) using default settings. Dendrograms were constructed in Newick format using TreeDyn 198.3 (www.phylogeny.fr/one_task.cgi?task_type=treedyn). Nucleotide sequences of *mecA* and *ccrB* genes determined in this study were submitted to

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GenBank under accession numbers KT021005–KT021015, KT003668, and KT003669.

Results

Detection and diversity of the staphylococcal mec *genes from wallabies*

Initially, the presence of *mecA* or *mecC* was set as the gold standard to consider an isolate as a MRS irrespective of its phenotype. The *mecA* gene was detected and subsequently sequenced from 11 staphylococcal isolates. These 11 isolates originated from 7 out of the 98 sampled animals, corresponding to a MRS carriage rate of 7.1%. The *mecA1* gene purportedly present in every *S. sciuri* isolate was detected in only 2 out of the 4 *S. sciuri* isolates recovered in this study (data not shown) and the *mecC* gene was not identified in any isolates.

Sequencing and subsequent bioinformatic analysis of the 11 *mecA* and 2 *mecA1* genes revealed that irrespective of resistance phenotype, all 7 methicillin-resistant *S. fleurettii* isolates contained 19 nucleotide variations in the *mecA* gene and that these variations were comparable to those found in the NCBI database. The remaining 4 *mecA* sequences from *S. cohnii*, *S. epidermidis*, and *S. warneri* were comparable with human-derived MRSA strains found in NCBI. Finally, the *S. sciuri mecA1* genes recovered in this study demonstrated 97 to 99% sequence homology to type strains K11 and ATCC 700061 across 1016 bp (data not shown).

Antimicrobial susceptibility testing

All MRS isolates were found to be susceptible to gentamicin, streptomycin, vancomycin, and chloramphenicol (Table 1) as assessed by Kirby–Bauer disc diffusion. Interestingly, two isolates of *S. fleurettii* (M11, M47) were identified to be β-lactam sensitive by disc diffusion and three cefoxitin-sensitive *S. fleurettii* isolates were identified. Despite their phenotypic resistance profiles, all five isolates demonstrated oxacillin MIC values greater than 16 μg/ml (Table 1). Conversely, *mecA*- and *mecC*-negative isolates M21 (*S. saprophyticus*) and M54 (*S. succinus*) which demonstrated resistance toward ampicillin, penicillin, oxacillin, and cefoxitin had MIC values of 32 μg/ml (Table 1). These two isolates were included in subsequent SCC*mec*-typing protocols to aid in the determination of the genetic mechanism behind this irregular phenotypic profile.

*Typing of SCC*mec *elements from MRS*

In total, 4 different staphylococcal species were identified from 11 *mecA*-positive isolates (Table 1) with the majority dominated by *S. fleurettii* from the *S. sciuri* species group. Overall, five different *ccr*-*mec*-complex combinations were detected in these 11 MRS isolates. These combinations could be broadly separated into three categories based on the method of classification. First, SCC*mec* types III (M72) and V (A16 and A17) were identified by multiplex PCR. Second, two novel variants were identified. Novel variant one (A31) was identified by the unique combination of a novel *ccrAB1* allele 117 and a class A *mec* element. Novel variant two was characterised by the detection of a type 4 *ccrB* element from the aforementioned SCC*mec* III strain M72. Finally, a single non-typeable variant was identified. This non-typeable element accounted for more than half (63.6%) of all SCC*mec* elements identified and all were found in *S. fleurettii* strains (Table 1). No SCC*mec* element genes could be detected for the two *mecA*- and *mecC*-negative cefoxitin-resistant strains (M21 and M54).

