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Genotypic and Phenotypic Characterization of *Staphylococcus aureus* Isolates from Wild Boars

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Eight *Staphylococcus aureus* isolates collected from 117 wild boars were characterized and compared to livestock isolates. They belonged to sequence types ST133, ST425, and the new type ST1643. The *spa* types were t1181, t6782, and the new types t6384, t6385, and t6386. Antimicrobial susceptibility testing and microarray-based genotyping confirmed the absence of important virulence/resistance genes.

Staphylococcus aureus is an abundant bacterium occurring as commensal flora of humans and various animal species (1). Beyond asymptomatic carriage, *S. aureus* is associated with a variety of diseases (2). Due to their ability to cause clinical conditions including life-threatening infections, staphylococci, especially methicillin-resistant *S. aureus* (MRSA), are deemed to comprise one of the most important nosocomial pathogens in humans and are considered a major public health concern (3, 4). More recently, their importance in veterinary medicine has also been described (5). Besides companion animals and horses (6), MRSA was found to colonize or infect important livestock species, including cows, pigs, and poultry (1, 7, 8). During recent years, a particular focus was laid on MRSA strains from domestic pigs, which belong predominantly to the clonal complex 398 (CC398) (9). These strains are highly prevalent among domestic pig herds and other livestock species in Europe and North America (10), and recent evidence implies their potential to cause infections in humans (7, 11, 12). Although some animal clones, like CC398, can colonize or infect multiple host species, modern typing techniques and genetic analyses of *S. aureus* populations have demonstrated the existence of several host-specific clonal lineages and imply an adaptive evolutionary host restriction (2, 7).

Nevertheless, studies determining the prevalence of *S. aureus* in wild game and game meat are rare (13, 14). Given the recent rise of CC398 MRSA in domestic pigs, it would be interesting to determine whether wild boars harbor methicillin-susceptible CC398 precursor strains or if they are already affected in the CC398 MRSA epidemic, too.

Nasal swabs were collected from 117 wild boars hunted in eight different regions across Germany during the years 2008 and 2009. Swabs were plated on Columbia blood agar (Oxoid, Wesel, Germany), Columbia blood agar with colistin and nalidixic acid (Heipha Dr. Müller, Eppelheim, Germany), and selective MRSA CHROMagar (Becton, Dickinson, Heidelberg, Germany). Colonies were identified using the Slidex Staph Plus and API ID32 Staph systems (both from bioMérieux, Marcy-l'Étoile, France). In addition, an *S. aureus*-specific PCR assay targeting the *eap* gene was carried out as described previously (15). Among the 117 nasal swabs, 8 (6.8%) were positive for *S. aureus* originating in four federal states in Germany in 2008 and 2009 (Table 1). In a previous investigation, neither *S. aureus* nor MRSA strains were detected in

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TABLE 1 Characteristics and geographic origins of *S. aureus* isolates from wild boars

Isolate	Origin		PFGE type	MLST	*spa* type	Microarray-based analysis		
	Federal state	Date[a]				*agr* group	Resistance gene[b]	Capsule type
1	Lower Saxony	10/16/2008	A	ST425	t6386	II	*fosB*	5
2	Saarland	10/24/2008	B1	ST133	t1181	I	*fosB*	8
3	Hesse	10/25/2008	B2	ST133	t6384	I	*fosB*	8
4	Saarland	11/08/2008	C	ST1643	t6385	II	—[c]	5
8	Saarland	11/08/2008	C	ST1643	t6385	II	—	5
9	Lower Saxony	12/11/2008	A	ST425	t6386	II	*fosB*	5
67	Rhineland-Palatinate	12/14/2009	D	ST425	t6782	II	*fosB*	5
68	Rhineland-Palatinate	12/14/2009	B3	ST133	t1181	I	*fosB*	8

[a] Month/day/year.
[b] No correlation between the presence of the *fosB* gene and elevated MICs of fosfomycin could be detected.
[c] —, not detected.

