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Running title: Methicillin-resistant staphylococci in cow's milk

Highlights

- Methicillin-resistant staphylococci surveyed on dairy farms in England and Wales
- Present on *c*. 5% of dairy farms
- Six species were found including potential zoonotic MRSA
- Genome sequencing revealed a range of SCC*mec* types and other resistance determinants
- Evidence for the transfer of primordial *mec* gene complex between different species

Abstract

Objectives To investigate the prevalence and characteristics of methicillin-resistant staphylococci on

dairy farms in England and Wales including zoonotic MRSA.

Methods Bulk tank milk was sampled from 363 dairy farms in 2015-2016 and methicillin-resistant

staphylococci were isolated by salt broth enrichment and plating on MRSA Brilliance selective agar.

Isolates were characterised through antimicrobial susceptibility testing and whole-genome sequencing.

Results Methicillin-resistant staphylococci were isolated from ~5% of dairy farms and belonged to six different species, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lentus*, *Staphylococcus saprophyticus*, *Staphylococcus fleurettii* and *Staphylococcus sciuri*. Whole-genome sequencing revealed a large variety of antimicrobial resistance genes and SCC*mec* elements were present, including *mecA* and *mecC* alleles. Potentially zoonotic methicillin-resistance *S. aureus* were found at a low prevalence (0.83% of sampled dairy farms). Whole-genome sequencing also provided evidence for the mobility of a primordial *mec* gene complex, independently of a SCC*mec* element, which appears to have been acquired by *S. saprophyticus* from *S. fleurettii*.

Conclusions These data give new insight into the epidemiology of veterinary methicillin-resistant staphylococci to inform future surveillance and zoonotic risk evaluation. Our data indicate that MRSA has likely decreased in prevalence since earlier survey work in England and Wales during 2011-12 and highlights the diversity of methicillin resistance and other resistance determinants among bovine-associated staphylococci with implications for veterinary and human medicine.

Introduction

The emergence and spread of antimicrobial resistance is a significant and growing public health concern throughout the world [1]. In this context, antimicrobial-resistant bacteria in food-producing animals are a major concern as a potential source for human infection [2]. Such zoonosis could occur either through direct contact with animals, via production or consumable of animal products such as

foodstuffs or indirectly through environmental dissemination. Concern is also be raised not simply by the potential transmission of resistant organisms themselves but by the horizontal gene transfer of resistance determinants from the animal microflora to human pathogens. Antimicrobial resistance is also economically important to farmers as a cause of treatment failure and prolonged duration of disease and treatment with an additional for animal welfare [3].

Staphylococcus aureus and particularly methicillin-resistant *S. aureus* (MRSA) is one example of an antimicrobial resistant pathogen found in livestock and apparently able to transfer to humans. Indeed, *S. aureus* and MRSA have a wide host range facilitated by horizontal gene transfer and core genome diversification [4]. MRSA are resistance to all but the latest generation of β-lactams antimicrobials by virtue of a modified penicillin-binding protein, PBP2a, encoded by the *mecA* gene which is carried on the mobile genetic element, SCC*mec* [5]. MRSA has been found in a wide range of animals [6-10] including various livestock species [11] and can pose a zoonotic risk to humans. Indeed, livestock-associated MRSA is now recognised as a third epidemiological form of MRSA in humans alongside health care-associated MRSA and community-associated MRSA with clonal complex (CC) 398 among the most prominent lineages responsible for LA-MRSA [12-14].

Interest in MRSA in the UK dairy herd has risen following the detection of LA-MRSA CC398 in bulk tank milk [15] and the isolation of MRSA encoding *mecC*, a distinct variant of *mecA*, from a dairy farm in England [16,17]. A subsequent prevalence study found *mecC* MRSA to be present on 2.15% of dairy farms across England and Wales [18] and in a wide range of host species [17], with evidence for zoonotic transmission from livestock to humans [19,20].

While previous work has focussed on MRSA in dairy cattle in the UK, methicillin resistance encoded by *mecA* and *mecC* has been described in a wide range of other staphylococcal species found in humans and animals. In the case of bovine mastitis this includes (among others); *mecA*-positive *Staphylococcus epidermidis* [21-27]; *Staphylococcus sciuri* [21,22,28]; *Staphylococcus saprophyticus* [25] and *Staphylococcus haemolyticus* [21,22,25,27,28]. By comparison to *mecA*, reports of *mecC*-positive non-*aureus*

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staphylococci in bovine milk are less frequent but do include *Staphylococcus xylosus* [29], *Staphylococcus pseudoxylosus* [30] and *S. saprophyticus* [31] with several other *mecC*-positive *Staphylococcus* species being isolated from other animal hosts and the environment [32-34]. Unfortunately, in many studies methicillin-resistant coagulase-negative or non-*S. aureus* staphylococci are isolated from bovine milk but are not identified to the species level and/or their genetic basis of resistance investigated [35-37].

