Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant Staphylococcus aureus ST398 from diseased swine

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Objectives: Fifty-four methicillin-resistant Staphylococcus aureus (MRSA) ST398 isolates from unrelated diseased swine collected all over Germany were comparatively investigated for their antimicrobial resistance and virulence properties, and for their genomic relatedness.

Methods: MICs of 30 antimicrobial agents were determined by broth microdilution. Resistance and virulence genes were detected via a diagnostic DNA microarray and specific PCRs. The genomic relationships were determined by ApaI-PFGE, spa typing and SCCmec typing.

Results: Twenty-two distinct resistance patterns were observed. All 54 isolates were tetracycline resistant, mediated by tet(M), tet(K) and/or tet(L), with 14 isolates being only resistant to β-lactam antibiotics and tetracyclines. Trimethoprim resistance, seen in 28 isolates, was mostly due to the gene dfrK or dfrG. Among the 24 macrolide/lincosamide-resistant isolates, the genes erm(A), erm(B) and/or erm(C) were detected. The two chloramphenicol/florfenicol-resistant isolates harboured the gene fexA. The eight gentamicin-resistant isolates carried the gene aacA/aphD. Fifty-three isolates harboured SCCmec type V elements while the remaining one carried mecA and ugpQ, but no recombinase genes. All isolates were PVL negative, but one and three isolates, respectively, were positive for the enterotoxin B and enterotoxin K and Q genes. Eight different spa types were identified with t011 being the most predominant. Six ApaI-PFGE clusters with up to nine individual patterns were detected.

Conclusions: MRSA ST398 isolates varied slightly in their virulence properties and spa types but differed distinctly in their antimicrobial resistance pheno- and genotypes as well as their ApaI-PFGE patterns. These data underline the ability of ST398 to acquire genetic material that might increase antimicrobial resistance and virulence.

Keywords: MRSA, SCCmec, zoonosis, genotyping

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is an important pathogen that causes healthcare- and community-associated infections in humans worldwide.1 During recent years, companion animals as well as food-producing animals have gained attention as carriers and shedders of MRSA isolates.2–6 Among the different clonal lineages identified in animals, MRSA isolates of the clonal lineage ST398 have been considered a matter of public health concern. Although such isolates have also been detected in horses, cattle and dogs,7 they seem to have their main reservoir in pigs.8,9 Previous studies have shown that such isolates can readily cross species barriers and also colonize and cause infections in humans. In particular, veterinarians, farmers and other persons with exposure to swine are at risk.10 To date, infections due to ST398 isolates have been detected in humans11–13 as well as in swine14,15 and an outbreak in the Netherlands17 has raised awareness of the zoonotic potential of ST398.

Previous studies in the Netherlands have shown that MRSA ST398 isolates from swine may differ in their spa types as well as their susceptibility profiles.8,14 These data point towards the

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ability of MRSA ST398 isolates to horizontally acquire genetic material from other bacteria. Such foreign genetic material may change the antimicrobial susceptibility status of the MRSA isolates and hence may have an impact on therapeutic approaches conducted to control infections due to such isolates. It is also possible that such additional genetic material increases the virulence of MRSA isolates. First proof towards acquired virulence properties was described in 2008 by the detection of MRSA ST398 isolates that harboured the Panton-Valentine leucocidin (PVL) genes.16,17 Moreover, the first MRSA ST398 isolate that carried the plasmid-borne gene cfr encoding transferable resistance to oxazolidinones has been reported recently.18 In addition, novel plasmid-borne resistance genes such as the trimethoprim resistance gene dfrK and the lincosamide/pleuromutilin/streptogramin A exporter gene vga(C) have also recently been described among porcine MRSA ST398 isolates.19,20

The aim of the present study was to provide a comprehensive comparison of unrelated MRSA ST398 isolates from swine collected all over Germany for their genetic diversity. For this, classic typing techniques, such as spa typing and PFGE with different restriction endonucleases, were used. In addition, particular emphasis was put on the detection of virulence and resistance genes by an S. aureus-specific diagnostic microarray that was supplemented by additional specific PCRs and Southern blotting. The data obtained by these approaches were intended to provide more detailed information about the genomic diversity and variability in acquired virulence and resistance properties among porcine MRSA ST398 isolates.

