Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study


Summary

Background Animals can act as a reservoir and source for the emergence of novel meticillin-resistant *Staphylococcus aureus* (MRSA) clones in human beings. Here, we report the discovery of a strain of *S aureus* (LGA251) isolated from bulk milk that was phenotypically resistant to meticillin but tested negative for the mecA gene and a preliminary investigation of the extent to which such strains are present in bovine and human populations.

Methods Isolates of bovine MRSA were obtained from the Veterinary Laboratories Agency in the UK, and isolates of human MRSA were obtained from diagnostic or reference laboratories (two in the UK and one in Denmark). From these collections, we searched for mecA PCR-negative bovine and human *S aureus* isolates showing phenotypic meticillin resistance. We used whole-genome sequencing to establish the genetic basis for the observed antibiotic resistance.

Findings A divergent mecA homologue (meCA gazi ) was discovered in the LGA251 genome located in a novel staphylococcal cassette chromosome mec element, designated type-XI SCCmec. The meCA gazi was 70% identical to *S aureus* mecA homologues and was initially detected in 15 *S aureus* isolates from dairy cattle in England. These isolates were from three different multilocus sequence type lineages (CC130, CC705, and ST425): spa type t843 (associated with CC130) was identified in 60% of bovine isolates. When human mecA-negative MRSA isolates were tested, the meCA gazi homologue was identified in 12 of 16 isolates from Scotland, 15 of 26 from England, and 24 of 32 from Denmark. As in cows, t843 was the most common spa type detected in human beings.

Interpretation Although routine culture and antimicrobial susceptibility testing will identify *S aureus* isolates with this novel mecA homologue as meticillin resistant, present confirmatory methods will not identify them as MRSA. New diagnostic guidelines for the detection of MRSA should consider the inclusion of tests for meCA gazi.

Introduction

*Staphylococcus aureus* causes a wide range of diseases in human beings, from minor skin infections to severe illnesses such as septicemia, toxic shock, endocarditis, and pneumonia. First described in 1961, the increasing incidence of meticillin-resistant *S aureus* (MRSA), and its spread in hospitals and the community, has posed a major challenge for infectious disease medicine. The evolution of meticillin resistance in *S aureus* is, in part, conferred by the acquisition of one of several staphylococcal cassette chromosome mec elements (SCCmec), which carry a gene (mecA) that encodes a penicillin binding protein (PBP2a) with low affinity for β-lactam antibiotics.

Most isolates of MRSA in the UK are identified with antimicrobial susceptibility testing by measuring zones of growth inhibition around antibiotic-impregnated discs on agar plates or measurement of minimum inhibitory concentrations (MIC), as recommended by the British Society for Antimicrobial Chemotherapy guidelines.

Meticillin resistance breakpoints are zone diameters of 14 mm or less around 1 μg oxacillin-impregnated discs, zone diameters of 21 mm or less around 10 μg cefoxitin-impregnated discs, an oxacillin MIC of more than 2 mg/L, or a cefoxitin MIC of more than 4 mg/L. Detection of the mecA gene by PCR or the detection of PBP2a in a slide agglutination assay can be used to confirm a diagnosis of MRSA when antimicrobial susceptibility results are borderline.

Before 2003, most MRSA identified belonged to multilocus sequence type clonal complexes (CC) associated with human carriage and infection. The emergence of MRSA CC398 (known as livestock-associated MRSA) in farm animals and human beings has shown that some *S aureus* lineages might not be strongly host-species restricted. A survey of slaughter pigs in the Netherlands showed that 39% harboured MRSA sequence type (ST)398 and another survey showed that 27% of people working at, or living on, a livestock farm in the Netherlands carried
livestock-associated MRSA. MRSA ST398 can cause infection in people, with close animal contact being the main risk factor, suggesting that farm animals could provide a reservoir of MRSA.

Here we report MRSA strains obtained from cattle and human beings, which carry a new SCCmec that is undetectable by molecular diagnostic tests used for identification of MRSA.

