Research articles

SINGLE-NUCLEOTIDE POLYMORPHISM IN THE SCCMEC-ORFX JUNCTION DISTINGUISHES BETWEEN LIVESTOCK-ASSOCIATED MRSA CC398 AND HUMAN EPIDEMIC MRSA STRAINS

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A number of real-time PCR assays for direct detection of methicillin-resistant (MRSA) in clinical specimens are targeting staphylococcal cassette chromosome *mec* (SCCmec) right extremity sequences and the *S. aureus* chromosomal *orfX* gene sequences located to the right of the SCCmec integration site. When testing 184 MRSA strains of human and animal origin from geographically distinct locations, we identified several characteristic single-nucleotide polymorphisms (SNPs) within the SCCmec-*orfX* junction of livestock-associated (LA) MRSA CC398 which serve as suitable strain markers for screening purposes. Within an assay time of 60 minutes and an additional 10 minutes for the melting curve analysis, all MRSA CC398 isolates were correctly identified by their characteristic T_m value in the commercial LightCycler MRSA Advanced test. Studies to confirm the diagnostic accuracy of the SNP-based strain identification assay with a larger collection of clinical and LA-MRSA strains are ongoing.

Introduction

Rates of methicillin-resistant *Staphylococcus aureus* (MRSA) infections have steadily increased during the past two decades and the occurrence and spread of MRSA strains in healthcare facilities as well as in the community is a growing problem worldwide [1,2,3]. Although classically considered as a nosocomial pathogen, reports of MRSA carriage or its acquisition in the community have become increasingly common during the past decade [2,4]. More recently, studies have demonstrated that especially swine and swine farmers in Austria [5], Denmark [6], Germany [7,8], the Netherlands [9-11], Portugal [12], the United States [13], and many other countries are colonised with MRSA. Since it was realised that livestock animals may form a new, separate reservoir, these strains are now called livestock-associated MRSA (LA-MRSA). Molecular characterisation revealed that a distinct clone of MRSA is predominant within this collective: LA-MRSA strains are grouping within the new clonal complex 398 (CC398) with sequence type 398 (ST398) as the most prevalent type. Animals carrying MRSA represent a reservoir for transmission to humans [13,14,15]. The MRSA strains from animal origin have been shown to be pathogenic for humans and can cause severe infections such as endocarditis and ventilator-

associated pneumonia [16]. A number of diagnostic strategies have been published on the molecular characterisation of the MRSA CC398 clonal lineage, using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) or other laborious techniques based on genome sequencing [1,19,20].

Active surveillance is needed

Since livestock animals may act as a reservoir for this bacterium, the development of rapid molecular methods for screening and identification of MRSA CC398 will have important implications for surveillance, epidemiology and future infection control initiatives. As with many other bacterial pathogens, the rapid and reliable detection of certain MRSA clones is of the utmost importance to prevent the spread of infection. A number of real-time PCR assays targeting staphylococcal cassette chromosome *mec* (SCCmec) right extremity sequences and the *S. aureus* chromosomal *orfX* gene sequences located to the right of the SCCmec integration site have recently been established for direct detection of MRSA in clinical specimens. In the course of the present study, such assays were evaluated for their performance to detect and distinguish LA-MRSA strains of the new clonal complex 398 (CC398).

Materials and Methods

Study population, survey methods and diversity of investigated strains

During an on-going study conducted by the Bavarian Health and Food Safety Authority to explore the epidemiology of MRSA CC398 in Bavaria, the MRSA colonisation status among swine bred in Bavaria and the involved farmers was investigated by sampling the nares of 634 swine and 116 farmers on 60 geographically distinct farms. Epidemiological results will be available when this particular study is completed. Additional representatives of the MRSA CC398 lineage from other geographic locations and other sources including horses, dogs, guinea pigs, chicken, poultry and humans with contact to colonised animals, as well as MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates of presumed to be related spa-types were kindly provided by a number of supporting laboratories listed in the Table and in the Acknowledgements section.