Discussion

A systematic review of the literature investigating antimicrobial-resistant bacteria in wildlife populations yielded 210 novel studies up until mid-2015 (30). Studies investigating the presence of *Escherichia coli* (115 studies), *Salmonella* spp (54 studies), and *Enterococcus* spp (43 studies) comprised the bulk of the literature. However, analysis of staphylococcal carriage involving free-ranging animals are scarce as the majority of studies conducted have focused on captive animals which have had regular contact with humans (31–33). However, in recent years, a trend of sampling free-ranging animals for the purpose of determining the prevalence of MRS has emerged (7, 34, 35). Most recently, analysis of faecal pellets from captive and free-ranging brush-tailed rock wallabies revealed the presence of class 1 integrons via PCR amplification (36). However, the present study is the first to investigate the carriage of MRS using nasal swabs collected from apparently healthy, captive, and free-ranging wallabies.

Worldwide, a large range of MRS incidence rates have been reported ranging from 15.4% in Turkish dogs (37), 28.6% in Polish riding horses (38), 29.5% in Belgian pigs (39), and up to 43.0% in Danish goats and sheep (40). Previous studies in Australia have found MRS carriage rates to be 4.0% in South Australian cats and dogs (18), 3.7% in horses admitted to a veterinary intensive care unit in New South Wales (41), and 0.9% in a nationwide study of pigs (42). In the present study, surveillance of healthy wallabies revealed MRS carriage rates of 7.4% for captive wallabies (5 out of 68 animals) and 10.0% for free-ranging animals (3 out of 30 animals). These carriage rates, however, were not considered statistically significant and thus indicate that the carriage of MRS was not affected by the captivity status of the wallabies. This finding is significant as we had expected wallabies with close association with humans to carry MRS more readily compared with those in a low-human environment.

The CLSI now recommends the use of cefoxitin discs as opposed to oxacillin discs for the detection of methicillin resistance in staphylococci by Kirby–Bauer disc diffusion and the amplification of the *mecA* gene by PCR as the

Table 1. Phenotypic and genotypic characterisation of methicillin-resistant staphylococcal isolates from wallabies

		Resistance profile												SCC*mec*-typing				
														ccr complex				
Strain	OX MIC (μg/ml)	AMP	OX	PEN	AMC	FOX	CN	S	VA	CTX	TE	E	C	Multiplex PCR[a]	*ccrB* typing[b,c]	*mec*	SCC *mec*	*mecA* accession number
S. aureus (MRSA)	8	R	R	R	S	R	R	S	S	S	S	S	S	A2B2	ND	A	II	NA
S. aureus ATCC 6538	0.047	S	S	S	S	S	S	S	S	S	S	S	S	–	–	–	NA	NA
S. cohnii (A31)[†]	0.625	R	R	R	S	R	S	S	S	R	S	R	S	–	A1B1 (117)	A	I	KT021007
S. epidermidis (M72)[‡]	1.5	R	R	R	S	R	S	S	S	iR	S	S	S	A3B3 + C	A4B4 (602)	C C	III	KT021015
S. fleurettii (A59)[‡]	16	R	R	R	S	R	S	S	S	iR	S	S	S	–	–	A	NT	KT021008
S. fleurettii (A61)[‡]	32	R	R	R	S	R	S	S	S	iR	S	S	S	–	–	A	NT	KT021009
S. fleurettii (A69)[‡]	32	R	R	R	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021010
S. fleurettii (A72)[‡]	32	R	R	R	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021011
S. fleurettii (M11)[‡]	16	S	S	S	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021012
S. fleurettii (M31)[‡]	16	R	R	R	S	S	S	S	S	iR	S	S	S	–	–	A	NT	KT021013
S. fleurettii (M47)[‡]	16	S	R	S	S	S	S	S	S	S	S	S	S	–	–	A	NT	KT021014
S. warneri (A16)[†]	4.5	R	R	R	S	R	S	S	S	iR	R	R	S	5	–	C	V	KT021005
S. warneri (A17)[†]	4	R	R	R	S	R	S	S	S	iR	R	R	S	5	–	C	V	KT021006
S. saprophyticus (M21)[‡]	32	R	R	R	S	R	S	S	S	S	S	R	S	–	–	–	–	NT
S. succinus (M54)[‡]	32	R	R	R	S	R	S	S	S	S	S	S	S	–	–	–	–	NT

[†]Staphylococci from free-ranging animal.