Materials and methods

MRSA strains

A total of 54 MRSA ST398 isolates of swine origin were included in this study. Forty-nine of these isolates were provided by local diagnostic laboratories from all over Germany during the National Resistance Monitoring programme GERM-Vet in 2008 on the basis of one isolate per herd. The remaining five isolates were from the BfT-GermVet study 2004–200615 and have been included mainly for confirmatory reasons. In these two complementary monitoring studies, only isolates from acutely diseased animals were monitored. Based on the background information provided by the diagnostic laboratories, 17 of the 54 isolates were obviously the causative agents of the respective infections [skin infections (n=8), urinary–genital tract infections (n=5), septicaemia (n=2), polyserositis (n=1) and necrotizing enteritis (n=1)]. The remaining 37 isolates originated from swine suffering from respiratory tract infections and it is most likely that these isolates represented colonizers of the nasal cavity that were accidentally co-isolated from the nasal swabs together with respiratory tract pathogens, e.g. Pasteurella multocida. The locations of the farms from which the 54 MRSA ST398 isolates originated are given in Figure 1. The geographical distribution of the isolates reflected the density of pork production in Germany with the majority of the isolates coming from the high-density regions in the Western part near the Dutch border (North-Rhine Westphalia, n=21) and the Southern part (Bavaria, n=15).

DNA microarray analysis and molecular typing

Initial analysis of the 54 isolates for their resistance and virulence properties was conducted by using a previously described diagnostic DNA microarray.21,22 This array recognizes a total of 330 different sequences (genes and alleles thereof), including species-specific genes, accessory gene regulator (agr) alleles, genes encoding virulence factors (toxins, enterotoxins, putative toxins, haemolysins, proteases, biofilm formation) and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule type-specific genes, as well as antimicrobial resistance genes. Arrays were mounted in microtiter strips (ASP system by CLONDIAG) and processed according to the manufacturer’s protocols. In principle, all targets were amplified and labelled in a primer elongation reaction. Since a single primer per target was used, this reaction was linear rather than exponential and facilitated the coverage of all targets simultaneously in a single multiplex reaction. This reaction was also used to incorporate biotin-16-dUTP into the amplicons, which were then hybridized to the array. After washing and blocking steps, horseradish peroxidase–streptavidin conjugate and, after incubation and further washing steps, Serumun Green precipitating dye (Seramun, Heidesee, Germany) were added, resulting in a specific hybridization pattern for each isolate. An image of the array was recorded and analysed using a designated reader and software (Arraymate, Iconoclust, both by CLONDIAG).

In addition, all MRSA were subjected to spa sequence typing (http://spaserver.ridom.de). PFGE was conducted with the enzymes SmaI (according to the harmony protocol) and Apal. The pulse times for Apal digests were increased from 2 to 5 s for 20 h. The Apal fragment patterns were analysed using the GelCompar software package (Applied Maths, Kortrijk, Belgium). The similarities between the profiles were calculated using the Dice coefficient, with a maximum position tolerance of 1.2%. The patterns were clustered by using the unweighted pair group method with arithmetic averages (UPGMA).

Although determination of the SCCmec type was included in the diagnostic microarray, selected isolates were retested for their SCCmec types by a previously described multiplex PCR assay.23

Susceptibility testing and detection of resistance genes

Antimicrobial susceptibility testing by broth microdilution followed the recommendations of the CLSI.24 For this, the microtitre plate panels used in the GERM-Vet programme were applied. In total, the susceptibility of the MRSA ST398 isolates to 30 antimicrobial agents was determined. These comprised penicillins (penicillin G, ampicillin, amoxicillin/clavulanic acid 2:1, oxacillin), cephalosporins (cefalotin, cefotaxime, cefoperazone, cefquinome, ceftiofur), tetracyclines (tetracycline, doxycycline), macrolides (erythromycin, tilimicosin, tylosin, tulathromycin, spiramycin), lincosamides (clindamycin, pirilmicin), folate pathway inhibitors (trimethoprim, sulfamethoxazole/trimethoprim 19:1), an aminoglycoside (gentamicin), aminocyclitolis (apramycin, spectinomycin), phenicols (chloramphenicol, florfenicol), a glycopeptide (vancomycin), (fluoro)quinolones (nalidixic acid, enrofloxacin), a pleuromutilin (tiamulin) and a polypeptide (colistin). In addition, isolates that were positive in the DNA microarray for the aminoglycoside resistance gene aadD were tested by broth macrodilution25 to determine their MICs of kanamycin. The reference strain S. aureus ATCC 29213 served as a quality control strain in the MIC determinations.