Diagnostic culture and template DNA preparation

The *S. aureus* strains collected from pigs and farmers in Bavaria were maintained on Columbia blood agar and identified by colony morphology, Gram-stain characteristics, catalase reaction, coagulase production, and the results of the API Staph system (bioMérieux). Oxacillin susceptibility was determined by the agar screening method with Mueller-Hinton agar containing 2% NaCl and 6 mg/l of oxacillin for *S. aureus* [3,19]. An identical protocol was applied for characterisation and maintaining the various *S. aureus* strains provided by supporting laboratories. Template DNA for PCR was prepared from individual bacterial colonies by a simple and rapid ‘boiling’ procedure [20]. Briefly, colonies were suspended in 200 µl of lysis buffer containing 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA, and incubated for 10 min at boiling temperature. After centrifugation for 2 min at 10.000 x g to sediment the debris, a 2 µl aliquot of the clear supernatant was directly transferred to PCR. Alternatively, the reagents and the protocol of the LightCycler Advanced Lysis kit (Roche Diagnostics) were used for template DNA preparation. For cultured bacterial organisms, the efficiencies of the commercial lysis kit and the ‘boiling’ procedure were found to be comparable for the extraction of amplifiable *S. aureus* DNA (data not shown).

PCR amplification, DNA sequencing and molecular strain typing

Amplification and sequencing of the *SCCmec-orfX* junction was performed according to Hagen et al. [21]. PCR reactions and subsequent hybridisation probe melting curve analyses were carried out on a Roche LightCycler 2.0 device. Amplicons of the expected size were purified (HighPure PCR Cleanup Micro kit,

Roche Diagnostics) and sequenced on an automated ABI 310 sequencer using BigDye v. 1.1 chemistry.

Real-time PCR amplification and detection reactions were carried out according to the protocol of the LightCycler MRSA Advanced test (Roche Diagnostics). In the case of a negative result, an in-house duplex PCR assay was performed targeting a segment of the *mecA* gene and the *S. aureus*-specific genomic fragment Sa 442 [22]. For selected *S. aureus* strains, accessory testing was performed with well-established commercial PCR tests designed for direct detection of MRSA from clinical specimens, namely the GenoType MRSA Direct (Hain Lifescience), the BD GeneOhm MRSA (Becton Dickinson) and the Xpert MRSA (Cepheid) assays.

The presence of Panton-Valentine leukocidin (PVL) [23] was investigated by PCR testing for the *lukS-PV* and *lukF-PV* genes [2,4]. Typing of the *S. aureus* protein A gene (*spa*) was performed for all isolates obtained in this study using a standard protocol [24]. Clustering of *spa* types into *spa* clonal complexes (*spa*-CC) was performed using the Based Upon Repeat Pattern (BURP) algorithm of the Ridom StaphType software (Ridom GmbH) with the following preset parameters as recommended previously [25]: *Spa* types were clustered into the same group if the cost was four or less; *spa* types which were shorter than five repeats were excluded. When an isolate was indicated to be closely related to a *spa* type presumed to be associated with CC398, but the *T_m* values observed in the LightCycler MRSA Advanced test did not correspond to the *T_m* values expected for CC398 isolates, MLST- and *SCCmec*-types of the isolates were determined [1] or provided by the supporting

TABLE

MRSA isolates investigated in the present study (n=184)

