Multiple cases of methicillin-resistant CC130 *Staphylococcus aureus* harboring *mecC* in milk and swab samples from a Bavarian dairy herd

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ABSTRACT

The discovery of a new *mecA* homolog, *mecC*, necessitates a modification of diagnostic procedures for the identification of methicillin-resistant *Staphylococcus aureus* (MRSA), as most assays used for the genotypic and phenotypic *mecA* detection cannot currently recognize *mecC*. Although the prevalence, distribution, and importance of *mecC* are not yet completely understood, an exchange of *mecC*-MRSA between humans and animals seems possible. All previously reported observations of *mecC*-positive strains have been sporadic. To the best of our knowledge, this is the first report about multiple cases of *mecC*-positive *Staph. aureus* in 1 dairy herd. Clonal complex 130 *Staph. aureus* harboring *mecC* were found in milk samples from 16 of 56 lactating cows kept in a herd in Bavaria, Germany. Almost all quarter milk samples positive for *mecC*-MRSA had the lowest possible California Mastitis Test score; composite somatic cell counts obtained from monthly milk recordings showed a mean of 51,600 cells/mL in *mecC*-MRSA affected cows. Additionally, *mecC*-positive clonal complex 130 *Staph. aureus* were detected in swab samples from the mammary skin and a teat lesion of 1 cow from this herd. This report suggests that *mecC*-carrying strains are able to spread among livestock, and that they have the ability to cause multiple cases in single herds. Therefore, future studies targeting MRSA in dairy cows need to consider *mecC*.

Key words: methicillin-resistant *Staphylococcus aureus*, *mecC*, dairy cow, microarray

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) are among the most important nosocomial infectious

agents in human medicine worldwide. These bacteria are increasingly reported in veterinary medicine as well. Their resistance to almost all β -lactam compounds in current clinical use is caused by the alternate penicillin-binding protein PBP2a that is encoded by some but not all alleles (Monecke et al., 2012) of the gene *mecA*.

In 2011, however, a previously unknown *mecA* homolog, *mecC*, was found in *Staph. aureus* isolated from 2 patients in Irish hospitals (Shore et al., 2011). At the same time, *mecC* was detected in 15 isolates from English cattle as well as in 51 human isolates from Scotland, England, and Denmark (García-Álvarez et al., 2011). These *mecC*-positive MRSA isolates could not be detected by conventional confirmatory MRSA tests. Since then, the novel allele has also been identified in *Staph. aureus* from other human cases (Kriegeskorte et al., 2012; Basset et al., 2013; Petersen et al., 2013), from milk samples (Unnerstad et al., 2013), and a nasal swab (Vandendriessche et al., 2013) from dairy cows and from other livestock (Eriksson et al., 2013; Vandendriessche et al., 2013) in Belgium, Denmark, France, Germany, Sweden, Switzerland, the Netherlands, and the United Kingdom. Moreover, *mecC* was detected in isolates from various companion animals and wildlife (Walther et al., 2012; Paterson et al., 2012; Loncaric et al., 2013; Monecke et al., 2013; Robb et al., 2013). The distribution, prevalence, and importance of *mecC* have not yet been fully understood. It is assumed that the MRSA prevalence in many countries is underestimated because *mecC*-MRSA isolates have not been correctly identified so far (García-Álvarez et al., 2011). As *mecC*-containing MRSA isolates were found to belong to lineages typically reported in cattle and other animals [i.e., clonal complex (CC) 130 and sequence type 425], a zoonotic reservoir can be assumed (García-Álvarez et al., 2011), warranting further studies. In Denmark, 2 human cases of *mecC*-MRSA infection could be linked to a ruminant reservoir using molecular typing techniques and whole genome sequencing (Petersen et al., 2013; Harrison et al., 2013).

Received August 13, 2013.

Accepted February 4, 2014.

