Title

Detection of *mecC*-MRSA isolates in river water: a potential role for water in the environmental dissemination.

Authors

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Running title

Presence of *mecC*-MRSA in river water.

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a public health concern due to limited treatment options. The recent description of a *mecA* homologue, *mecC* in human and cattle, led to studies to detect this new variant in human and other animal species. Detection of *mecC* in wild boar and fallow dear in a Spanish game estate led us to further investigate the presence of *mecC*-MRSA at this location. Samples from cattle, wild animals, workers and river water were tested. A further three *mecC*-MRSA isolates were obtained from river water. Molecular characterization (MLST and *spa* typing) and antimicrobial susceptibility testing (broth microdilution) showed that isolates were similar to those detected in wild animals. Whole genome sequencing confirmed that the isolates from the river water and wild animals in the same geographic area were all closely related isolates of ST425 *mecC*-MRSA. The presence of *mecC*-MRSA in the river water highlights the potential role of water in the dissemination of *mecC*-MRSA.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequent causes of invasive bacterial disease in Europe (Grundmann *et al.*, 2010; Gagliotti *et al.*, 2011). Resistance to β-lactam antibiotics in MRSA is mediated by the expression of an alternative penicillin-binding protein (PBP2a) which is encoded by the *mecA* gene. In 2011, a divergent form of the *mecA* gene named *mecC*, was described in humans and cattle (García-Álvarez *et al.*, 2011), and more recently has been detected in a range of animal species, including wild animals (García-Álvarez *et al.*, 2011; Paterson *et al.*, 2012; Walther *et al.*, 2012; Porrero *et al.*, 2014). More recent studies in Denmark using traditional epidemiology and whole genome sequencing have shown that *mecC*-MRSA can be transmitted from livestock to humans (Harrison *et al.*, 2013; Petersen *et al.*, 2013). Although previous studies have identified direct contact as the most plausible mechanism of MRSA transmission between animals and humans (Verkade and Kluytmans, 2014), the potential role of other mechanisms of dissemination remains unknown.

The presence of antibiotic resistant bacteria in water has been previously recognized and is likely due to selective pressure linked to antimicrobial residues in water and high concentration of microorganisms, which promote exchange of genetic material (Baquero *et al.*; 2008, Lupo *et al.*, 2012). Specifically, MRSA has been reported to survive in river and water environments (Tolba *et al.*, 2008). In municipal wastewater and recreational marine water, detection of MRSA has been associated with shedding by colonized people (Borjesson *et al.*, 2009; Plano *et al.*, 2011; Plano *et al.*, 2013). However, although wastewater treatment plants diminish the MRSA proportion, wastewater discharge might act as a reservoir for MRSA (Borjesson *et al.*, 2009; Rosenberg Goldstein *et al.*, 2012). Recently, *mecC*-MRSA have been detected in an urban effluent in Spain (Porrero *et al.*, 2014).

A number of human *mecC*-MRSA cases have been identified (Basset *et al.*, 2013; Romero-Gómez *et al.*, 2013; García-Garrote *et al.*, 2014), with no obvious epidemiological links to livestock or wild animals. We previously identified three *mecC*-MRSA isolates from wild boar (n=1) and fallow deer (n=2) in the same game estate (Porrero *et al.*, 2014). This, combined with the low prevalence of MRSA in wild animals in Spain (Porrero *et al.*, 2013), led us to further investigate the presence of *mecC*-MRSA in the game estate, so we returned to more comprehensively sample wildlife, domestic animals, workers and river water. *mecC*-MRSA isolates were detected and subjected to whole genome sequencing to understand the phylogenetic relationship between isolates. Analysis identified that closely related isolates were circulating in both wildlife and river water, highlighting a possible mechanism for spreading between animals and potentially even transmission on to humans.