The detection of resistance genes was performed mainly via the diagnostic microarray. Additional specific Southern blot and/or PCR assays served to detect genes recently described to occur in MRSA ST398 isolates, such as the tetracycline resistance gene tet(L) and the trimethoprim resistance gene dfrK, or which have been detected in other methicillin-resistant staphylococci, such as the trimethoprim resistance gene dfrG.19,25
Results

Molecular typing and microarray analysis

A total of eight different \( \text{spa} \) types was seen with \( t011 \) (\( n = 39 \)) as the dominant type. The \( \text{spa} \) types \( t034, t1451 \) and \( t2510 \) occurred in five, three and three isolates, respectively, whereas \( t571, t1197, t1250 \) and \( t1456 \) were detected only in single isolates. Further analysis of the \( \text{spa} \) types, however, revealed that most of them were closely related to one another despite their strikingly different designations. Most likely, \( t011 \) can be considered as the basic
spa type (repeats 08-16-02-25-34-24-25) from which the other types developed mostly by the loss of either a single repeat (e.g. t1451: loss of repeat 24) or multiple repeats (e.g. t1456: loss of the terminal repeats 34-24-25), but also by duplication of certain repeats (e.g. t034: duplication of repeats 02-25). Moreover, a single G→C exchange that turns repeat 34 into repeat 46 was found to change the spa type from t011 to t1197.

As previously observed in ST398, all 54 isolates were non-typeable by Smal-directed PFGE. However, Apal digestion produced a wide range of fragment patterns. In total, six major Apal-PFGE clusters A–F (with up to nine individual patterns) were distinguished using a cut-off at 80% similarity (Figure 2). Among these six major clusters, pattern A isolates \( (n=38) \) were dominant over those assigned to clusters B \( (n=2) \), C \( (n=7) \), D

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**Figure 2.** (a) Apal fragment patterns obtained after PFGE analysis. The letters refer to the different fragment patterns in Table 1. Lanes marked with an M contain the Smal-digested *S. aureus* strain NCTC 8325. (b) Cluster analysis of the Apal fragment patterns. The numbers in brackets after the different fragment patterns indicate the numbers of isolates that exhibited the respective fragment pattern. A cut-off at 80% similarity was used to distinguish the six major clusters A–F.
of the host could be detected. All isolates were positive for infections concerning the presence of genes encoding MSCRAMMs. Combined resistance to macrolides and lincosamides was seen recently described resistance gene clfA, seb, and clfB and sasG. One isolate was positive for the enterotoxin B gene (seb) and another three isolates were positive for the enterotoxin K and Q genes (sek and seq) (Table 1).

The detection of the SCCmec types via the diagnostic microarray identified a type V SCCmec cassette in 53 isolates. The remaining isolate also carried mecA and uppQ, but no recombinase genes. Additional signals for ccrA1 in four isolates might indicate the presence of an atypical SCCmec element.

Antimicrobial susceptibility and detection of resistance genes
Susceptibility testing revealed a wide variety of 22 different susceptibility patterns. While all isolates were resistant to β-lactams and tetracyclines, 14 (25.9%) isolates did not reveal any additional resistance properties. Another 16 (29.6%) isolates exhibited resistance to a member of one more class of antimicrobial agents and another 9 (16.7%) isolates showed resistance to members of two more classes of antimicrobial agents. Resistance to members of five and six or more classes of antimicrobial agents were seen in nine (16.7%) and six (11.1%) isolates, respectively. The most predominant resistance patterns were resistance to β-lactams + tetracyclines (n = 14), β-lactams + tetracyclines + trimethoprim (n = 9) and β-lactams + tetracyclines + macrolides/lincosamides (n = 4). Each of the 19 remaining resistance patterns was observed in only one or two isolates. All isolates were susceptible to vancomycin (MICs of 0.5–1 mg/L), but revealed high MICs of >16 mg/L of colistin and of 32 to >128 mg/L of nalidixic acid.