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In dairy cows, *Staph. aureus* is one of the most important causal agents of bovine mastitis. Therapy costs and milk loss turn mastitis into the most cost-intensive disease in dairy herds worldwide (Seegers et al., 2003). Generally, *Staph. aureus* isolates associated with mastitis are methicillin-sensitive *Staph. aureus* (MSSA; Vanderhaeghen et al., 2010), but even if the MRSA prevalence in mastitis is presumed to be low, this needs to be reconsidered given the new knowledge on *mecC*. So far, only sporadic *mecC* mastitis cases have been reported (García-Álvarez et al., 2011; Laurent et al., 2012), and to the best of our knowledge no outbreak situation has yet been described. As part of a larger project that aimed to investigate antimicrobial susceptibility among *Staph. aureus* from bovine mastitis, several *mecC*-positive *Staph. aureus* were found in milk and swab samples from a dairy herd in southern Germany. This survey describes the first finding of multiple cases of *mecC*-MRSA in 1 location.

MATERIALS AND METHODS

Herd Characteristics

The affected Bavarian herd included 59 Simmental cows (56 lactating cows and 3 dry cows), 29 heifers, 57 fattening bulls, and 31 calves. The cows were milked twice a day in a 2 × 3 tandem milking parlor. At the time of the investigation, the bulk milk SCC was 127,000 cells/mL of milk.

Milk and Swab Samples

Quarter milk samples ($n = 213$; 11 nonlactating quarters) from all 56 milk-producing cows were taken according to the standard procedure (NMC, 1999; DVG, 2009). Additionally, 28 swab samples were collected from locations considered typical for *Staph. aureus*: bovine nasal mucosa ($n = 10$), nasal mucosa from fattening bulls ($n = 3$), mammary skin from cows which had at least 1 *Staph. aureus*-positive milk sample ($n = 2$), teat lesions from cows that yielded at least 1 *Staph. aureus*-positive milk sample ($n = 2$), ankle abrasions from 2 cows ($n = 2$), vaginal discharge from 1 cow ($n = 1$), holders for the milking clusters as part of the milking system ($n = 3$), leaked milk on the floor ($n = 3$), and hand ($n = 1$) and nasal swabs ($n = 1$) from 1 farmer. Samples were collected by wiping the area with a sterile cotton swab (Mastaswab, Mast Diagnostica GmbH, Reinfeld, Germany), which was transferred to Amies medium for transport to the laboratory.

Isolation of *Staph. aureus*

Cultivation from milk samples was carried out according to the standard procedure (NMC, 1999; DVG,

2009). Specimens were spread on esculin blood agar (Oxoid, Wesel, Germany) and incubated at 37°C. The plates were examined after 24 and 48 hr of incubation. To identify *Staph. aureus*, the coagulase test was performed using rabbit plasma (bioMerieux, Marcy-l'Étoile, France). In cases of unique morphology (hemolysis, pigmentation) of the grown staphylococci, the presence of coagulase was tested in 1 isolate per sample. In case of heterogeneous morphology, the coagulase test was performed in all grown staphylococci phenotypes.

The swab samples were spread on both esculin blood agar (Oxoid) and Baird-Parker agar (Baird-Parker medium base supplemented with 5% egg yolk tellurite emulsion; TN 1104 and TN1310; Sifin, Berlin, Germany). Colonies that morphologically resembled *Staph. aureus* were subcultured and tested for the presence of coagulase using rabbit plasma (bioMerieux). If the colonies on a plate looked alike, 1 staphylococcal isolate per swab sample was tested. Otherwise the coagulase test was performed in all grown phenotypes.

Detection of MRSA and Further Characterization with Microarray

Staphylococcus aureus were subcultured on Brilliance MRSA 2 Agar (Oxoid). The growth of bluish colonies was regarded as possible indication for the presence of MRSA and these isolates were considered as presumptive MRSA. One presumptive MRSA isolate per cow from the milk samples and 1 presumptive MRSA isolate per cow from the swab samples were selected for further testing. This included the screening for PBP2a and *mecA* as well as characterization using microarrays. The presence of the penicillin-binding protein characterizing MRSA was tested using a PBP2a Latex Test (DR 0900 A; Oxoid) according to manufacturer's instructions. The *mecA* screening was performed by PCR (modified to McDonald et al., 2005; primer "mecA for" 5'-GGC AAT ATT AAC GCA CCT CA-3' instead of 5'-GGC AAT ACC GCA CCT CA-3') in 2 randomly chosen PBP2a-negative presumptive MRSA isolates from milk of different cows. Microarray characterization was performed using the StaphyType kit (Alere Technologies GmbH, Jena, Germany), covering 333 target sequences that correspond to approximately 170 distinct genes and their allelic variants. These genetic targets include species markers, capsule type, SCC*mec* and *agr* group-typing markers, resistance genes, and genes encoding exotoxins as well as adhesion factors. Primer and probe sequences were described previously (Monecke et al., 2011). In addition to the selected set of presumptive MRSA isolates from milk and swab samples, the presumptive MSSA isolates recovered from milk and swabs