Results and discussion

The presence of *mecC*-MRSA in wild animals led us to perform additional studies in the same game estate where *mecC*-MRSA was isolated in wild boar (n=1) and fallow deer (n=2) (Porrero *et al.*, 2014). None of the animals examined in the present study [wild boars (n=10), red deer (n=10), veal calves (n=20) and beef cattle (n=48)] were positive for *mecC*-MRSA (or *mecA*-MRSA) corroborating the low frequency of *mecC*-MRSA isolates in wild animals (Porrero *et al.*, 2014). None of the persons working within the game estate (n=14) were positive for MRSA. However, three additional *mecC*-MRSA were isolated from two water subsamples of one sample (1 L) out of the six river water taken inside the game state. An additional river water sample taken outside the game estate (4.4 km upstream) was negative. This is the first report of isolation of *mecC*-MRSA from an environmental source. The three *mecC*-MRSA isolates from the river´s water had

identical MLST and *spa* type (ST425-t11212) to those isolated from wild animals (Porrero *et al.*, 2014) indicating that the same genotype of *S. aureus* was shared by wild animals and river water of the game estate.

Whole genome sequencing was carried out to further elucidate the genetic relationship between the wild animals and river water isolates. Phylogenetic analysis using SNPs present in the core genome (the core genome been defined as the all the genomic regions not part of mobile genetic elements). indicated that all the isolates were closely related, differing by a total of 132 SNPs (Fig. 1). Wild animal and river water isolates clustered into three separate clades which were differentiated by ~100 SNPs (Figure 1) One of the clades contained the two isolates from fallow deer (ZTA12/02038STA and ZTA12/02038STB) differing from each other by 1 SNP. These isolates differed from the isolate from the wild boar (ZTA09/03698-9ST) by ~70 SNPs. The three river water isolates (ZTA13/00933–98ST, ZTA13/00933–98SA and ZTA13/00933–60SA) clustered in a different clade (Fig. 1); isolates ZTA13/00933–98SA and ZTA13/00933–98ST were identical and differed from isolate ZTA13/00933–60SA by 33 SNPs. The three isolates from the river water differed from the isolate from the wild boar (ZTA09/03698-9ST) by ~50 SNPs and from isolates of fallow deer by ~100 SNPs. Recently, up to 40 SNPs have been identified between isolates from a single colonised human (Golubchik *et al.*, 2013). All six isolates displayed a highly conserved accessory genome, except for the loss of a phage in isolate ZTA13/00933–60SA. Overall, phylogenetic analysis indicated that all the isolates were highly related which suggests the existence of a closely related *mecC*-MRSA ST425-t11212 population circulating between the different animal species and the water environment in the investigated game state. Water represents a major pathway for the dissemination of bacteria (Vaz-Moreira *et al.*, 2014) and MRSA are able to survive for several days in contaminated water environments (Tolba *et al.*, 2008). Therefore, the

presence of *mecC*-MRSA in the river water highlights the potential role of water in the dissemination of *mecC*-MRSA. In addition, the fact that both wild animals and livestock can share a common water source might also facilitate the transmission of MRSA between different animal species. Further studies will be necessary to evaluate the role of water in the maintenance and spread of MRSA between different environmental compartments.

Antimicrobial resistance profiles were also similar, with isolates from the water being resistant to benzylpenicillin and cefoxitin but susceptible to all other antimicrobials tested as identified for wild animals (Porrero *et al.*, 2014). Those antimicrobial susceptibility profiles fit with those previously described for *mecC*-MRSA isolates (Paterson *et al.*, 2014). Further analysis of the genome sequence of the *mecC*-MRSA isolates from the river water and wild animals (n=6) only identified the resistance genes *mecC* and *blaZ*$_{LGA251}$, which fit with the observed antimicrobial resistance phenotypes.