All 54 β-lactam-resistant isolates carried mecA with all but one also harbouring the β-lactamase gene blaZ. The absence of the blaZ operon had no impact on the MICs of the penicillins and cephalosporins tested. Among the 54 tetracycline-resistant isolates, 40 carried the genes tet(M)+tet(K), 11 tet(M)+tet(K)+tet(L), while single isolates revealed the presence of tet(M)+tet(L), tet(M) or tet(L). Trimethoprim resistance was seen in 28 isolates with 14 of them carrying the recently described resistance gene dfK and another nine isolates carrying the gene dfG. No resistance was identified in the remaining five isolates with high MICs of >128 mg/L. Combined resistance to macrolides and lincosamides was seen in 24 isolates with the genes erm(A), erm(B) and erm(C) being present in 3, 6 and 12 isolates, respectively. Single isolates harboured the combination erm(A)+erm(B) or erm(A)+erm(C) while one isolate was negative not only for these genes, but also for other staphylococcal macrolide and lincosamide resistance genes, such as msr(A), mph(C) and lnu(A). Gentamicin resistance was detected in eight isolates for which the MICs were 32 to >128 mg/L, all of which carried the gene aacAaphD. The kanamycin/neomycin resistance gene aadD was detected in four isolates for which kanamycin MICs were 32 to >128 mg/L. The two chloramphenicol-resistant isolates (MICs 64–128 mg/L) also exhibited high MIC values of 64 mg/L of florfenicol. Both carried the Tn558-associated phenicol exporter gene fexA.

Discussion
The DNA microarray results obtained in this study showed that epidemiologically unrelated porcine MRSA ST398 isolates collected all over Germany had a similar set of basic species-specific, metabolic and regulatory genes, but also shared the same SCCmec type, capsule type, haemolysin genes, genes for biofilm production and genes encoding MSCRAMMs. With regard to virulence genes, the 54 isolates included in this study did not differ distinctly from one another, except for isolates carrying enterotoxin genes. The detection of the enterotoxin genes seb, sek and seq in these isolates was a novel observation and might indicate an evolutionary trend towards an increased pathogenicity for humans. In contrast to studies from China and Sweden, none of the isolates carried the PVL-encoding genes lukF-PV and lukS-PV. Since these PVL-positive isolates were obtained from humans without exposure to animal husbandry, they might indicate the presence of a distinct lineage within ST398 being more virulent towards humans.

Analysis of the susceptibility status revealed an unexpected diversity of resistance patterns. In total, 22 different resistance patterns were seen with 19 of them being present in only one or two isolates. Interestingly, 30 (55.6%) of the 54 isolates were resistant to either only β-lactams and tetracyclines or exhibited resistance to these two classes of antimicrobial agents plus one additional resistance trait. Expanded resistance patterns (i.e. resistance to compounds from six or more classes of antimicrobial agents) were seen in only six (11.1%) isolates. When checking the resistant isolates for the presence of specific resistance genes, it became obvious that for certain resistance properties, more than one resistance gene was present. All but one isolate carried a blaZ β-lactamase operon in addition to the gene mecA. Tetracycline resistance was commonly based on the presence of either two [tet(K)+tet(M), tet(L)+tet(M)] or even three [tet(K)+tet(L)+tet(M)] resistance genes. Among the macrolide-resistant isolates, the combinations of the methylase genes erm(A)+erm(C) and erm(A)+erm(B) were seen. Whenever more than one gene for a certain resistance property was present, some of these resistance genes were associated with plasmids [tet(K), tet(L), erm(C)] or transposons [blaZ, tet(M), erm(A), erm(B)] and might have been acquired by the respective isolates at different times. The stable maintenance of redundant genes encoding the same resistance property might be due either to a chromosomal integration of e.g. tet(K)-carrying plasmids and tet(M)-carrying transposons or to co-selection caused by a physical linkage with other resistance genes as recently shown for the genes tet(L) and dfK. The gene tet(L) has rarely—if at all—been detected among MRSA isolates. In the present study, most of the tet(L)-carrying isolates also harboured dfK and the physical linkage of both genes was confirmed by PCR.
Table 1. Comparison of the characteristics of the 54 MRSA ST398 isolates

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<th>Isolate(s)</th>
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<th>PFGE type</th>
<th>Resistance pattern</th>
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<th>Resistance genes</th>
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APR, apramycin; BLA, β-lactam antibiotics; CHL/FFN, chloramphenicol/florfenicol; ENR, enrofloxacin; GEN, gentamicin; KAN, kanamycin; ML, macrolides/lincosamides; SPE, spectinomycin; SXT, sulfamethoxazole/trimethoprim; TET, tetracyclines; TIA, tiamulin; TMP, trimethoprim.

Despite the lack of CLSI-approved breakpoints, strains that showed high MIC values of enrofloxacin (≥4 mg/L), spectinomycin (≥512 mg/L), tiamulin (≥32 mg/L) and apramycin (≥64 mg/L) were considered resistant.