**Methods**

**Bacterial isolates**

*S. aureus* LGA251 and *S. aureus* LGA254 were isolated from a bulk milk sample from a farm in southwest England in May, 2007.24 24 MRSA were obtained from a collection of 940 *S. aureus* isolates, submitted from 465 different herds, after antibiotic susceptibility testing of all milk samples from cows with mastitis. The samples had been submitted to one of 14 regional Veterinary Laboratories Agency centres between April, 2006, and September, 2007, for bacteriological characterisation.11 None of these isolates tested positive after standard PCR tests for *mecA*.7 MRSA isolated from human beings, as detailed in the webappendix (p 11), were provided by the Health Protection Agency (HPA; Addenbrooke’s Hospital, Cambridge, UK); the Staphylococcal Reference and Antibiotic Resistance Monitoring Laboratories, HPA (Colindale, London, UK); the Scottish MRSA Reference Laboratory (Glasgow, UK); and the National MRSA Reference Laboratory, Statens Serum Institut (Copenhagen, Denmark). Every centre identified likely candidate isolates for PCR testing for the *mecA* homologue, as detailed in the webappendix (p 11). Submission of all Danish MRSA to the Statens Serum Institut has been mandatory since November, 2006, and all MRSA and meticillin-susceptible *S. aureus* isolates, submitted since 2001, have been *spa* typed; 678 isolates in 2007, 857 in 2008, 817 in 2009, and 1090 in 2010 were MRSA.

**Procedures**

Species identification of selected isolates was confirmed by PCR with primers based on the 16S–23S rRNA spacer region for *S. aureus*.25 Testing of strains for the presence of PBP2a was done with the Mastalex test (Mast Group, Boole, UK), which is a slide agglutination assay that detects PBP2a in MRSA by use of latex sensitised with a monoclonal antibody directed against PBP2a.26 Molecular detection of *mecA*, *femB*, and the SCCmec–*orfX* junction was done with PCR, as described previously.22–24 A comparison of the primer sequences used to test for *mecA* and the target sequences in *mecA* and *mecA* were shown in the webappendix (p 2). Isolates were genotyped for multilocus sequence type and *spa* type, as described previously.11,20,22

For all test isolates, the MIC of oxacillin and cefoxitin were measured by either Etest (AB Biodisk, Solna, Sweden) or agar dilution,22 depending on which laboratory did the test. The disc diffusion technique was used to establish susceptibility of LGA251 and LGA254 to penicillin, oxacillin, cefoxitin, gentamicin, neomycin, ciprofloxacin, tetracycline, erythromycin, clindamycin, fusidic acid, chloramphenicol, ticoplanin, rifampicin, trimethoprim, linezolid, and mupirocin.22 To establish whether β-lactam resistance was a result of hyperproduction of β-lactamase, tests were done with and without adjacent discs impregnated with both amoxicillin and clavulanic acid.22 The *S. aureus* NCTC 12493 strain was used as a control for MRSA and the *S. aureus* NCTC 6571 strain was used as a control for meticillin-susceptible *S. aureus* (both strains from the National Collection of Type Cultures, HPA, Salisbury, UK). Growth on chromogenic MRSA screening agar, MRSA ID (bioMérieux, Basingstoke, UK), was measured by standard plating and incubation for 18 h at 35°C.