Comparison of the SCC*mec* type XI of the six *mecC*-MRSA isolates with that of the strain LGA251 revealed that it was highly conserved (99.94% identity over 29,422 bp) in all the isolates. The isolate from wild boar ZTA09/03698-9ST contained an extra ~14.4 kb insertion (Figure 2) that corresponds to a putative conjugative transposon inserted in one hypothetical protein (SARLGA251_00430; NCBI accession no: NC_017349). This transposon exhibited higher than 95% nucleotide identity with transposons present in a number of mobile genetic elements (plasmid pSK53; NCBI accession no: GQ915272; a SCC*mec* type II; NCBI accession no: AB435014) or the chromosome (NCBI accession no: HE681097) of other *S. aureus* isolates (Han *et al.*, 2009; Holden *et al.*, 2013). It contains a number of genes involved in conjugative transposition (Table S1) including *tcpA, tcpE* and *tcpC*. The presence of a conjugative transposon inserted in the SCC*mec* type XI element in the isolate from the wild boar is of interest, as it could potentially mediate the transfer of the SCC*mec* type XI cassette between bacteria as an alternative

mechanism of transfer than transduction, which has been demonstrated for other SCC*mec* elements (Scharn *et al*, 2013). Overall, our study demonstrates for the first time the presence of *mecC*-MRSA in an environmental water source and highlights the potential role of water in the dissemination of *mecC*-MRSA.

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Figure legends

Figure 1. Phylogenetic relationships between the ST425 *mecC*-MRSA isolates in the game estate. Figure shows an unrooted maximum likelihood tree generated from SNPs in the core genome. The branch length for LGA251 has been trimmed. Bootstrap values for branches are shown in black. The number of differentiating SNPs for each branch is shown below each branch in bold. Figure references: Wild boar_101109_9HST (ZTA09/03698-9HST); Fallow_deer_201212_HSTA (ZTA12/02838HSTA); Fallow_deer_201212_HSTB (ZTA12/02838HSTB); Stream_In_100613_60SA (ZTA13/00933-60SA); Stream_In_100613_98SA (ZTA13/00933-98SA); Stream_In_100613_98ST (ZTA13/00933-98ST).

Figure 2. Comparison of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) type XI of strain LGA251 and strain ZTA09/03698-9ST (SCC*mec* element of strain ZTA09/03698-9ST was submitted to the EMBL database under accession no: LK024544).

Figure 1

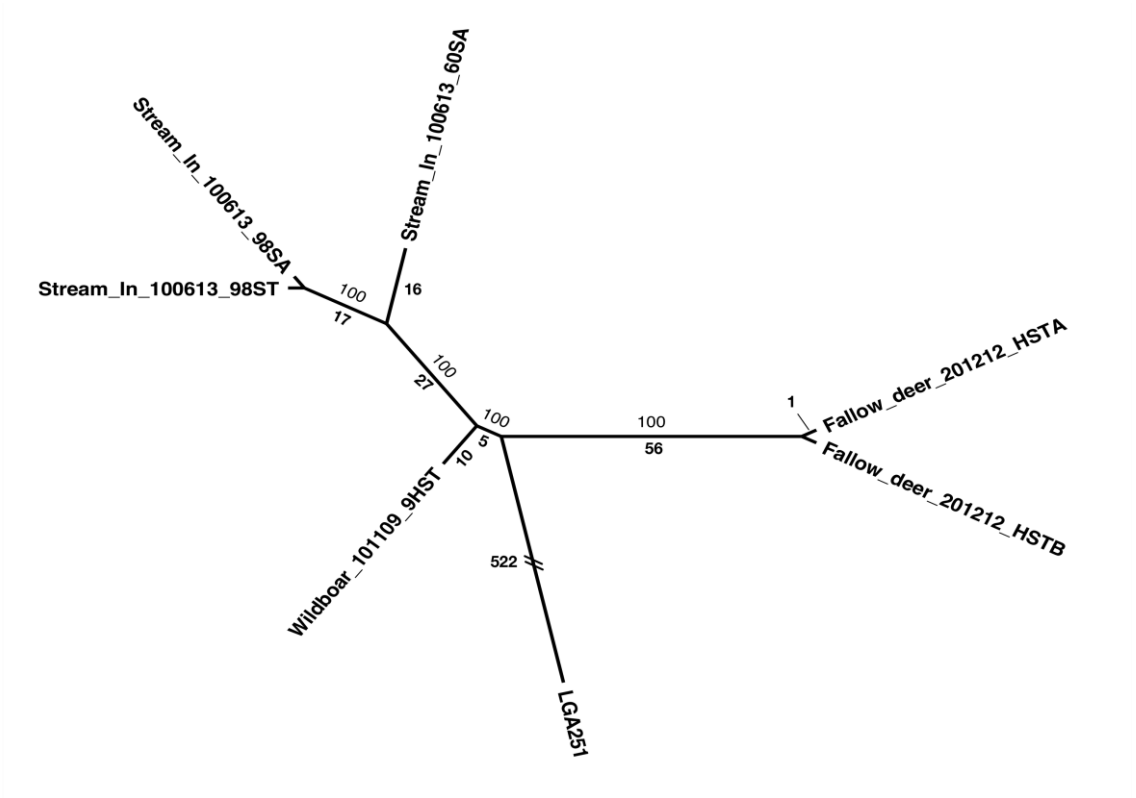
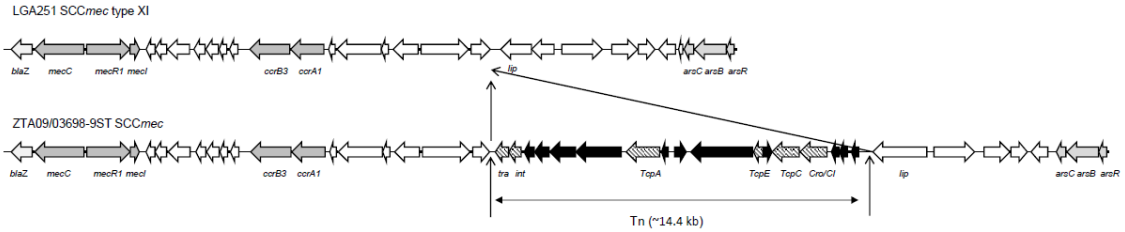