The isolation of staphylococcal species, including methicillin-resistant isolates, from dairy cattle or in milk is potentially important as a cause of mastitis, a source of resistant zoonotic infection and as a reservoir for antimicrobial resistance genes. However, there is currently little data on how common methicillin-resistant staphylococci are among British dairy herds, which species they belong to and the resistance genes that they encode. We therefore undertook a study to isolate and characterise not just MRSA but all methicillin-resistant staphylococci (MRS) from dairy milk collected in England and Wales.

Materials and Methods

Isolation of methicillin-resistant staphylococci from bulk tank milk

Bulk tank milk samples were supplied by National Milk Laboratories Ltd. (Chippenham, UK) and processed as described previously [18]. In brief, samples were collected aseptically by trained technicians

for quality assurance purposes and stored at 4°C for up to 5 days before freezing at -20°C prior to testing. One hundred and thirty-two samples were collected in September 2015 and 231 collected in February 2016. The frozen samples were thawed at 37°C and 1 mL of milk was added to 4 mL of Mueller–Hinton broth (Oxoid, Basingstoke, UK) supplemented with 6.5% (w/v) NaCl and incubated at 37°C for 24 h with shaking at 200 rpm. After which, 50 µL of culture was spread onto MRSA Brilliance 2 plates (Oxoid, Basingstoke UK) and incubated at 37°C for 24 h. From each sample, colonies with distinct morphologies were sub-cultured on Columbia blood agar with 5% horse blood (Oxoid). To confirm the apparent methicillin resistant phenotype of isolates they were then tested for cefoxitin resistance by disc diffusion following European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2015 clinical guidelines. Isolates not displaying cefoxitin resistance by disc diffusion were not studied further. In the initial absence of a species identification, the zone diameter breakpoints S≥25, R<25 mm were used as per EUCAST. Results were re-interpreted using the appropriate species breakpoint once a staphylococcal isolate was identified following genome sequencing and in all such cases these isolates were all still deemed to be cefoxitin resistant by disc diffusion. *S. aureus* strains NCTC12493 and NCTC12973 were used as positive and negative controls respectively for cefoxitin resistance. Following disc diffusion, cefoxitin-resistant isolates then underwent PCR with primers specific for the 16S rRNA gene of *Staphylococcus* using the primers (5'CCTATAAGACTGGGATAACTTCGGG 3' and 5'CTTTGAGTTTCAACCTTGCGGTCG3') as described previously [38]. Isolates positive for staphylococcal 16S rRNA gene were then whole genome-sequenced and characterised further. Those isolates negative or producing only a faint product with the *Staphylococcus* 16S rRNA gene primers underwent a further PCR with the universal 16S rRNA gene primers fD1 (5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3') and fD2 (5'CCGAATTCGTCGACAACAGAGTTTGATCATGGCTCAG3') as described previously [39]. The ensuing PCR products were partially sequenced by Sanger sequencing (Source BioScience, Nottingham) using the PCR primers and the isolates identified by BLAST analysis of the resultant partial 16S rRNA gene sequence. Isolates confirmed by this analysis to belong to genera other than *Staphylococcus* were not

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studied further here. Antimicrobial sensitivity testing was performed on resultant methicillin-resistant *Staphylococcus* isolates by Vitek2 using the AST-P635 card following the manufacturer's instructions with the MIC of cefoxitin determined using the ETEST® (both BioMérieux, Basingstoke UK).

Whole genome sequencing and analysis

Whole genome sequencing (WGS), using Illumina HiSeq technology with 2x250 bp paired-end reads, read trimming and assembly was performed by Microbes NG (University of Birmingham, UK). Reads were trimmed using Trimmomatic version 0.30 [40], using a sliding window quality cut-off of 15. Genome assembly was done *de novo* using SPAdes, version 3.7 [41], with default parameters for 250 bp Illumina reads. Assemblies were annotated by the NCBI Prokaryotic Genome Annotation Pipeline. Acquired resistance genes were identified and multilocus sequence types where extracted from the genome sequence using ResFinder-3.1 [42] and MLST 2.0 [43] respectively. SCC*mec* typing from the genome sequences was performed by SCC*mec*Finder 1.2 [44]. Schematic comparison of the *mecA* region was done with EasyFig [45].