We developed a PCR assay to amplify a region of *mecA* and the novel homologue described here, *mecA* (version 1.1.4) for melting temperatures and self-complementarity,24 and pDraw32 (version 1.1.101) was used to confirm the amplicon size and melting temperatures. Primers were as follows: Fw, 5’ TCACCGAGTTTCAAC[CAAAA]C’; and Ry, 5’ CCTGAATC[W]GCTAATAATTTT3’. Primers for the amplification of the *femB* control gene were obtained from a previously described protocol.13 A 25 μL PCR reaction contained final concentrations of 2–5 units of Taq DNA polymerase (Qiagen, Crawley, UK); 1xQ solution (Qiagen); 1xQiagen CoralLoad PCR buffer (Tris-Cl, KCl, [NH₄]₂SO₄, 15 mmol/L MgCl₂, gel-loading reagent, orange dye, and red dye; pH 8.7; Qiagen); 4 mmol/L of MgCl₂; 0.8 μmol/L of each primer (Operon, Cologne, Germany); and 50 ng of DNA template. A negative control, with no target DNA, was included in the PCR run in the GeneAmp PCR System 9700 (Applied Biosystems). The amplification programme consisted of an initial denaturation step at 94°C for 5 min; 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were analysed by electrophoresis on a 1% agarose gel, previously stained with ethidium bromide at 0·14 μg/ml (Sigma, Gillingham, UK), and run at 5 V/cm for 45 min. The molecular marker used was a 100 bp ladder (Promega, Southampton, UK). The sizes of the
PCR products sequenced after PCR were 356 bp for the mecA gene, and 651 bp for the femB gene. We designed a duplex-PCR to detect the mecA regulatory genes (primers: mecIF, 5’ GACACGTGAAAGCTATGATATAT 3’; mecIR, 5’ ATTCTTCAATATCATCTTGGAC 3’; mecRIF, 5’ GGCTCAGTTAACCATAAGGTTT 3’; mecRIR, 5’ AAATTGCGCTTACCATAGCTTGT 3’), a duplex-PCR to identify the two cassette recombinase genes (primers: ccrAF, 5’ GCAATAGGTTATCTACGTCAAAG 3’; ccrAR, 5’ TCTAATGATTGCGTTGATTCC 3’; ccrBF, 5’ TTCGTGTATCGACAGAAATGCAG 3’; ccrBR, 5’ CATCTTTAGAATATCAATACGG 3’), and a single target PCR to amplify the β-lactamase gene blaZ (primers: blaZF, 5’ AGTCGTGTTAGCGTTGATATTAA 3’; blaZR, 5’ CAATTTCAGCAACCTCACTTACTA 3’). The sizes of the expected PCR products were 344 bp for mecI, 710 bp for mecR1, 932 bp for ccrA, 1449 bp for ccrB, and 809 bp for blaZ. Except for use of an annealing temperature of 58°C instead of 55°C, we used the same method as for the other PCR assay described above.

The whole genome of the S aureus isolate LGA251 was sequenced with both capillary sequencing (on ABI 3730xl analysers; Applied Biosystems) and pyrosequencing (on 454 instruments; Life Sciences, Roche Diagnostics Corporation, Branford, CT, USA). A total of 29 300 high quality capillary reads were produced mostly from two subclone libraries (a 2–3 kb insert library and a 3–4 kb library, both with the vector pOTW12). The average read length was 650 bp and these reads represented 6·8 times coverage. The 454 sequencing produced 59·07 Mb data in reads with an average length of 225 bp. The assembly of these reads with Newbler 1.1.03.24 gave 81 contigs greater than 500 bp with a combined length of 2699 627 bp in six scaffolds.

A combined assembly of the capillary reads, with Phrap (Version 17.0), and the consensus sequences from the 454 assembly (which were converted into overlapping 500 bp sequences) produced 26 contigs (overlapping sequences or clones from which a sequence can be obtained) greater than 2 kb with an N50 of 532 kb. A further 2310 high quality reads were produced to close gaps and to improve the quality of the sequence to finished standard. The sequence was finished and annotated as described previously.25 The sequences and annotations of the S aureus strain LGA251 genome have been entered in the EMBL database (accession numbers FR821779 for the chromosome and FR821780 for the plasmid).

**Statistical analysis**

Temporal trends for annual incidence of detection of mecA(LGA251) in Denmark for the years 2007–10, with all MRSA as the denominator, were assessed with the Cochran-Armitage trend test. Statistical analysis was done with StatXact version 9.

**Role of the funding sources**

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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**Figure 1:** Comparison of the type XI SCCmec of LGA251 with other SCC elements

Schematic diagram of type II SCCmec of meticillin-resistant Staphylococcus aureus MRSA252 (top), type IV SCCmec of MW2 (upper middle), type XI SCCmec of LGA251 (lower middle), and the SCCmec-like element of Macrococcus caseolyticus JSC7095 (bottom). The names of the coding sequences associated with drug and metal resistance are provided. Coding sequences are marked in the direction of transcription as arrows. Coding sequences belonging to the mec and ccr complexes, and IS431 are coloured, with homologues given the same colour; the individual genes belonging to these groups are named. Light pink shading joins regions that are conserved between elements. The coloured boxes at the end of the SCC elements mark the integration site sequence (blue, attL; green attR).
Results
LGA251 was resistant to penicillin, oxacillin, and cefoxitin, but susceptible to gentamicin, neomycin, ciprofloxacin, tetracycline, erythromycin, clindamycin, fusidic acid, chloramphenicol, teicoplanin, rifampicin, trimethoprim, linezolid, and mupirocin. LGA251 gave negative reactions in the latex agglutination test for PBP2a, a PCR assay with primers for mecA, and a PCR that amplifies a region of the SCCmec (SCCmec-orfX). Culture of LGA251 on agar plates with and without adjacent discs impregnated with both amoxicillin and clavulanic acid indicated that resistance was not mediated by β-lactamase hyperproduction.