Stream_In_100613_98SA
Stream_In_100613_60SA
Stream_In_100613_98ST
100
16
17
100
27
100
10
5
100
56
1
Fallow_deer_201212_HSTA
Fallow_deer_201212_HSTB
Wildboar_101109_9HST
522
LGA251

Figure 2


LGA251 SCCmec type XI
blaZ
mecC
mecR1 mecI
corB3
corA1
lip
arsC arsB arsR
ZTA09/03698-9ST SCCmec
blaZ
mecC
mecR1 mecI
corB3
corA1
tra
int
TopA
TopE
TopC
Cro/CI
lip
arsC arsB arsR
Tn (~14.4 kb)

SI Experimental procedures

Samples

We tested wild boars (n=10), red deer (n=10), veal calves (n=20) and beef cattle (n=48) that share pastures in a game estate of >3,000 ha located in the centre of Spain in which *mecC*-MRSA had been detected (Porrero *et al.*, 2014). Samples were collected between November 2012 and August 2013. Samples were also collected from workers of the game state (n=14) and from the water of a river (n=7) that runs through the game estate at two different locations; one approximately 0.5 km from its entry into the estate (n=6) and a second sample 4.4 km upstream, outside the game estate (n=1).

Isolation and identification

Nasal swabs were cultured in 9 mL of Mueller–Hinton broth (6.5% NaCl, Oxoid) and incubated at 37 ºC for 16–20 h. One mL was then transferred to 9 mL tryptone soy broth (Oxoid) with cefoxitin (3.5 mg/L, Sigma–Aldrich) and aztreonam (75 mg/L, Sigma–Aldrich) and incubated at 37 º C for 16–20 h. Finally, 25 µL was streaked onto Brilliance MRSA plates (Oxoid) and incubated for 24–48 h at 37 ºC (Porrero *et al.*, 2013). Denim blue colonies were confirmed as MRSA (*mecA* or *mecC* positive) or methicillin susceptible *S. aureus* (MSSA; *mecA* and *mecC* negative) by PCR using primers *spa*-1113f-*spa*-1514r, *mecA* P4-*mecA* P7 and *mecA*$_{LGA251}$ MultiFP- *mecA*$_{LGA251}$ MultiRP (Stegger *et al.*, 2012). All water samples consisted of 1 L of stream water and were divided into 100 x 1 mL subsamples that were processed separately. Subsamples were cultured in 9 mL Mueller–Hinton broth (6.5% NaCl, Oxoid) and incubated at 37 ºC for 16–20 h. After this enrichment, 25µL were streaked onto Baird Parker (bioMerieux) and 1mL was transferred to 9 mL tryptone soy broth (Oxoid) with cefoxitin (3.5 mg/L, Sigma–Aldrich) and aztreonam (75 mg/L, Sigma–Aldrich) as described for nasal swabs (Porrero *et al.*, 2013).