Number of tested strains	MLST	<i>spa</i> type	<i>T_m</i> value (°C)	<i>mecA</i>	Sa 422	Xpert MRSA	BD GeneOhm MRSA	Source	Geographic origin	Culture result	Comment
8	ST 398	t011	55	n.d.	n.d.	pos.	pos.	Piglets	Germany (center 1)	MRSA	Bavarian LA-MRSA survey
11	ST 398	t034	55	n.d.	n.d.	pos.	pos.	Piglets	Germany (center 1)	MRSA	Bavarian LA-MRSA survey
11	ST 398	t011	55	n.d.	n.d.	pos.	pos.	Fattening pigs	Germany (center 1)	MRSA	Bavarian LA-MRSA survey
5	ST 398	t034	55	n.d.	n.d.	pos.	pos.	Fattening pigs	Germany (center 1)	MRSA	Bavarian LA-MRSA survey
5	ST 398	t011	55	n.d.	n.d.	pos.	pos.	Humans (pig farmers)	Germany (center 1)	MRSA	Bavarian LA-MRSA survey
4	ST 398	t034	55	n.d.	n.d.	pos.	pos.	Humans (pig farmers)	Germany (center 1)	MRSA	Bavarian LA-MRSA survey
1	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Pig	Germany (center 2)	MRSA	
1	ST 398	t2510	55	n.d.	n.d.	n.d.	n.d.	Pig	Germany (center 2)	MRSA	
1	ST 398	t1451	55	n.d.	n.d.	n.d.	n.d.	Pig	Germany (center 2)	MRSA	
1	ST 398	t108	55	n.d.	n.d.	n.d.	n.d.	Pig	Germany (center 2)	MRSA	
13	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Human (pig farmers conference)	Austria (center 3)	MRSA	SCCmec type V
1	ST 398	t2576	55	n.d.	n.d.	n.d.	n.d.	Human (veterinarian conference)	Austria, Germany, Switzerland (center 3)	MRSA	SCCmec type V
7	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Human (veterinarian conference)	Austria, Germany, Switzerland (center 3)	MRSA	SCCmec type V
3	ST 398	t034	55	n.d.	n.d.	n.d.	n.d.	Human (veterinarian conference)	Austria, Germany, Switzerland (center 3)	MRSA	SCCmec type V
13	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Human (pig farmers and veterinarians)	Austria (center 3)	MRSA	SCCmec type V
24	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Dust (from pig breeding facilities)	Austria (center 3)	MRSA	

1	ST 398	t034	55	n.d.	n.d.	n.d.	n.d.	Dust (from pig breeding facility)	Austria (center 3)	MRSA	
10	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Pigs	Germany (center 8)	MRSA	
1	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Human (wound swab)	Germany (center 1)	MRSA	
1	ST 398	t1456	55	n.d.	n.d.	n.d.	n.d.	Human (wound swab)	Germany (center 1)	MRSA	
1	ST 398	t1456	55	n.d.	n.d.	n.d.	n.d.	Human (wound swab)	Germany (center 1)	MRSA	
1	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 5)	MRSA	
7	ST 398	t108	55	n.d.	n.d.	n.d.	n.d.	Humans	Netherlands (center 7)	MRSA	
2	ST 398	t034	55	n.d.	n.d.	n.d.	n.d.	Humans	Netherlands (center 7)	MRSA	
9	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Humans	Netherlands (center 7)	MRSA	
2	ST 398	t034	55	n.d.	n.d.	n.d.	n.d.	Humans	Denmark (center 6)	MRSA	SCCmec type V
1	ST 398	t108	55	n.d.	n.d.	n.d.	n.d.	Human	Denmark (center 6)	MRSA	SCCmec type V
2	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Humans	Denmark (center 6)	MRSA	
1	ST 398	t5706	55	n.d.	n.d.	n.d.	n.d.	Human	Denmark (center 6)	MRSA	
1	ST 398	t108	55	n.d.	n.d.	n.d.	n.d.	Human	Denmark (center 6)	MRSA	PVL positive, SCCmec type V
1	n.d.	t1793	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	PVL positive
1	n.d.	t1250	55	n.d.	n.d.	n.d.	n.d.	Pig	Germany (center 2)	MRSA	
2	n.d.	t011	55	n.d.	n.d.	n.d.	n.d.	Poultry	Germany (center 2)	MRSA	
1	n.d.	t011	55	n.d.	n.d.	n.d.	n.d.	Guinea pig	Germany (center 2)	MRSA	
1	n.d.	t011	55	n.d.	n.d.	n.d.	n.d.	Dog	Germany (center 2)	MRSA	
3	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Horses	Germany (center 2)	MRSA	
1	n.d.	t1457	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t1580	55	n.d.	n.d.	n.d.	n.d.	Human (pharynx)	Germany (center 2)	MRSA	
1	n.d.	t2011	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t1451	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t2346	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t2370	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t2576	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t2741	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t3423	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t1255	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t1197	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t571	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t108	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t2582	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	n.d.	t034	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	ST 398	t011	55	n.d.	n.d.	n.d.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	ST 30	t138	59	pos.	pos.	pos.	n.d.	Human (nose)	Germany (center 2)	MRSA	
1	ST 9	t1430	59	pos.	pos.	pos.	n.d.	Chicken (wing)	Germany (center 4)	MRSA	SCCmec type IVa
1	ST 1	t127	59	pos.	pos.	pos.	pos.	Piglet (nose)	Germany (center 1)	MRSA	
1	ST 398	t034	neg.	pos.	pos.	neg.	neg.	Human	Denmark (center 6)	MRSA	SCCmec type IVa
1	ST 398	t034	neg.	pos.	pos.	neg.	neg.	Human	Denmark (center 6)	MRSA	SCCmec type VII
1	ST 398	t571	neg.	pos.	pos.	neg.	neg.	Human	Denmark (center 6)	MRSA	PVL positive, SCCmec non-typeable
1	ST 398	t1606	neg.	pos.	pos.	neg.	neg.	Human (nose)	Germany (center 2)	MRSA	SCCmec non-typeable
1	ST 753	t898	neg.	pos.	pos.	neg.	neg.	Human (nose)	Germany (center 2)	MRSA	SCCmec non-typeable
1	ST 398	t567	neg.	pos.	pos.	neg.	neg.	Human (nose)	Germany (center 2)	MRSA	SCCmec non-typeable
1	ST 30	t021	neg.	pos.	pos.	neg.	neg.	Human (nose)	Germany (center 2)	MRSA	SCCmec non-typeable