MICs of 24 mg/L and oxacillin Etest (AB Biodisk NA, Culver City, CA, USA) MICs of 12 mg/L, and although resistant to penicillin, was susceptible to gentamicin, neomycin, ciprofloxacin, tetracycline, erythromycin, clindamycin, fusidic acid, chloramphenicol, teicoplanin, rifampicin, trimethoprim, linezolid, and mupirocin. Both LGA251 and LGA254 were sequence type 425 by multilocus sequence type but the spa type of LGA251 was t6300 (spa repeat: 14-44-12-17-23-18-110-17-17-23-24) and the spa type of LGA254 was t6292 (spa repeat: 14-44-12-17-23-18-110-17-17-23-24).

The genetic basis of the meticillin resistance in LGA251 was investigated by whole-genome sequencing.
Comparative analysis of the 2·8 Mb genome revealed a 29·4 kb SCC\textit{mec} element inserted in the chromosome at the same locus (3´ region of\textit{orfX}) as other SCC\textit{mec} elements, flanked by sequences that match the SCC\textit{mec} integration site sequence. The element contained 29 coding sequences, including homologues of the site-specific recombinase genes\textit{ccrA} and\textit{ccrB}, and the\textit{mecA} gene encoding the penicillin-binding protein PBP2\text{\textalpha} (figure 1). The site-specific recombinases\textit{CcrA} and\textit{CcrB} were divergent by comparison with those encoded by other SCC\textit{mec} elements;\textit{CcrA} was classified as\textit{ccrA1} group and\textit{CcrB} was classified as\textit{ccrB3} group (webappendix p 13). The combination of\textit{ccrA1} and\textit{ccrB3} is novel and has been designated as type-8\textit{ccr}. 

Comparison of the PBP2\text{\textalpha} encoded by the LGA251\textit{mecA} (MecA LGA251) with proteins in the public sequence databases (Universal Protein Resource; UniProt) indicated that it is divergent in comparison with all other MecA homologues; MecA\textsubscript{LGA251} was most similar to MecA from the type III SCC\textit{mec} (accession number Q93IC2), having 63% identity at the aminoacid level (70% at the DNA level; webappendix p 13). Phylogenetic analysis of MecALGA251 showed that it belongs to the PBP2\text{\textalpha} family (data not shown) and is found on a branch outside the clade that includes\textit{S} aureus homologues and homologues from other\textit{Staphylococcus} species (webappendix p 6).

The\textit{mecA} LGA251 gene was part of a complex (\textit{mecI}–\textit{mecR1}–\textit{mecA}–\textit{blaZ}) that has a different organisation to other SCC\textit{mec} elements, and has been designated as class E\textit{mec} gene complex. A complex with the same organisation has been identified in a plasmid,\textsuperscript{27} and an SCC\textit{mec}-like element carried by\textit{Macrococcus caseolyticus},\textsuperscript{28} although the sequence conservation of the two complexes is low (<60% identity at the DNA level).

The LGA251 SCC\textit{mec} element was novel and holds only two of the three joining regions normally seen (J1 and J2). For more on UniProt see http://www.uniprot.org/
candidates of about 120 000 clinical isolates: the only one tested from Cambridge, 14 of 25 tested from London, 12 of 16 tested from Glasgow, and 24 of 32 tested from Copenhagen. The results of tests are listed in the table and the geographical distribution of the mecA homologues is shown in figure 2 for England and in the webappendix for Scotland (p 7) and Denmark (p 8). The oxacillin MICs ranged from 0·75 mg/L to 32 mg/L, with an MIC of 4 mg/L and MIC of 16 mg/L. The cefoxitin MICs ranged from 4 mg/L to 64 mg/L, with an MIC of 8 mg/L and MIC of 24 mg/L. All the British isolates were multilocus sequence typed together with seven of 24 Danish isolates. In the Danish isolates mecA was detected in none of 678 isolates in 2007, one of 857 isolates in 2008 (0·12%, 95% CI 0·0–0·35%), four of 817 isolates in 2009 (0·49%, 0·01–0·97), and 13 of 1090 isolates (0·67%, 0·61–1·95) in 2010 (trend for increase: p=0·0002).