The gene ccrAA is a gene adjacent to ccrC, accession number AM292304.1.
byible to define distinct lineages or subclones by the microarray or indistinguishable from each other (Table 1), and it was not poss-

Figure 3. Splitstree diagram visualizing relationships between MRSA ST398 isolates. Hybridization results for variable genes (as in Table 1) were converted into binary sequences and used for tree reconstruction (Splitstree 4.5).34 The putative ‘founder variant’ is shown. Isolates carrying enterotoxin genes and selected resistance genes [fexA, aacA/aphD and erm(B)] are also shown.

(datum not shown). The trimethoprim resistance genes \( drfK \) and \( drfG \) are another two resistance genes that have rarely been detected so far among MRSA isolates. The macrolide/lincosamide resistance gene \( erm(B) \) has previously been described in just one other epidemic MRSA strain (‘Taiwan Clone’, ST59-MRSA-VII).29 The Tn558-associated phenicol exporter gene \( fexA \) has been detected in the only \( efr \)-positive MRSA ST398 isolate yet described12 as well as in a few other staphylococci of animal origin.30 A comparison of the MRSA ST398 isolates for their resistance genes (Table 1) revealed distinct differences that strongly pointed towards the ability of MRSA isolates to readily acquire resistance genes from other bacteria.

A comparison of all characteristics identified 11 MRSA ST398 isolates to be largely indistinguishable by their microarray data, their resistance patterns and their assignment to PFGE cluster A. Six of these isolates were nasal colonizers obtained from pigs in the Western and North-Eastern parts of Germany whereas the remaining five isolates were from a urinary–genital tract infection (\( n = 1 \)), a skin infection (\( n = 1 \)) or from cases of septicemia (\( n = 3 \)) in the South-Eastern or Northern parts of Germany. Another three isolates were closely related but either differed in the PFGE type or showed additional trimethoprim resistance. These 14 isolates might represent a ‘founder variant’ from which other variants have derived by gene acquisitions and losses (Figure 3). Besides this cluster, only a few isolates were indistinguishable from each other (Table 1), and it was not possible to define distinct lineages or subclones by the microarray or by \( spa \) typing or other methods. This might be attributed to a very recent or rapid proliferation of MRSA ST398 isolates. Moreover, no correlations could be observed between PFGE type, \( spa \) type and disease condition/carer status. In comparison with nasal colonizers, the isolates from defined disease conditions also did not differ in their virulence profiles or their resistance profiles.

A comparison of the ApaI-PFGE results with the results of other typing techniques was performed to gain information on the preferential occurrence of isolates with specific characteristics in certain geographical regions. Isolates assigned to the most predominant PFGE cluster (A) exhibited five \( spa \) types (t011, t034, t1451, t1456 and t2510), and were disseminated all over Germany. In contrast, the seven isolates assigned to PFGE cluster C originated from the Eastern part of Germany, and they represented seven of the eight gentamicin-resistant isolates. Since there are currently no figures on the consumption of veterinary antibiotics available for the different regions in Germany, it is not known whether this resistance property has evolved as a consequence of a specific selective pressure imposed by a possible preferential use of gentamicin in the Eastern part of Germany. Another interesting observation was the geographical distribution of the macrolide/lincosamide resistance genes: all seven \( erm(B) \)-carrying isolates originated from the Western part whereas 12 of the 13 \( erm(C) \)-carrying isolates were found in the central and Eastern parts of Germany. A closer look at these isolates revealed that the \( erm(B) \)-positive isolates had three different \( spa \) types (t011, t034 and t2510) and were assigned to PFGE type A0, A1, A3 or E0 (Table 1). The \( erm(C) \)-carrying isolates also proved to be rather heterogeneous by displaying nine different PFGE types (A0, A1, A2, A4, A6, B0, C0, C1 and E2) and three different \( spa \) types (t011, t034 and t1451). The presence of the genes \( erm(B) \) and \( erm(C) \) in structurally different MRSA ST398 isolates might be explained by their location on mobile elements, such as \( erm(B) \)-carrying transposons or \( erm(C) \)-carrying plasmids, both of which are known to be exchanged not only across staphylococcal strain and species boundaries, but also between staphylococci and other Gram-positive bacteria.31–33
Antimicrobial resistance of porcine MRSA ST398

In conclusion, the comprehensive characterization of porcine MRSA ST398 isolates from Germany with regard to their genomic relationships as well as their virulence and resistance properties provided insight into the genetic variability and the gene acquisition capacities of MRSA ST398 isolates from pigs. This is of particular relevance since MRSA ST398 shows a low host specificity and can colonize various animal hosts as well as humans. The methods applied also showed that a clustering of these isolates into subtypes is possible and that these methods may also be used for better outbreak tracing.

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Transparency declarations

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