BD GeneOlm MRSA: test result of a commercial MRSA-specific PCR assay (Becton Dickinson); MLST: multilocus sequence typing; MRSA: methicillin-resistant *Staphylococcus aureus*; mecA: test result of an in-house realtime PCR assay targeting the *mecA* gene [20]; neg: no specific amplification products observed or negative test result for MRSA; n.d. not done; pos: specific amplification products observed or positive test result with the applied realtime PCR assays designed for direct detection of MRSA by targeting the *SCCmec-orfX* integration site; pSA422: test result of an in-house realtime PCR assay targeting a *S. aureus*-specific species marker gene *Sa422* [20]; T_m : T_m -value observed with the LightCycler MRSA Advanced Test (Roche Diagnostics); Xpert MRSA: test result of a commercial MRSA-specific PCR assay (Cepheid).

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FIGURE 1

Multiple sequence alignment of a selected *S. aureus* *orfX* segment

FJ830606	CCGCATCATTG G TGTGG A AATGTCATTTTGCTGAATGATA	porcine MLST 398
AB033763	A.....G.....	SCCmec Type I
D86934	A.....G.....	SCCmec Type II
AB047089	A.....G.....	SCCmec Type III
AB063172	A.....G.....A.....	SCCmec Type IVa
AB063173	A.....G.....	SCCmec Type IVb
AB096217	A.....G.....	SCCmec Type IVc
DQ106887	A.....G.....	SCCmec Type IVg
AB121219	A.....G.....A.....	SCCmec Type V
AF411935	A.....G.....	SCCmec Type VI
AB373032	A.....G.....	SCCmec Type VII
FJ390057	A.....G.....	SCCmec Type VIII
AM292304	A.....G.....	SCCmec Type unknown
AB425823	A.....G.....	SCCmec Type IVa
U10927	..T.....	SCCmec Type unknown
Pos.	300 312 318 330 340	in FJ830606