Results and discussion

Isolation of methicillin-resistant staphylococci from bulk tank milk

Raw bulk tank milk samples from 363 unique dairy farms across England and Wales were tested for the presence of putative methicillin-resistant staphylococci by salt broth enrichment and culture on

MRSA Brilliance plates. No growth was present from 122 bulk tank samples with the remaining 241 samples producing growth on the MRSA Brilliance plates. A wide range of colonies types were present, often within the same sample, and a resultant total of 305 isolates were collected. Confirmation of methicillin-resistance was performed using cefoxitin disc diffusion with 99 isolates from 76 farms showing resistance. These 99 were then examined by staphylococcal-specific 16S PCR to confirm if they belonged to the *Staphyloccccus* genus or not. Seventy-five isolates were negative for the staphylococcus 16S amplicon or produced a weak band suggesting that these belonged to other genera. Amplification and sequencing of their 16S rDNA using universal primers showed this to be the case for all 75 isolates which were; *Enterocococus faecium* (26 isolates), *Macrococcus caseolyticus* (14 isolates), *Bacillus* species (10 isolates), *Enterococcus faecalis* (9 isolates), other enterococci (7 isolates), *Burkholderia* species (4 isolates) and other species (5 isolates). The *M. caseolyticus* isolates have since been confirmed to be methicillin resistant and to encode the *mec* variants *mecB* and *mecD* [46]. The isolation of a range of species show that MRSA Brilliance when used in this context is not specific for the isolation of staphylococci and so care is needed to subsequently identify isolates. We investigated all colony types, even those considered not likely to staphylococci to ensure that no cultured staphylococci were inadvertently missed from subsequent analysis.

Together this resulted in a total 24 methicillin-resistant staphylococci (as confirmed by cefoxitin disc diffusion and 16S amplification with staphylococcal-specific primers) from 18 dairy farms. Isolates were identified and antibiograms generated by Vitek 2. Where identification and antibiograms were identical between isolates from the same farm only a single isolate from each farm was taken for further study and whole genome sequencing resulting in a final total of 18 isolates from 18 different dairy farms representing 4.96% of the 363 sampled, Table 1.

Identification and SCC*mec* typing of methicillin-resistant staphylococci

The 18 isolates of methicillin-resistant staphylococci belonged to 6 species with the most abundant being *S. sciuri* (6 isolates), the others being *S. epidermidis* (4 isolates), *S. aureus* (3 isolates), *S.*

saprophyticus (3 isolates), *Staphylococcus fleuretti* (1 isolate) and *Staphylococcus lentus* (1 isolate). Thirteen of the isolates encoded *mecA*, a single isolate of S. *aureus* possessing *mecC* with 3 isolates of *S*. *sciuri* encoding both *mecA* and *mecC*. A variety of SCC*mec* types were present, template coverage matches of >75% were identified to SCC*mec* type IVa(2B) in *S*. *aureus* (1 isolate) and *S*. *epidermidis* (2 isolates), type IVc(2B) in *S*. *epidermidis* (1 isolate), type XI in *S*. *aureus* (2 isolates), type III(3A) in *S*. *lentus* (1 isolate) and *S*. *sciuri* (1 isolate), Table 1. Weaker matches to SCC*mec* type III(3A) (63-66%) were present in *S*. *saprophyticus* (2 isolates) and *S*. *sciuri* (2 isolates) and to type I(1B) *S*. *epidermidis* (1 isolate), indicative of variant SCC*mec* elements being present in these isolates. The 3 *S*. *sciuri* isolates encoding both *mecA* and *mecC* carry the hybrid SCC*mec-mecC* previously described in this species [47]. These 3 isolates have since been analysed along with other *mecA/C*-positive *S*. *sciuri* isolates in further work which shows this *SCCmec-mecC* is highly conserved but can be differentiated into two variants on the basis of the encoded *ccr* genes [48]. Isolates *S*. *saprophyticus* EF72a and *S*. *fleuretti* EF187 encoded *mecA* but no SCC*mec* element was detected and these were therefore examined in more detail, Figure 1. *S*. *fleuretti* EF187 encoded *mecA* on the chromosome not associated with a SCC*mec* element but in an arrangement as described previously in methicillin-resistant *S*. *fleuretti* [49]. Five structural types, A-E, has been described for the genomic organisation of such *mecA* regions in *S*. *fleuretti*, the example in isolate EF187 belonging to Type B which lacks a transposase present in the commoner Type A between the genes *mvaS* and *ugpQ* as seen in *S*. *fleuretti* CCUG 43834^T, Figure 1. The 5' portion of this *mecA* region was highly conserved between *S*. *fleuretti* CCUG 43834^T and *S*. *saprophyticus* EF72a with the latter possessing the transposase absent in *S*. *fleuretti* EF187, Figure 1. However, the 3' region after the *xylR* gene was entirely different in *S*. *saprophyticus* EF72a compared to the two *S*. *fleuretti* comparators. *S*. *saprophyticus* EF72a carrying the *icaADBC* locus encoding for the polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) involved in staphylococcal biofilm formation [50]. Consistent with the inability of *S*. *saprophyticus* to ferment xylose, *xylABE* were not present elsewhere in the EF72a genome. The *mvaACS* genes of *S*. *saprophyticus* EF72a are more closely related to those from *S*. *fleuretti* CCUG 43834^T than to those of *S*. *saprophyticus* ATCC15305^T