Molecular and phenotypic characterization

All confirmed *S. aureus* were characterized by *spa* typing (Harmsen *et al.*, 2003) and Multilocus Sequence Typing (MLST) (Enright *et al.*, 2000; Porrero *et al.*, 2013; Porrero *et al.*, 2014). Antimicrobial susceptibility testing was carried out by microdilution and Minimum Inhibitory Concentrations (MICs) were interpreted according to the European Committee on Antibiotic Susceptibility Testing (EUCAST) epidemiological cut-offs (Porrero *et al.*, 2012). Antimicrobials tested were benzylpenicillin, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, quinupristin-dalfopristin, rifampicin, streptomycin, sulfamethoxazole, tetracycline, tiamulin, trimethoprim and vancomycin (EUST plates, Trek Diagnostics).

Genome sequencing and bioinformatics

To perform epidemiological investigations (Harrison *et al.*, 2013), whole genome sequencing (WGS) was carried out in all *mecC* positive isolates (n=6). Overnight cultures were grown in tryptic soy broth (TSB) at 37º C with 200 rpm shaking. Genomic DNA was then extracted from 1 ml of the overnight cultures using a MasterPure Gram Positive DNA Purification Kit (Cambio, UK). Illumina library preparation was carried out as described by Quail and collegues (Quail *et al.*, 2008). Hi-seq sequencing was carried out following the manufacturer's standard protocols (Illumina, Inc, USA).

The whole genome based phylogeny was generated by mapping the fastq files against the LGA251 reference genome (EMBL accession no: FR821779) using SMALT (www.sanger.ac.uk/smalt). Single Nucleotide Polymorphisms (SNPs) located in mobile genetic elements were removed (Harrison *et al.*, 2013) and a maximum likelihood tree was generated using the SNPs present in the core genome (the core genome been defined as the all the genomic regions not part of mobile genetic elements) in RAxML (Stamatakis

et al., 2005). Genomes were assembled *de novo* using Velvet (Zerbino and Birney, 2008) and contigs reordered against LGA251 using Mauve (Darling *et al.*, 2004).

Staphylococcal Cassette Chromosome *mec* (SCC*mec*) of those isolates carrying *mecC* were compared with SCC*mec* type XI of strain LGA251 using ACT (Carver *et al.*, 2005), Artemis (Rutherford *et al.*, 2000) and BLAST (Zhang *et al.*, 2000). Identification of acquired antimicrobial resistance genes in sequenced isolates was also done using Resfinder (http://cge.cbs.dtu.dk/services/ResFinder/) (Zankari *et al.*, 2012) and manually using BLAST.

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Table S1

Putative conjugative transposon (~14.4 kb)

Product	Size (bp)	Orientation
Putative transposase (pseudogene)	1023	-
Hypotetical protein	411	-
Hypothetical protein	591	-
Putative traG membrane protein	1047	-
Hypothetical protein	1839	-
FtsK/SpoIIIE family protein	1359	-
Hypothetical protein [*Staphylococcus aureus*]	267	-
Hypothetical protein	477	+
Hypothetical protein	2496	-
Putative TcpE family protein	384	-
Putative conjugative transposon protein TcpC	1101	-
Replication initiation factor family protein	1092	-
Hypothetical protein	303	-
Hypothetical protein	321	-
Hypothetical protein	294	-