The evolutionary relation of the MRSA sequence types that carry mecA compared was within this population and with the S aureus population as a whole, considering the most frequently reported sequence types (ten or more isolates reported per sequence type). Analysis of the population framework shows that mecA homologues are present in phylogenetically distinct S aureus, indicating that multiple independent acquisitions of the gene have occurred (webappendix p 9).

Discussion

A novel mecA homologue, mecA associated with resistance to β-lactam antibiotics was present in clinical MRSA isolates from the UK and Denmark, and bovine milk samples from the UK. Our search for mecA MRSA has been limited to existing collections comprising isolates collected for various reasons and for which incomplete clinical data or strain typing were available. Therefore, interpretations made from our results are tentative and need to be confirmed by more systematic studies with appropriate methodology.

The detection of this gene in MRSA isolates cultured from blood and infected wound sites is strong circumstantial evidence that these organisms are capable of causing clinical disease. Whether disease caused by mecA is identical to that caused by conventional MRSA is not clear without evidence from formal epidemiological and virulence studies. Isolate collections were systematically searched for isolates that seemed to be β-lactam resistant, but tested negative by PCR for mec or by slide agglutination test for PBP2a, and from a search for MRSA isolates that had spa types associated with mecA MRSA (webappendix p 11). The Danish isolate archive is a complete national collection. These results reveal that detection rates of mecA MRSA increased substantially between 2007 and 2010. These detection rates are good incidence estimates for those years in Denmark because the archive for 2007–10 contains all Danish MRSA, and because all MRSA isolates were spa-typed.
In this study, we provide evidence of an association between SCCmec and β-lactam resistance—which includes meticillin resistance—in *S. aureus*. Genetic manipulation of *S. aureus* strains to show that the insertion or removal of the mecA homologue leads to acquisition or loss of β-lactam resistance will need to be done to show direct linkage, and such studies are underway. However, we were able to search the entire genome of LGA251 for evidence of other genes that might confer resistance. Although a β-lactamase gene (*blaZ*) is present in SCCmec, the inability of clavulanate (a β-lactamase inhibitor) to ablate resistance indicates that resistance is unlikely to be caused by β-lactamase. This finding is consistent with the fact that penicillinase hyperproducers do not show heteroresistance, as was seen with LGA251 (panel). The expected complement of genes that encode PBPs is present (PPB1, PPB2, PPB3, and PPB4) in a highly conserved form compared with other sequences from other *S. aureus* isolates (data not shown), and we recorded no additional PBPs. The most likely explanation for the β-lactam resistance seen in LGA251 is that it is mediated by the mecA homologue present in the type XI SCCmec.

The discovery of this new mecA homologue raises issues about the detection and confirmation of MRSA. Irrespective of whether an infection is caused by mecA MRSA or conventional MRSA, after culture and antimicrobial susceptibility testing, appropriate decisions about care of patients can be made. However, when existing PCR or monoclonal antibody methods are used as the only method to detect MRSA, or when these methods are used to confirm provisional detection of MRSA, then mecA MRSA *S. aureus* will be wrongly diagnosed as meticillin susceptible. The use of new PCR primers such as those described in this paper, and the production of new monoclonal antibodies, would address this problem.

Another important question concerns the potential role of cattle in the epidemiology of human MRSA infections caused by mecA MRSA. Data from the Veterinary Laboratories Agency MRSA survey suggest that mecA MRSA could be present in up to 1·4% (95% CI 0·6–2·1%) of *S. aureus* bovine mastitis isolates, and is present in up to 2·8% (1·3–4·3) of herds. The data described in this paper do not provide direct evidence of transmission between cattle and people. However, four pieces of circumstantial evidence suggest that cattle could be the epidemiological source. First, the human isolate strain types were either CC130 (48 of 66)—a lineage that is consistent with the fact that penicillinase hyperproducers are used to confirm provisional detection of MRSA, then mecA MRSA—and “MRSA” for any evidence of livestock-associated meticillin-resistant *S. aureus* (MRSA) that were not accounted for by known SCCmec types. None were found.