(99.9-100% and 60.8-69.9 respectively). This indicates their likely acquisition by *S. saprophyticus* EF72a from *S. fleuretti* and probably also that of the associated *mec* gene complex. We believe this is the first description of this non-SCC*mec mec* gene complex in *S. saprophyticus*. It has been postulated to have been a progenitor for the generation of the SCC*mec* element and here we provide further evidence for its transmission between staphylococcal species.

<u>Antimicrobial resistance and multi-locus typing</u>

All 18 isolates were resistant to benzylpenicillin as assessed by Vitek2. Despite the cefoxitin disc diffusion results in which all isolates were resistant, two of the isolates, EF187 and EF220, were susceptible in the Vitek2 cefoxitin screen, Table 1. All but two isolates were resistant to oxacillin. The two in question being the *mecC*-positive *S. aureus* isolates which displayed the Vitek2 profile of cefoxitin-resistant/oxacillin-sensitive which is common among such isolates and indicative of a MRSA isolate being *mecC* positive [51,52]. As is also very common for *mecC* MRSA these 2 isolates lacked resistance to non-β-lactams antimicrobials [17]. In contrast, the other methicillin-resistant staphylococci encoded resistance genes to additional antimicrobial classes and several displayed multidrug resistance illustrating the reservoir of antimicrobial resistance present in bovine-associated staphylococci. The most common non-β-lactams phenotypic resistance was to fucisdic acid present in 10 isolates, followed by tetracycline and clindamycin, both seen in 6 isolates. There was no perfect agreement between antimicrobial resistance genotypes and phenotypes due to a number of factors including antimicrobials not being included in the Vitek2 panel, exclusion of mutation-based resistance in the ResFinder database and the absence of some acquired resistance genes such as *fusD*. Work is ongoing to resolve any discrepancies and it is possible that isolates may encode hitherto unreported antimicrobial resistance genes.

To place these methicillin-resistance staphylococci into the context of their wider populations, multi-locus sequence typing was performed for the species where such a scheme exists, namely *S. aureus* and *S. epidermidis* in this case. The 2 *mecC* MRSA isolates belonged to ST130 and ST425 which are the

predominant lineages among *mecC* MRSA and have been isolated from a wide range of hosts species including humans and dairy cattle [17]. Previously *mecC* MRSA belonging to ST425 and ST130 had been detected on 2.15%, (95% CI 1.17%–3.91%) of 465 sampled dairy farms in England and Wales between November 2011 and October 2012 [18]. The prevalence in this current study is much lower at 0.55% (95% CI 0.21% to 1.31%), although not statistically different, and indicates that prevalence has not increased since the earlier study and indeed most likely has fallen. This is consistent with studies of human MRSA isolates in England finding only negligible numbers of *mecC* MRSA [53,54]. The single *mecA* MRSA isolate belonged to the livestock-associated lineage ST398 and carried the canonical SNPs associated with this lineage [55]. In many countries this lineage poses an occupational risk to those in contact with livestock and in some regions is a predominant lineage among human MRSA isolates [56-58]. In the United Kingdom, it has been isolated sporadically from various animals [59-62] and their products [63,64] including bulk tank milk [15,18] but human isolates appear limited at present [53,54]. The finding of a single isolate from 363 dairy farms in this study is similar to a previous study finding a single among 465 dairy farms [18] and indicates that LA-MRSA CC398 continues to be present albeit at a low level in bulk tank milk in England and Wales.