FJ830606	GTGCGTAGTTACTGCGTTGTAAGAC A TCCTTGTGCAGGCCG	porcine MLST 398
AB033763	G.....	SCCmec Type I
D86934	G.....	SCCmec Type II
AB047089	G.....	SCCmec Type III
AB063172	A.....G.....	SCCmec Type IVa
AB063173	G.....	SCCmec Type IVb
AB096217	G.....	SCCmec Type IVc
DQ106887	G.....	SCCmec Type IVg
AB121219	A.....G.....	SCCmec Type V
AF411935	G.....	SCCmec Type VI
AB373032	G.....	SCCmec Type VII
FJ390057	G.....	SCCmec Type VIII
AM292304	G.....	SCCmec Type unknown
AB425823	A.....G.....	SCCmec Type IVa
U10927	G.....	SCCmec Type unknown
Pos.	341 350 360 366 381	in FJ830606

FJ830606	TTTGATCCGCCAATGACGAATACAAAGTCGCTTGGCCCTTG	porcine MLST 398
AB033763	A.....	SCCmec Type I
D86934		SCCmec Type II
AB047089		SCCmec Type III
AB063172		SCCmec Type IVa
AB063173	A.....	SCCmec Type IVb
AB096217		SCCmec Type IVc
DQ106887		SCCmec Type IVg
AB121219		SCCmec Type V
AF411935		SCCmec Type VI
AB373032		SCCmec Type VII
FJ390057		SCCmec Type VIII
AM292304		SCCmec Type unknown
AB425823		SCCmec Type IVa
U10927		SCCmec Type unknown
Pos.	382 390 400 410 422	in FJ830606

Multiple sequence alignment of a selected *S. aureus orfX* segment located close to the *SCCmec-orfX* junction (position 253 in GenBank FJ830606). The most similar sequences found in BLAST search show either a sequence identical to GenBank sequence entries of *S. aureus* isolates carrying one of the eight *SCCmec* types or differ from the MRSA ST 398 isolates of the study by at least two nucleotides at positions 312 and 366 (GenBank FJ830606).

laboratories. Typing of *SCCmec* elements of types I to VII was carried out according to previously published PCR procedures [26].

Results

Molecular characteristics of MRSA isolates derived from the Bavarian LA-MRSA survey

By sampling the nares of 634 swine and 116 farmers on 60 geographically distinct farms in Bavaria during the course of an ongoing study, a total number of 245 MRSA strains from pigs and 34 MRSA strains from farmers were grown from the collected swabs. From this collection, 44 MRSA isolates from geographically distinct farms were chosen for further analyses (Table, rows 1 to 6). The distribution of *spa* types among these isolates was as follows: t011 (n=24) and t034 (n=20). MLST-typing of all selected MRSA isolates revealed that they belonged to MLST ST398. All 44 MRSA strains tested negative for PVL-encoding genes.

Novel single nucleotide polymorphisms in the *SCCmec-orfX* integration site of LA-MRSA isolates

By systematic sequencing of the *SCCmec-orfX* integration sites of MRSA isolates of animal origin, all of the 44 sequences obtained from Bavarian porcine isolates (Table, rows 1 to 6) were found to be identical in a multiple alignment (using pileup from the HUSAR sequence analysis package from the German Cancer Research Center (DKFZ), <http://genius.embnet.dkfz-heidelberg.de>, data not shown). As the sequence differed from previously published *SCCmec-orfX* integration site sequence motifs, it was deposited in

GenBank with accession number FJ830606 (to be released after publication).