**Interpretation**

Advice on the detection and treatment of infections with mecA MRSA by antimicrobial susceptibility testing is no different to the detection and treatment of infections with other MRSA strains. However, clinicians should be aware that molecular techniques of detection of MRSA with PCR or slide agglutination tests do not detect mecA MRSA. This means that when these tests are used either for primary detection, or for confirmation of MRSA, a small chance exists that a false-negative result is obtained. The data presented in this paper suggest that the prevalence of mecA MRSA is likely to be in the range of one in 100 to one in 500 of total MRSA in the UK and Denmark. The discovery of the same mecA MRSA in dairy cows suggests that these animals might provide a reservoir of infection and close links with farms or contact with dairy cattle could be risk factors that increase the likelihood of mecA MRSA carriage or infection in patients. Until better evidence is generated from appropriate observational or experimental studies, this study provides the best evidence to inform clinical decisions concerning this new discovery.

**Panel: Research in context**

**Systematic review**

The discovery of a novel mecA homologue prompted a search of public sequence databases (UniProt) with the predicted aminoacid sequence for similar mecA homologues. No identical sequence was found and the closest match had 63% identity at the aminoacid level. We searched PubMed with the terms “cattle”, “MRSA”, “livestock”, and “MRSA” for any evidence of livestock-associated meticillin-resistant *S. aureus* (MRSA) which might have originally been a human strain. The absence of diversity between the mecA MRSA *S. aureus* isolates from Denmark and those from the UK could be a result of a searching bias (ie, that spa-type t843 was used as one of the searching criteria, and by testing for known spa types only we might have missed mecA MRSA that had different spa types). The discovery of an isolate from a sample obtained from a patient in 1975 possessing the same spa-type shows that the mecA homologue has been in *S. aureus* for at least 36 years, and that the Danish lineage could have changed very little during this period. This early Danish isolate also suggests that mecA MRSA *S. aureus* might have originally been a human strain. The presence of type XI SCCmec in four separate multilocus sequence type lineages, and the fact that it is bounded by integration site sequence repeats and has intact site-specific recombination components, suggests that it has the potential to be transferred to other *S. aureus* lineages in the future.

The search for mecA MRSA also yielded several isolates that had an MRSA phenotype but no mecA gene.
that could be detected by PCR. These isolates might possess other mecA homologues or other mechanisms leading to β-lactam resistance. The discovery of this previously undetected mecA homologue is potentially of public health importance. Diagnostic protocols, whether for clinical or epidemiological purposes, should consider the ramifications of not detecting S aureus strains that carry this new mecA homologue.

Contributors
LG-A and MAH collected the original bovine isolates. LG-A and HL did most of the laboratory work. DFJB, MDC, and EW repeated PCR and antimicrobial susceptibility tests and initial identification of the first human isolate. Sequencing and genetic analysis were done by MTGH, SDB, JP, KB, and DJP. CT was responsible for collection and initial characterisation of bovine isolates from the Veterinary Laboratories Agency. GFE and EKG were responsible for identification and initial characterisation of the Scottish isolates. AMK, RLRH, and BP were responsible for the identification and characterisation of the English isolates. RLS and ARL were responsible for the identification of the Danish isolates. Supervision and management of the study were done by MAH, CRW, SJP, and DJM. All authors were involved in compiling the report and approved the final version.

Conflicts of interest
All authors declare that they have no conflicts of interest.

Acknowledgements
During the publication process we became aware that Shore and colleagues had submitted a paper for publication describing human MRSA isolates from Ireland from the clonal complex 130, which contains the type XI SCCmec (Shore AC, Deasy EC, Slickers P. Detection of staphylococcal cassette chromosome mec type XI encoding highly divergent mecA, mecCl, mecRI, blaz and ccr genes in human clinical clonal complex 130 methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother [in press]).

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