were characterized using the StaphyType DNA microarray system.

The assay was performed according to manufacturer's instructions. An image of the microarray was taken and analyzed using the reader and the current software script provided by Alere Technologies GmbH. The automated comparison of a given hybridization pattern to a reference database allowed to determine its affiliation to clonal complexes as defined by multilocus sequence typing (Enright et al., 2000; Monecke et al., 2008) and, in cases of MRSA, to epidemic strains as defined by multilocus sequence typing and SCCmec carriage. Strains that were found to belong to CC130 were further characterized using another array for detection and identification of *mecA* alleles and *mecC* (Monecke et al., 2012). Moreover, the presence of the novel exfoliative toxin homolog, putatively named *etD2*, was investigated by PCR (according to Monecke et al., 2013; forward primer 5'-TCA AGA CAC CAC TAG AAG TC-3'; reverse primer 5'-GTT TTC AGC TAA TCG TGC-3').

Additional Testing

At the time of sampling, the California Mastitis Test (CMT) was carried out on functional quarters ($n = 213$) of all lactating cows ($n = 56$). The CMT reaction of each quarter was recorded in an ordered scale as either, 0, 1, 2, or 3, with 0 indicating no reaction and 3 being a markedly positive reaction (almost-solid gel forms). Moreover, we had access to the recording data for each cow based on Fossomatic (composite milk samples). The recording was performed 5 d after the quarter milk sampling.

The antimicrobial susceptibility of all detected *Staph. aureus* was investigated by broth microdilution (penicillin, oxacillin, cefazolin, cefoperazone, cefquinome, pirlimycin, marbofloxacin, amoxicillin/clavulanate, and kanamycin/cephalexin) following the recommendations given in the Clinical and Laboratory Standards Institute (CLSI) document M31-A3 (CLSI, 2008). Clinical breakpoints according to CLSI-M31-A3 (penicillin, oxacillin, cefazolin, pirlimycin, and amoxicillin/clavulanate) and according to manufacturer's instructions (cefoperazone, cefquinome, marbofloxacin, and kanamycin/cephalexin), respectively, were used for interpretation of microdilution results. For cefoperazone, cefquinome, marbofloxacin, and kanamycin/cephalexin, MIC values of ≤ 4 , ≤ 2 , ≤ 1 , and $\leq 4/0.4$ were categorized as susceptible, respectively. Additionally, the antimicrobial susceptibility of all detected *Staph. aureus* isolates was investigated using VITEK2 system (test card AST-P580; bioMérieux; benzylpenicillin, oxacillin, gentamicin, tobramycin, levofloxacin, moxifloxa-

cin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, fosfomycin, nitrofurantoin, fusidic acid, mupirocin, and rifampicin) according to the manufacturer's instructions. Results of VITEK2 system were interpreted according to the clinical breakpoints established by EUCAST (2013).

RESULTS

Detection of *Staph. aureus* in Milk and Swab Samples

Staphylococcus aureus was found in 33 (15.5%) quarter milk samples from 18 different cows. One quarter was affected in 9 cows, 2 quarters were affected in 4 cows, and 3 quarters were affected in 4 cows. In 1 cow, *Staph. aureus* was detected in 4 quarter milk samples. Moreover, 5 *Staph. aureus* were isolated from 5 different swab samples (Table 1).