A detailed investigation of the novel sequence revealed some nucleotide positions uncommon in *S. aureus* GenBank sequences and at least three characteristic single nucleotide polymorphisms (SNPs) in the *S. aureus* chromosomal *orfX* gene sequence located to the right of the *SCCmec* integration site: guanine at position 312, adenine at position 366, and cytosine at position 441 (GenBank FJ830606). At least two of these SNPs were found exclusively in the investigated MRSA strains of animal origin and may serve as a diagnostic marker for the presence of MRSA CC398. A BLAST search (National Center for Biotechnology Information (NCBI), <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the complete amplicon sequence revealed GenBank accession number AM292304 (*S. aureus* *SCCmec*ZH47 mobile element) as the most similar hit with five mismatches. GenBank AB425823 and U10927, the next similar sequences found in the BLAST search, were either identical to GenBank entries of one of the eight acknowledged MRSA *SCCmec* types deposited in GenBank, or had at least one nucleotide difference at position 366 compared with the sequence FJ830606 obtained from the investigated MRSA ST398 strains of porcine origin (Figure 1).

Practical application of the identified single nucleotide polymorphisms

In addition to the broad spectrum of unpublished in-house PCR protocols, also the proprietary sensor hybridisation probe of

FIGURE 2

Specificity of the Roche LightCycler MRSA Advanced test for differentiating MRSA CC398 and non-CC398 strains in hybridisation probe melting curve analysis

The curves represent the MRSA strain ATCC 33592 ($T_m \approx 60^\circ\text{C}$) and 30 MRSA ST 398 strains of porcine origin with characteristic T_m values around 55.5°C . A methicillin-sensitive strain of *S. aureus* (clinical isolate) was used as negative control.

the recently developed LightCycler MRSA Advanced Test (Roche Diagnostics) covers one of these two particular nucleotide positions. This real-time PCR assay detects MRSA strains with different molecular sequences surrounding the right extremity junction of the *SCCmec* cassette with the *S. aureus orfX* gene. As a practical application of the SNPs identified in our study, we present the use of this commercial real-time PCR kit for the direct detection of MRSA and simultaneous identification of LA-MRSA CC398. For all 44 investigated Bavarian MRSA ST398 strains, specific amplification products were generated with the LightCycler MRSA Advanced test, and they all harboured at least one of the identified SNPs in the *SCCmec-orfX* junction represented by a characteristic T_m of 55.5 °C in the subsequent LightCycler hybridisation probe melting curve analysis (Figure 2). Since we have not yet observed such a T_m -shift with any non-ST398 MRSA strains of human or animal origin, this point mutation may serve as a molecular marker for the presence of MRSA CC398.

As an approved in vitro diagnostics (IVD) product, the Roche LightCycler MRSA Advanced test has already been validated with a comprehensive collection of MRSA strains of human origin for the limit of detection, inclusivity and exclusivity. The results of systematic studies on the assay's diagnostic performance will be published soon (personal communication, Roche Diagnostics). According to the product information of the test kit, the range of T_m values observed in these multicenter validation studies with various epidemic MRSA clones of human origin was from 57.0 to 62.0 °C. Therefore a T_m value of 55.5 °C observed with MRSA CC398 should be discriminative with respect to most of the clinical MRSA strains, and melting curve analysis represents a reliable surrogate marker for screening purposes.

From a technical point of view, it should be noted that melting points outside the expected range of 57.0 to 62.0 °C have to be examined manually in the LightCycler software. When testing MRSA CC398 strains of the present study, the calculation algorithms embedded in the automated assay interpretation software of the LightCycler MRSA Advanced test (Micro Analysis Software; MAS) reported "MRSA result: not detected" with a specific comment "Peak(s) outside Target TM range".

In the course of the study, we also applied a number of other commercial PCR tests targeting the *SCCmec-orfX* junction. These included the GenoType MRSA Direct (results not shown), the BD GeneOhm MRSA, and the Xpert MRSA test. The 44 investigated Bavarian MLST CC398 strains, which had all tested positive in the LightCycler MRSA Advanced test, also tested positive for MRSA in these other assays (see Table) - but these PCR test platforms either did not have an option to perform a hybridization probe melting curve analysis or did not allow viewing such melting curve data. Since clinical sensitivity of real-time PCR assays may also depend on the annealing temperatures of the respective probes, it is currently unclear whether the point mutations in the target region will have an impact on the sensitivity when testing samples from patients or animals.