[‡]Staphylococci from captive animal.

[a]Multiplex PCR performed as recommended by Kondo et al. (22).

[b]*ccrB*-typing performed as recommended by Oliveira et al. (25).

[c]*ccrB* sequence submitted to GenBank under accession numbers: KT003668 and KT003669.

R, resistant; iR, intermediate resistant; S, susceptible; –, no amplification; NA, not available; NT, not typeable; AMP, ampicillin; OX, oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; CN, gentamicin; S, streptomycin; VA, vancomycin; TE, tetracycline; E, erythromycin; C, chloramphenicol.

gold standard (43). However, similar to other authors (44), we detected staphylococcal isolates which contained the *mecA* gene but were susceptible to both oxacillin and cefoxitin by disc diffusion. Our strains had a non-induced oxacillin MIC of 32 μg/ml compared to 1 μg/ml observed by Cuirolo et al. (44). This demonstrates the limitations of using cefoxitin as a predictor of *mecA*-mediated methicillin resistance.

Many SCC*mec*-typing strategies have been developed since the publication of its discovery in 2000 (45). However, the majority of studies have been conducted with human samples and thus most methodologies were designed specifically for the detection of MRSA. As all MRS identified in this study were CNS, a range of multiplex PCR strategies were selected from the literature and trialled in this study. No PCR amplicons could be

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obtained from the 11 MRS isolates using PCR methodologies from Lim et al. (23) or Zhang et al. (24). SCC*mec*-typing by the Kondo multiplex PCRs (22) showed the carriage of known types (III and V). However, the majority of *mec* elements, identified as containing class A *mec* elements, were unable to be classified by this method.

MRCNS are considered to be a source of SCC*mec* elements by horizontal gene transfer to *S. aureus* and the diversity of SCC*mec* element types among CNS is larger than that among *S. aureus* (14). Although *S. aureus* was not found in coexistence with MRCNS in our nasal swabs, native wildlife have already been shown to potentially act as a reservoir for multidrug-resistant staphylococci (15) and could function as a reservoir for the evolution of novel SCC*mec* types. This is extremely worrisome given that *S. epidermidis* and the *S. sciuri* species group are also opportunistic pathogens and their zoonotic potential cannot be discounted (46). In this study, two novel variants of the existing SCC*mec* types were identified by sequencing the *ccrB* gene. Variant one contained a class A *mec* element with a previously undescribed variant of *ccrAB1* thus leading to the formation of a novel SCC*mec* I variant (*S. cohnii* strain A31). Variant two was identified by the discovery of a type 4 *ccrAB* element in *S. epidermidis* SCC*mec* III strain M72. Furthermore, two MRS isolates (*S. warneri* strains A16 and A17) from a single free-ranging wallaby were identified as harbouring community-acquired SCC*mec* type V. This SCC*mec* type was first identified in the Australian indigenous population in 2005 (47), and the discovery of this element in free-ranging wildlife living in indigenous land is further evidence of its community origin.

Conclusions

To be best of our knowledge, this is the first report of MRS in captive and free-ranging wallabies in Australia. Our data demonstrate the absence of MRSA and a low prevalence of MRCNS in both captive and free-ranging wallabies, indicating that MRS occurs naturally even in the absence of human intervention. However, the presence of multidrug-resistant staphylococci carrying the *mecA* gene isolated from indigenous land is worrisome and may have implications for wildlife rehabilitation and subsequent antimicrobial treatment in cases of wound infections, localised inflammation and systemic bacterial infections. This study highlights the need for further longitudinal and environmental studies involving a larger range of native wildlife species in order to increase our understanding regarding the epidemiology of resistance genes.

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Conflict of interest and funding

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