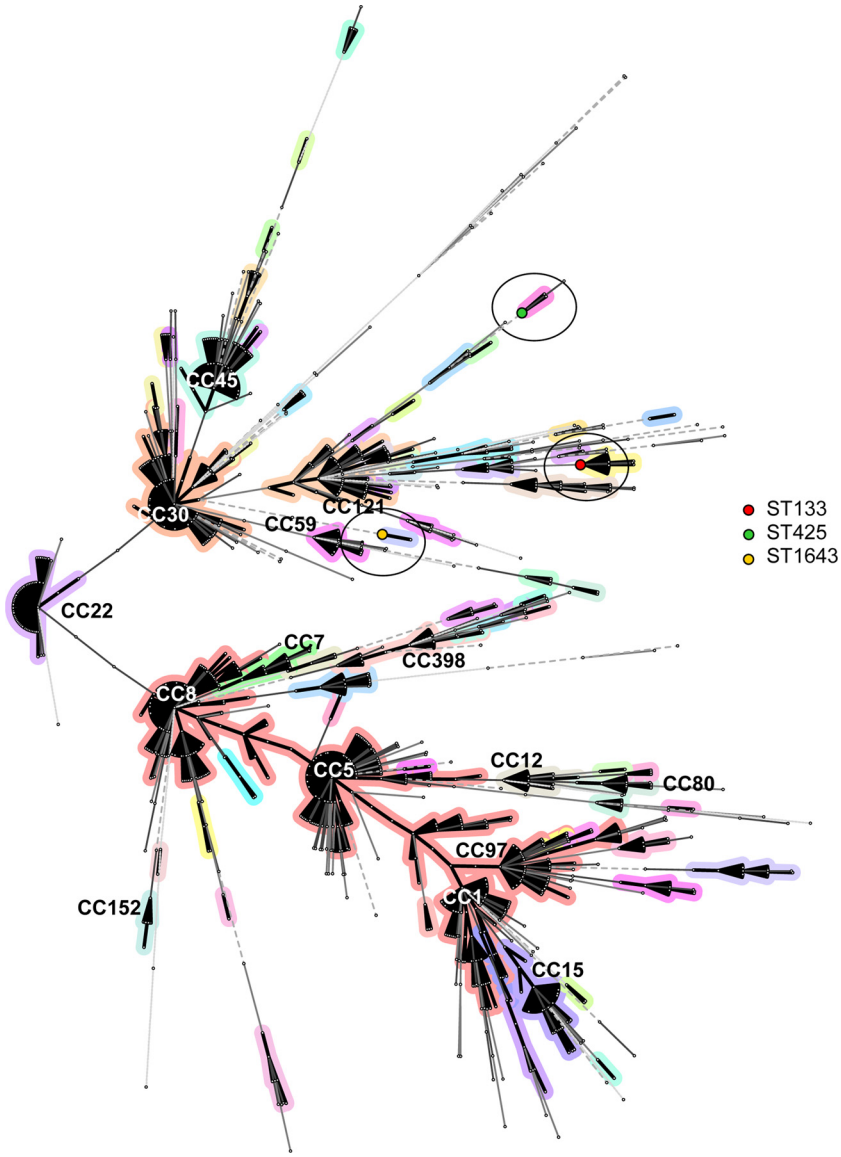

FIG 1 *S. aureus* population (31 January 2012), represented as a minimum spanning tree based on MLST profiles obtained from the MLST database (http://saureus.mlst.net/) and generated using the software program Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium). Affiliations with clonal complexes were determined by using the software program eBURST. Each sequence type present in the database is represented by a sphere. The length of connecting lines is proportional to the number of different MLST alleles. The positions of the methicillin-susceptible *S. aureus* strains from wild boars within the population are indicated by green, red, and yellow dots, respectively.

nasal swabs from 120 wild boars (16). The small number of *S. aureus* strains isolated in the present study corroborates the rarity of *S. aureus* as a nasal colonizer of wild boars.