S. epidermidis isolates belonged to sequence types ST1, ST59, ST88 and a novel *pyrR* single locus variant of ST59. The *S. epidermidis* MLST website (https://pubmlst.org/ sepidermidis/) [65] shows that ST1, ST59 and ST88 have all been isolated from humans and thus may be lineages able to transmit between host species. Indeed, *S. epidermidis* ST59 has also been isolated from bovine mastitis and ST88 from a cat according to the *S. epidermidis* MLST website.

Prompted by the discoveries of *mecC* MRSA and livestock-associated MRSA on British dairy farms this current study has investigated the prevalence and characteristics of all methicillin-resistant staphylococcal species isolated from bulk tank milk in England and Wales. The study of 363 dairy farms found nearly 5% to be positive for methicillin-resistant staphylococci which belonged to six different species and which encoded a variety of SCC*mec* types and other antimicrobial resistance genes. This

gives new insight into the epidemiology of veterinary methicillin-resistant staphylococci, informs future surveillance and the evaluation of zoonotic risks. Our data indicate that CC398 LA-MRSA and *mecC* MRSA have not increased in prevalence in bulk tank milk in England and Wales since earlier work, indeed the latter has likely declined. Finally and of particular note, we provide evidence for the horizontal gene transfer between of staphylococcal species of the *mec* gene complex independent of a SCC*mec* element.

DECLARATIONS

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Ethical Statement

No humans or animals were specifically sampled for this study, the milk samples investigated were taken for the purposes of routine milk quality assurance of UK dairy farms operating under relevant UK and EU welfare legislation and thus no further ethical approval was considered.

Conflict of Interest Statement

None to declare.

Acknowledgements

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Figure 1

Genomic organisation of the *mecA* region (non-SCC*mec*) in *S. fleuretti* and *S. saprophyticus*. Comparison of the *mecA* regions of *S. fleuretti* 187 and *S. saprophyticus* 72a generated in this study with the archetypical region described previously in *S. fleuretti* CCUG 43834[T 49].
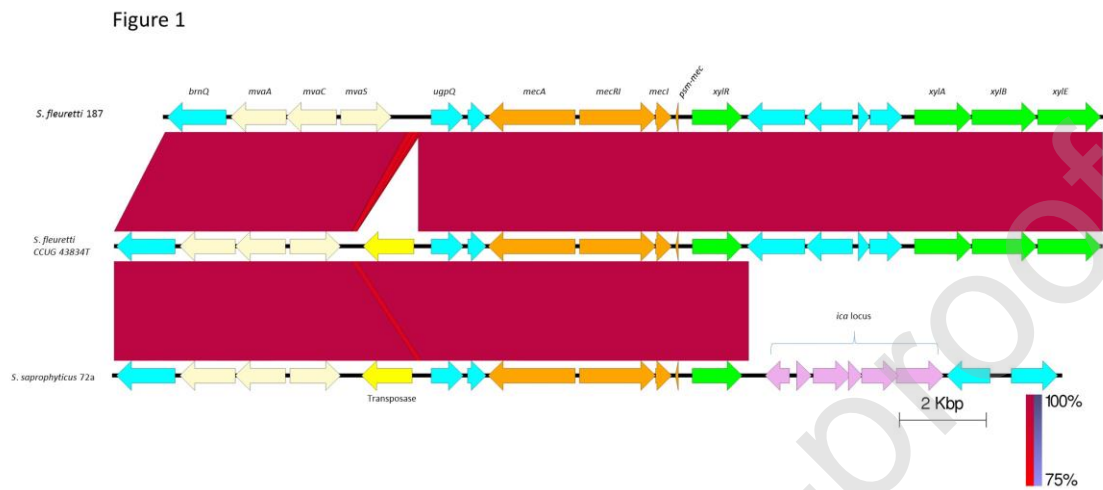

Figure 1

Table 1 Characteristics of methicillin-resistant staphylococci form bulk tank milk