Detection of MRSA

Thirty-one *Staph. aureus* isolated from milk samples from 16 different cows (28.6%) as well as 2 *Staph. aureus* isolated from swab samples (1 from a teat lesion and another from mammary skin) grew on Brilliance MRSA 2 Agar forming MRSA-like bluish colonies (Table 1). The remaining 2 *Staph. aureus* isolated from milk samples, as well as 3 *Staph. aureus* isolated from swab samples, did not grow on Brilliance MRSA 2 Agar.

Of the 16 presumptive MRSA isolates from milk selected for further characterization, 2 yielded a positive result in the PBP2a test, whereas the remaining 14, as well as the 2 presumptive MRSA isolates from swab samples, did not present with a visible agglutination in the PBP2a assay. The *mecA*-PCR was negative for both isolates tested.

Results of Microarray-Based Genotyping

Using DNA microarray analyses, the 18 presumed MRSA isolates were assigned to CC130, *agr* group III, and capsule type 8. All 18 isolates were *mecA*-negative, instead harboring *mecC* and the SCCmec XI-associated β -lactamase gene *blaZ*-SCCmec XI.

Hybridization patterns of all *mecC*-positive CC130 *Staph. aureus* samples were identical. Full array hybridization profiles are provided in the supplemental file (<http://dx.doi.org/10.3168/jds.2013-7378>). All 18 isolates harbored the *hlg*-locus (*hlgA*, *lukF/S*) and the leukocidin gene homolog *lukD*, whereas *lukE* gave variable, weak, or negative results. The Pantone-Valentine leukocidin genes and the animal-associated leukocidin genes *lukF-P83/lukM* were absent. Enterotoxin genes

Table 1. Overview of the typed *Staphylococcus aureus* isolates

Origin	Initial <i>Staph. aureus</i> isolate		Characterization of presumptive MRSA and MSSA ¹								
	n	Growth on MRSA agar	n	CC ²	<i>mecC</i>	Virulence factor gene					
						<i>lukF-P83</i>	<i>lukM</i>	<i>lukD</i>	<i>lukE</i>	<i>egc</i> ³	<i>etD2</i> ⁴
Milk	31	31	16 ⁵	130	16	—	—	16	1	—	16
Teat lesion	1	1	1 ⁵	130	1	—	—	1	1	—	1
Mammary skin	1	1	1 ⁵	130	1	—	—	1	1	—	1
Mammary skin	1	—	1 ⁶	479	—	1	1	1	1	1	— ⁷
Ankle abrasion	1	—	1 ⁶	479	—	1	1	1	1	1	— ⁷
Milk	2	—	2 ⁶	705	—	2	2	2	2	2	— ⁷
Nasal mucosa (bull)	1	—	1 ⁶	705	—	1	1	1	1	1	— ⁷
Σ	38	33	23* ⁸		18	5	5	23	8	5	18

¹MRSA = methicillin-resistant *Staph. aureus*; MSSA = methicillin-sensitive *Staph. aureus*.²CC = clonal complex.³*egc* = enterotoxin gene cluster (*seg*, *sem*, *sen*, *sei*, *seo*, *seu*).⁴*etD2* = exfoliative toxin homolog.⁵Presumptive MRSA.⁶Presumptive MSSA.⁷Not tested.⁸23* = 23 isolates from 21 different cows (in 2 cows, *mecC* was detected in milk as well as in a swab sample).

could not be detected. Moreover, all 18 isolates harbored *etD2*.

Apart from the nonspecific efflux protein *sdrM*, no other genes associated with antibiotic resistance detectable by the array were identified [i.e., *blaZ*, *blaI*, *blaR*, *tet(K)*, *tet(M)*, *erm(A)*, *erm(B)*, *erm(C)*, *lnu(A)*, *aphA3*, *sat*, *aadD*, *aacA-aphD*, *fosB*, *qacA*, *qacC*, *cfr*, *fexA*, *mupR*, *fusB*, *fusC*, *vanA*, *vanB*, *vanZ*]. The characterization of the 5 presumptive MSSA isolates showed that 4 isolates belonged to CC705, *agr* II, capsule type 8. One isolate was assigned to CC479, *agr* II, capsule type 8 (Table 1).