Macrorestriction analysis according to the Harmony protocol was performed to investigate the clonalities of the isolates (17). Four different macrorestriction patterns were detected, designated types A to D. Each type was represented by 1 to 3 isolates exhibiting indistinguishable or very similar fragment patterns (Table 1). Antimicrobial susceptibility testing by broth microdilution and Etest (bioMérieux) following Clinical and Laboratory Standards Institute (CLSI) recommendations (18, 19) revealed that the isolates were susceptible (or exhibited low MICs) to all 17 antibiotics/antibiotic combinations tested, including oxacillin. Thus, they differed from CC398 livestock isolates, which often are resistant to beta-lactams and tetracycline and which, in some cases, show additional resistance to macrolides, lincosamides and aminoglycosides (7, 9).

Microarray analysis was done using the StaphyType kit (Alere Technologies, Jena, Germany) according to the manufacturer's instructions (2). ST425 isolates were also tested using an experimental array that additionally harbored probes for *mecC* (20) and a SCC*mec*-XI-associated *blaZ* allele. Analyses confirmed the presence of *S. aureus* species markers. None of the isolates harbored virulence genes encoding staphylococcal enterotoxins, exfoliative toxins (*eta*, *etb*, and *etd*), the toxic shock syndrome toxin (*tst*), epidermal cell differentiation inhibitors (*edin-A*, *edin-B*, and *edin-C*), genes encoding immune evasion components (*sak*, *chp*, and *scn*), or the Panton-Valentine leukocidin (*lukF-PV* and *lukS-PV*). Except for *fosB* (a putative fosfomycin/bleomycin resistance gene) (21), which was present in six isolates (Table 1), no antibiotic

resistance genes were detected. However, the *fosB*-carrying isolates exhibited MICs of ≤4 mg/liter fosfomycin, indicating a susceptible phenotype. PCR experiments with the primers fosB-fw (5′-CTTTACTGACCCTGATGGT-3′) and fosB-rv (5′-TAATCT GTTCTCAAGTGTGC-3′) (61 bp) and subsequent sequencing of the amplicons confirmed the presence of *fosB*. Nevertheless, the mechanism responsible for the functional inactivity of *fosB* remains to be clarified.

To further characterize the isolates, multilocus sequence typing (MLST) and *spa* typing were performed (22, 23). New types were assigned by the *spa* and MLST database curators, respectively. Three MLSTs, ST133, ST425, and the novel type ST1643, were detected (Table 1). The relatedness to other MLSTs from animals and humans is shown in Fig. 1. Two of the ST133 isolates belonged to *spa* type t1181, whereas the remaining ST133 isolate belonged to the novel type t6384. ST133 appears to be an ungulate-animal-specific genotype largely without association with humans (24). Among ST425 isolates, the *spa* types t8782 and novel type 6386 were detected (Table 1). ST425 is a well-known animal-associated lineage, and MRSA isolates of this sequence type originating with bovine milk samples and humans were recently found to carry a *mecA* homologue, the *mecC* gene (25, 26). The *mecC* gene was absent from all three boar isolates from this study. In contrast, ST1643 was so far detected only in wild boars. A single *S. aureus* ST1643 strain, associated with a skin infection of wild boar, was isolated about 40 years ago in Germany (http://saureus.mlst.net/sql/burstspadvanced.asp; identification no. 3286). The allelic profile for this isolate was previously assigned as ST856 and was amended due to a change in the trim length of the gene *gmk*, which is used for MLST analysis (http://saureus.beta.mlst.net/trim.html). Both ST1643 isolates detected during this study carried the novel *spa* type t6385.