Isolate	Species	Regional location	ST	mec-gene	Cefoxitin MIC (µg / ml)	Other phenotypic resistance¹	Other resistance genes	SCC*mec* type (template coverage)	Genome nucleotide accession
EF39a	*S. aureus*	Devon	398	A	96	pen, gen, cip, tet, trim	*aac*(6')-*aph*(2''), *blaZ*, *mecA*, *tet*(M), *dfrK*	Type IVa(2B) (95.49%)	ERR3357335
EF9a	*S. aureus*	Cleveland	130	C	12	pen	*mecC, blaZ*	Type XI(8E) (99.19%)	ERR3357332
EF65b	*S. aureus*	Berkshire	425	C	16	pen	*mecC, blaZ, fosD*	Type XI(8E) (99.81%)	ERR3357296
EF181	*S. epidermidis*	Lancashire	1	A	48	pen, tei, tet, trim	*mecA, blaZ* (x2), *fosB*	Type I(1B) (50.85%)	ERR3357299
EF201	*S. epidermidis*	Somerset	88	A	32	pen, tet, fus, chl	*mecA, blaZ, fosB, fusB, fexA*	Type IVa(2B) (88.50%)	ERR3357302
EF275	*S. epidermidis*	Denbighshire	SLV59 (*pyrR*)	A	12	pen, ery, cli, tei, tet,	*mecA, fosB*	Type IVa(2B) (76.24%)	ERR3357304
EF295	*S. epidermidis*	Flintshire	59	A	12	Pen, tet	*str, mecA, blaZ, fosB, tet*(K)	Type IV(2B) (98.18%)	ERR3383514
EF187	*S. fleurettii*	Shropshire	-	A	8	pen, clin, fus, trim	*mecA*, sal(A)	No SCC*mec*	ERR3357300
EF220	*S. lentus*	Somerset	-	A	6	pen, cli, dap, tei, fus, trim, (ery) (tet) (rif)	*blaZ, mecA, mph*(C), *erm*(43), *tet*(K)	Type_III(3A) (89.33%)	ERR3357303
EF192	*S. saphrophyticus*	Pembrokeshire	-	A	16	pen, ICR, fus, (mup)	*mecA, msr*(A), *mph*(C), *lnu*(A)	Type_III(3A) (62.84%)	ERR3357301
EF72a	*S. saphrophyticus*	Isle of Wight	-	A	12	pen, ICR, tet, chl	*str, mecA, erm*(C), *fexA*	No SCC*mec*	ERR3357297
EF121	*S. saphrophyticus*	Cleveland	-	A	24	pen, clin, fus	*mecA, lnu*(A)	Type III(3A) (62.85%)	ERR3357298
EF7a	*S. sciuri*	Cheshire	-	A	>256	pen, dap, fus, (clin)	*mecA1, mecA, fosD,* sal(A)	Type III(3A) (81.62%)	ERR3357330
EF288	*S. sciuri*	Denbighshire	-	A	16	pen, dap, fus, (clin) (trim)	*mecA1, mecA, lnu*(A), sal(A)	Type III(3A) (66.26)%	ERR3383513
EF365	*S. sciuri*	Not applicable (transport tanker sample)	-	A	24	pen, clin	*mecA1, mecA, lnu*(A), sal(A)	Type III(3A) (65%)	ERR3383518
EF3a	*S. sciuri*	Lancashire	-	AC	>256	pen, dap, fus, (clin), (trim)	*str, mecA1, mecA, mecC, blaZ,* sal(A)	Hybrid SCC*mec-mecC*	ERR3357328
EF260	*S. sciuri*	Shropshire	-	AC	24	pen, clin, fus	*str, mecA1, mecA, mecC, blaZ,* sal(A)	Hybrid SCC*mec-mecC*	ERR3383511
EF324a	*S. sciuri*	Staffordshire	-	AC	128	pen, clin, fus, trim	*mecA1, mecA, mecC, blaZ,* sal(A)	Hybrid SCC*mec-mecC*	ERR3357305

¹Vitek2 AST-P635 panel: cefoxitin (screen), benzylpenicillin (pen), oxacillin, gentamycin (gen), ciprofloxacin (cip), inducible clindamycin resistance (ICR), erythromycin (ery), clindamycin (clin), daptomycin (dap), teicoplanin (tei), vancomycin (van), tetracycline (tet), tigecycline, fusidic acid (fus), mupirocin (mup), chloramphenicol (chl), rifampicin (rif) and trimethroprim (trim). Intermediate resistance indicated by parentheses.