Additional Results

The CMT was scored as 0 in 179 out of 213 quarters investigated, 22 quarters were scored as 1, 3 quarters were scored as 2, and 9 quarters showed a CMT 3 reaction. With regard to the 31 MRSA-positive quarters, the CMT was scored as having no reaction in 29 quarters, the milk of 2 quarters was scored as 3. The milk recording showed a mean cell count of 51,600 cells/mL of milk in *mecC*-affected cows (median = 37,000 cells/mL of milk, minimum = 16,000 cells/mL of milk, maximum = 109,000 cells/mL of milk).

The *mecC*-positive *Staph. aureus* proved to be resistant to penicillin and oxacillin by both techniques for susceptibility testing (MIC und VITEK2). Moreover, all isolates showed resistance to levofloxacin. Resistance to cefoperazone was observed in 17 (94.44%) of the 18 *mecC*-positive isolates, 16 isolates (88.89%) were

resistant to cefquinome, whereas MIC values for cefoperazone and cefquinome in susceptible isolates were marginal (4 and 2 µg/mL, respectively). Resistance to linezolid was observed in 7 isolates (38.89%), and 5.56% (1 isolate each) were resistant to teicoplanin and pirlimycin, respectively. The *mecC*-positive isolates were susceptible to all other antimicrobials tested.

DISCUSSION

Contrary to the sporadic single detections of *mecC* described before, here we had to take into account multiple cases of *mecC*-MRSA within 1 dairy herd. Because of identical hybridization patterns of all characterized *mecC*-MRSA, we concluded that it was a single strain spreading within the herd. Results of this study are scarcely comparable with other investigations. Whereas the *mecA*-MRSA cow-level prevalence has been adequately investigated (Vanderhaeghen et al., 2010; Spohr et al., 2011), such examinations are still missing for *mecC*. Similarly, very little is known about the *mecC* herd-level prevalence. Paterson et al. (2014) carried out the first formal prevalence study and detected *mecC*-MRSA on 10 of 465 (2.15%) dairy farms in England and Wales, but did not find it on 625 farms studied in Scotland. Future research is necessary to further elucidate the importance of *mecC*-MRSA, especially as a zoonotic potential of *mecC*-MRSA is indicated by different studies, resulting not only in asymptomatic colonization but also in clinical cases in humans (Shore et al., 2011; Petersen et al., 2013; Har-

rison et al., 2013). Consumers could come into contact with CC130 MRSA by raw milk or cheese consumption (Friedrich et al., 2011). The risk of foodborne disease associated with CC130 strains might be limited, as so far no enterotoxin genes have been detected in CC130-MRSA-XI (Cuny et al., 2011; Shore et al., 2011; Sabat et al., 2012; Monecke et al., 2013; Harrison et al., 2013). However, *Staph. aureus* is able to integrate mobile genetic elements on which enterotoxins are located (Argudín et al., 2010) and the enterotoxin C gene *sec* was already detected in *mecC*-carrying MRSA CC599 strains (Sabat et al., 2012). Therefore, the genetic properties of *mecC*-positive isolates should be monitored in the future. *Staphylococcus aureus* transfer through direct contact between humans and cows is considered to be more likely than transmission through untreated milk (Tenhagen et al., 2008). In the present study, the livestock owner did not show a detectable nasal *Staph. aureus* colonization.

The presence of *mecC* causes resistance to all β -lactams used in veterinary medicine (Monecke et al., 2012). This also applies to the isolates of the present study, even if they were partially tested susceptible to different β -lactams. The phenotypic absence of the resistance against different β -lactams in MRSA isolates has also been described by Brown (2001) and Fernandes et al. (2005). At least in *mecA*-MRSA isolates, growth conditions in routine diagnostics seem to be suboptimal for MRSA strains, and the expression of *mecA* is diminished. Moreover, it has been demonstrated that the *mecC*-encoded PBP2a is unstable at 37°C and that it has a higher relative affinity for oxacillin as compared with cefoxitin contrary to the *mecA*-encoded PBP2a (Kim et al., 2012). Therefore, the observed difference in resistance profile might be due to the distinct biochemical properties of the *mecC*-encoded PBP2a. As penicillins and cephalosporins are crucial in mastitis therapy, the therapy options in the affected herd are very limited. For the generally recommended dry-up therapy for *Staph. aureus* mastitis (Pyörälä, 2009), not a single antimicrobial agent is available and authorized in Germany that does not contain at least 1 β -lactam component (Vetidata, 2014). Thus, any intervention is restricted to infection control measures, especially during milking. Therefore, the livestock owner is advised to take hygienic precautions for milking (formation of a *Staph. aureus* group, teat dipping, wearing gloves, general disinfection of milking equipment) as well as for the removal of (possible) pathogen reservoirs (especially chronically diseased and permanently secreting cows). Even if an effective antibiotic treatment would be available, it should be applied only after careful consideration—particularly against the background of the current resistance debate. Culling the diseased animals