Testing of non-Bavarian MRSA strains within or related to MLST CC398

In addition to the 44 strains of the Bavarian porcine LA-MRSA survey, 140 MRSA strains recovered from animals and humans in other geographical regions or from other animal sources as well as *S. aureus* isolates of spa-types sharing similar spa repeat patterns, were included in the present study to further address the diversity among isolates within the MLST CC398 clonal complex. Overall, 133 of the 140 isolates were successfully detected by the LightCycler MRSA Advanced test. The collection of investigated strains is shown in detail in the Table, together with the characteristic T_m values observed in the LightCycler MRSA Advanced test and the corresponding results of supplementary *S. aureus*- and *mecA*-specific PCR assays, as well as the results obtained in other commercial PCR tests targeting the *SCCmec-orfX* junction. While seven isolates were not detectable, 130 isolates were associated with T_m values of 55.5 °C in the LightCycler MRSA Advanced test, indicative of the presence of the novel SNPs, and three isolates were associated with T_m values of 59 °C, known to be within the range observed for the epidemic MRSA clones of human origin.

All of the applied real-time PCR assays, which are designed for direct detection of MRSA by targeting the *SCCmec-orfX* integration site, failed to generate specific amplification products with seven (3.8%) of the investigated MRSA strains (Table). The MRSA phenotype of these strains was confirmed by diagnostic culture including oxacillin susceptibility testing. In addition, the MRSA genotype was confirmed by an in-house duplex PCR assay targeting the *mecA* gene and a *S. aureus*-specific species marker. *SCCmec* typing of these seven isolates revealed that one was associated with *SCCmec* IVa, one with *SCCmec* VII and five isolates were not typeable using a common *SCCmec* typing approach.

A population snapshot based on the BURP algorithm was performed for all MRSA isolates included in the study (Figure 3). For arithmetical reasons, three isolates characterised by a T_m of 55.5 °C (two t1456 isolates and one t2510 isolate, all typed as MLST ST398) were excluded from spa cluster formation by BURP because they were shorter than five repeats. The snapshot showed that all remaining 127 isolates associated with T_m values of 55.5 °C clustered into one spa-CC. This spa-CC comprised the major spa types t011 and t034 shown to be associated with MLST ST398. This spa-CC contained a further 20 spa types sharing closely related spa repeat patterns: t108, t567, t571, t898, t1197, t1250, t1255, t1451, t1457, t1580, t1606, t1793, t2011, t2346, t2370, t2576, t2582, t2741, t3423 and t5706.

FIGURE 3

Population snapshot of the tested isolates based on BURP analysis (n=127)

Each dot represents a single spa type and the diameter of the dot reflects the number of isolates associated with the respective spa type. Group founders are coloured in blue, Subgroup founders are coloured in light blue.

Moreover, six of the seven isolates not detected by the LightCycler MRSA Advanced test clustered in this spa-CC. MLST typing revealed that five isolates (two of spa type t034 and one each of types t567, t571 and t1606) were associated with ST398, and one isolate associated with spa type t898 was MLST ST753 (90-35-19-2-20-26-39), which is closely related to ST398 (3-35-19-2-20-26-39). Thus, all these six isolates were part of the CC398 complex. The remaining isolate not detected by the LightCycler MRSA Advanced test was associated with spa type t021 (ST30).

Those three strains that were characterised by a T_m of 59.0 °C in the LightCycler MRSA Advanced test showed spa types t127 (ST1), t138 (ST30) and t1430 (ST9).

Discussion and conclusions

Although a number of comprehensive studies have been published on the molecular characterisation and detection of the CC398 clonal MRSA lineage using PFGE, MLST or other techniques based on genome sequencing [1,16], this is the first report on a truly rapid detection and/or screening method for this livestock-associated clonal lineage based on characteristic SNPs within a popular target sequence of MRSA-specific PCR assay.