None of the wild boars carried MRSA CC398, which is widely distributed among industrially raised pigs and which can be spread from pig farms into the environment (27, 28). Nevertheless, the low concentrations of MRSA detected in the vicinity of pig barns and the absence of antibiotic selective pressure are two factors that might reduce the probability of transmission. A similar observation was made by Cuny et al. (2012), who could not detect MRSA CC398 or any other *S. aureus* on 25 organic pig farms that do not administer antibiotics to pigs of more than 25 kg in body weight (16). This finding may underline the role of antibiotic treatment and environmental conditions.

In conclusion, *S. aureus* seems to be a rare nasal colonizer of wild boars, and isolates differ distinctly in their genotypes and resistance phenotypes from common livestock isolates. Apparently, given the small sample size, CC398 MRSA appears not to be abundant yet among wild boars in Germany, but further studies are required to confirm this observation and to observe possible future developments.

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REFERENCES

1. **Graveland H, Duim B, van Duijkeren E, Heederik D, Wagenaar JA.** 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans. Int. J. Med. Microbiol. **301**:630–634.
2. **Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M, Chow H, Ip M, Jatzwauk L, Jonas D, Kadlec K, Kearns A, Laurent F, O'Brien FG, Pearson J, Ruppelt A, Schwarz S, Scicluna E, Slickers P, Tan HL, Weber S, Ehricht R.** 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One **6**:e17936. doi:10.1371/journal.pone.0017936.
3. **Gould IM, David MZ, Esposito S, Garau J, Lina G, Mazzei T, Peters G.** 2012. New insights into meticillin-resistant *Staphylococcus aureus* (MRSA) pathogenesis, treatment and resistance. Int. J. Antimicrob. Agents **39**:96–104.
4. **Deurenberg RH, Vink C, Kalenic S, Friedrich AW, Bruggeman CA, Stobberingh EE.** 2007. The molecular evolution of methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect. **13**:222–235.
5. **Köck R, Harlizius J, Bressan N, Laerberg R, Wieler LH, Witte W, Deurenberg RH, Voss A, Becker K, Friedrich AW.** 2009. Prevalence and molecular characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) among pigs on German farms and import of livestock-related MRSA into hospitals. Eur. J. Clin. Microbiol. Infect. Dis. **28**:1375–1382.
6. **Catry B, Van Duijkeren E, Pomba MC, Greko C, Moreno MA, Pyörälä S, Ruzauskas M, Sanders P, Threlfall EJ, Ungemach F, Törneke K, Munoz-Madero C, Torren-Edo J.** 2010. Scientific Advisory Group on Antimicrobials (SAGAM). Reflection paper on MRSA in food-producing and companion animals: epidemiology and control options for human and animal health. Epidemiol. Infect. **138**:626–644.
7. **Fitzgerald JR.** 2012. Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. Trends Microbiol. **20**:192–198.
8. **Cuny C, Friedrich A, Kozytska S, Layer F, Nübel U, Ohlsen K, Strommenger B, Walther B, Wieler L, Witte W.** 2010. Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. Int. J. Med. Microbiol. **300**:109–117.
9. **de Neeling AJ, van den Broek MJ, Spalburg EC, van Santen-Verheuvel MG, Dam-Deisz WD, Boshuizen HC, van de Giessen AW, van Duijkeren E, Huijsdens XW.** 2007. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Vet. Microbiol. **122**:366–372.
10. **Jamrozy DM, Fielder MD, Butaye P, Coldham NG.** 2012. Comparative genotypic and phenotypic characterisation of methicillin-resistant *Staphylococcus aureus* ST398 isolated from animals and humans. PLoS One **7**:e40458. doi:10.1371/journal.pone.0040458.
11. **Garcia-Graells C, Antoine J, Larsen J, Catry B, Skov R, Denis O.** 2012. Livestock veterinarians at high risk of acquiring methicillin-resistant *Staphylococcus aureus* ST398. Epidemiol. Infect. **140**:383–389.
12. **Witte W, Strommenger B, Stanek C, Cuny C.** 2007. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg. Infect. Dis. **13**:255–258.
13. **Membré JM, Laroche M, Magras C.** 2011. Assessment of levels of bacterial contamination of large wild game meat in Europe. Food Microbiol. **28**:1072–1079.
14. **Lozano C, López M, Gómez-Sanz E, Ruiz-Larrea F, Torres C, Zarazaga M.** 2009. Detection of methicillin-resistant *Staphylococcus aureus* ST398 in food samples of animal origin in Spain. J. Antimicrob. Chemother. **64**:1325–1326.
15. **Hussain M, von Eiff C, Sinha B, Joost I, Herrmann M, Peters G, Becker K.** 2008. *eap* gene as novel target for specific identification of *Staphylococcus aureus*. J. Clin. Microbiol. **46**:470–476.
16. **Cuny C, Friedrich AW, Witte W.** 2012. Absence of livestock-associated methicillin-resistant *Staphylococcus aureus* clonal complex CC398 as a nasal colonizer of pigs raised in an alternative system. Appl. Environ. Microbiol. **78**:1296–1297.
17. **Murchan S, Kaufmann ME, Deplano A, de Ryck R, Struelens M, Zinn CE, Fussing V, Salmenlinna S, Vuopio-Varkila J, El Solh N, Cuny C, Witte W, Tassios PT, Legakis N, van Leeuwen W, van Belkum A, Vindel A, Laconcha I, Garaizar J, Haeggman S, Olsson-Liljequist B, Ransjo U, Coombes G, Cookson B.** 2003. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J. Clin. Microbiol. **41**:1574–1585.
18. **Clinical and Laboratory Standards Institute.** 2012. Performance standards for antimicrobial susceptibility testing; 22nd informational supplement. CLSI document M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA.
19. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria

isolated from animals; approved standard, 3rd ed. CLSI document M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
20. **Monecke S, Müller E, Schwarz S, Hotzel H, Ehricht R.** 2012. Rapid microarray-based identification of different *mecA* alleles in staphylococci. Antimicrob. Agents Chemother. **56**:5547–5554.
21. **Zilhao R, Courvalin P.** 1990. Nucleotide sequence of the *fosB* gene conferring fosfomycin resistance in *Staphylococcus epidermidis*. FEMS Microbiol. Lett. **68**:267–272.
22. **Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **37**:3556–3563.
23. **Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38**:1008–1015.
24. **Smyth DS, Feil EJ, Meaney WJ, Hartigan PJ, Tollersrud T, Fitzgerald JR, Enright MC, Smyth CJ.** 2009. Molecular genetic typing reveals further insights into the diversity of animal-associated *Staphylococcus aureus*. J. Med. Microbiol. **58**:1343–1353.
25. **García-Álvarez L, Holden MTG, Lindsay H, Webb CR, Brown DFJ, Curran MD, Walpole E, Brooks K, Pickard DJ, Teale C, Parkhill J, Bentley SD, Edwards GF, Girvan EK, Kearns AM, Pichon B, Hill RL, Larsen AR, Skov RL, Peacock SJ, Maskell DJ, Holmes MA.** 2011. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect. Dis. **11**:595–603.
26. **Shore AC, Deasy EC, Slickers P, Brennan G, O'Connell B, Monecke S, Ehricht R, Coleman DC.** 2011. Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent *mecA*, *mecI*, *mecR1*, *blaZ*, and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **55**:3765–3773.
27. **Witte W, Cuny C, Klare I, Nübel U, Strommenger B, Werner G.** 2008. Emergence and spread of antibiotic-resistant Gram-positive bacterial pathogens. Int. J. Med. Microbiol. **298**:365–377.
28. **Schulz J, Friese A, Klees S, Tenhagen BA, Fetsch A, Rösler U, Hartung J.** 2012. Longitudinal study of the contamination of air and of soil surfaces in the vicinity of pig barns by livestock-associated methicillin-resistant *Staphylococcus aureus*. Appl. Environ. Microbiol. **78**:5666–5671.