would be the only reasonable way to eliminate the germ (Friedrich et al., 2011). However, especially from an owner's point of view, the culling of the animals is not warranted due to an absence of an SCC elevation. At the time of livestock examination, the bulk milk SCC was below 130,000 cells/mL of milk, and thus even below the Bavarian average of 158,000 cells/mL of milk (Milchprüfing Bayern, 2013). Other symptoms associated with mastitis (i.e., reduced milk yield) were also not reported by the farmer. If *mecC*-positive *Staph. aureus* are not necessarily associated with cell count increases, the livestock owners will not necessarily see a reason for a bacteriological examination of a herd and, hence, supposed *mecC* herds cannot be identified. A potential cause for the low cell counts might be the absence of certain virulence-associated genes, and thus a low pathogenic potential. With reference to Younis et al. (2005), the presence of leukocidin genes in *Staph. aureus*, for example, is associated with an inflammatory response in milk. Being superantigens, the toxic shock syndrome toxin and different enterotoxins can cause an overproduction of proinflammatory cytokines and can therefore influence the inflammatory response, too. In accordance to Cuny et al. (2011), Sabat et al. (2012), and Monecke et al. (2013), neither *lukF-P83/lukM* nor *lukS/F-PV* nor *tst-1* genes were detected in the *mecC*-positive *Staph. aureus* during the present investigation. Asymptomatic colonization without an increased SCC might be a factor in the spread of the organism in an animal population.

Only a few resistance genes detectable by means of microarray test could be found in the examined *mecC*-positive isolates. This observation may suggest a low selection pressure resulting from a restricted use of antibiotics in bovine practice. However, it has to be considered that StaphyType test targets focus on human medicine purposes, and therefore certain markers such as *erm*(T), *dfrK*, *tetL*, and *vgaE* might not have been detected. The absence of the multiresistance gene *cfr* indicates that resistance to linezolid in 7 isolates is likely based on mutations. Mutations in 23S ribosomal RNA are the most frequent reason for linezolid-resistance (Besier et al., 2008), and it is possible that a single mutated *Staph. aureus* has spread in the herd.

The present study shows that a detection of *mecC*-positive CC130 *Staph. aureus* might not only point to isolated, sporadic cases, but also to multiple cases in dairy cattle herds. Livestock from the affected herd will be examined frequently in the future, with the calves, heifers, and fattening bulls on the farm the focus of further investigation. Infection with *mecC*-positive *Staph. aureus* has so far barely shown any effect in the form of a cell count increase. This might be different under certain circumstances, such as stress (e.g., an

increase in stocking density, sudden changes in food), decreased immunity, poor nutritional status, and so on, and thus the clinical significance of CC130 *Staph. aureus* in dairy cattle needs to be further elucidated. Livestock monitoring with regard to *mecC* is of vital importance to be able to draw conclusions regarding the spread and effect of *mecC*-positive MRSA strains.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance by Ines Engelmann, Jana Sachschal (Jena) and Dominik Einert, Judith Buchenberg, Silvia Frahs, Zarife Preniqi, Susanne König-Wiedemann, and Silvia Müller (Poing), as well as the support by Eugen Ermantraut and the critical reading from Carly Scott. The investigations described herein were funded by the laboratories where they were performed; no external funding was used for this project. The authors acknowledge the Bavarian State Ministry for Food, Agriculture and Forestry and the Bavarian Livestock Insurance Agency for supporting and funding the routine operations of the laboratory of the Bavarian Animal Health Service.

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