Here, 184 different LA-MRSA isolates obtained from various geographic regions in several European countries and from different sources including pigs, horses, dogs, guinea pigs, chicken, poultry as well as associated in-contact humans were systematically investigated for a characteristic SNP-induced T_m -shift in the LightCycler MRSA Advanced test.

The novel SNPs within the *S. aureus* chromosomal *orfX* gene detected in the investigated LA-MRSA isolates seemed to represent a conserved sequence motif for these MRSA strains. Even if seven of 184 MRSA strains (six of which were LA-MRSA CC398) were not picked up by the assays due to the presence of uncommon *SCCmec* elements, it can be stated that the investigated commercial PCR tests targeting the *SCCmec-orfX* junction showed acceptable inclusivity rates for members of the MRSA CC398 complex. A spa type population snapshot applying the BURP algorithm showed that all MRSA isolates characterised by the SNP-induced T_m -shift in the LightCycler MRSA Advanced test clustered into a distinct spa clonal complex indicative for CC398. Therefore, the novel SNPs within the *S. aureus* chromosomal *orfX* gene sequences could serve as a discriminative marker for MRSA belonging to the CC398 complex.

It is a well known fact that primer and probe sequences of the current PCR assay concepts are designed to cover the most common *SCCmec* types encountered in clinical MRSA isolates. With our increasing knowledge about the enormous sequence diversity of *SCCmec* sequences, rational primer selection and assay design can only be a best compromise between the coverage of as many *SCCmec* variants as possible and loss of analytical sensitivity due to primer multiplexing problems in the PCR reaction mixture.

In the course of the present study, we identified a powerful additional feature of the commercial Roche LightCycler MRSA Advanced test. This observation is another example for the fact that the natural diversity of MRSA is also reflected on genomic level. The more isolates are tested for a given target sequence, the more nucleotide mutations or deletions may be encountered. This fact has also implications on the design of specificity panels when developing assays. The assay panel covering epidemiologically relevant clones frequently encountered in patients at risk for MRSA infection is not necessarily congruent with the spectrum

of variant isolates that may be found in a specific geographical or epidemiological setting (e.g. introduction of LA-MRSA lineages into a hospital setting). A recent study by Bartels et al. [25] highlighted this problem reporting on a variant *SCCmec* type IVa clone (spa t024 ST 8) circulating in Copenhagen, which was not detected by a commercial real-time PCR assay targeting the *SCCmec-orfX* junction.

Now that characteristic SNPs have been identified, colleagues may verify our findings with their collections of animal-associated MRSA strains and may check the primer and probe sequences of their individual in-house PCR protocols targeting the *SCCmec-orfX* junction for the ability to cover and/or to discriminate MRSA CC398 from human MRSA clones.

If the LightCycler MRSA Advanced test was implemented in a diagnostic laboratory for the intended purpose of direct detection of MRSA in clinical specimens, the occurrence of presumptive MRSA CC398 strains could be monitored without extra work or extra cost just by looking at the melting curve screen. In combination with the simple 'boiling'-protocol for template DNA preparation, it can be easily integrated into the workflow of any clinical or veterinary laboratory routinely using molecular techniques for diagnostic purposes. Once growth of staphylococci is observed on agar plates, a portion of the colony can be transferred to PCR and discriminative MRSA results can be available within 80 minutes. Moreover, knowing about our study results, users of this assay will no longer be confused by the comment "Peak(s) outside Target T_m range" generated by the automated assay interpretation software.

In conclusion, the characteristic SNP-induced T_m -shift found in the LightCycler MRSA Advanced test was shown to be suitable to rapidly identify LA-MRSA CC398 clones. By simultaneous screening for general MRSA carriage as well as for MRSA CC398 carriage, this commercial real-time PCR test or comparable assay designs may help to monitor the spread of MRSA CC398 in the human population and, in particular, its importation into healthcare settings. Moreover, this approach may be helpful in screening for MRSA CC398 carriage among animals, farmers or other risk groups.

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