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Short communication

First description of PVL-positive methicillin-resistant *Staphylococcus aureus* (MRSA) in wild boar meat

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ABSTRACT

Staphylococcus aureus is an important food-borne pathogen due to the ability of enterotoxigenic strains to produce staphylococcal enterotoxins (SEs) in food. Methicillin-resistant *S. aureus* (MRSA) is also an important pathogen for humans, causing severe and hard to treat diseases in hospitals and in the community due to its multiresistance against antimicrobials. In particular, strains harbouring genes encoding for the Panton–Valentine leukocidin (PVL) toxin are of concern from a public health perspective as they are usually capable of causing severe skin and soft tissue infections (sSSTIs) and occasionally necrotizing pneumonia which is associated with high mortality. This is the first report on the detection of MRSA with genes encoding for PVL in wild boar meat. Among the 28 MRSA isolated from wild boar meat in the course of a national monitoring programme in Germany, seven harboured PVL-encoding genes. Six of the isolates were identical according to the results of *spa*-, MLST-, microarray- and PFGE-typing. They could be assigned to the epidemic MRSA clone USA300. Epidemiological investigations revealed that people handling the food were the most likely common source of contamination with these MRSA. These findings call again for suitable hygienic measures at all processing steps of the food production chain. The results of the study underline that monitoring along the food chain is essential to closely characterise the total burden of MRSA for public health.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a threat for public health for decades, causing severe infections mainly in hospital settings that are difficult to treat due to the multiresistance of the bacteria. Severe infections caused by MRSA were also described outside hospitals. The clonal lineages involved in those infections occurring in the community, i.e. ST8, ST30, and ST80 in Germany (Robert Koch-Institute, 2011) and globally (Mediavilla et al., 2012), often harbour genes encoding for Panton–Valentine leukocidin (PVL), which is one of the major exotoxins of *S. aureus*. PVL is a well-known virulence factor of *S. aureus* causing severe disease (Thurlow et al., 2012; Vandenesch et al., 2003; Gillet et al., 2002). PVL-positive MRSA are also associated with chronic and recurrent sSSTIs (Stieber et al., 2014).

The frequent detection of specific clonal MRSA lineages (CC398 MRSA) among livestock, i.e. pigs, cattle, poultry, in recent years in several (European) countries is a matter of concern (de Neeling et al., 2007; Spohr et al., 2010; Richter et al., 2012). These so-called livestock-associated (LA)-MRSA are considered to be zoonotic (van Loo et al., 2007; Witte et al., 2007b; Cuny et al., 2013) and people with occupational contact to livestock, e.g. farmers, veterinarians, workers at abattoirs, are frequently exposed and colonised. Consequently European authorities recommended monitoring the prevalence of MRSA and its properties among livestock and food (EFSA, 2012).

S. aureus is also an important hazard from a food safety perspective as it is able to produce staphylococcal enterotoxins, preformed in food (Argudín et al., 2010). Food poisoning caused by staphylococcal enterotoxins is among the leading causes of food-borne outbreaks in the European Union (EFSA, 2013). Typically, staphylococcal food poisoning (SFP) occurs after ingestion of foods that are contaminated with *S. aureus* by improper handling and subsequent storage at temperatures supporting the growth of the bacteria. Staphylococcal food poisoning is rarely fatal, but it can lead to severe *S. aureus* infections such as osteomyelitis and pneumonia (Mulder and Verwiel, 1980; Duben et al., 1988).

Although *S. aureus* is mainly involved in SFP, intoxications caused by the ingestion of SE produced by MRSA so far were only described occasionally. One reason for this might be that poisoning can occur even if the bacterial cells are dead or un-culturable after cooking, as staphylococcal enterotoxins are heat stable (Schelin et al., 2011). People, inapparently colonised with *S. aureus* handling food that favours growth of bacteria, e.g. (raw) food of animal origin with high protein

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content such as milk, milk products, meat, meat products and salads, can introduce the bacteria into the food chain (Hennekinne et al., 2012).

Since its first description in livestock in 2005, clonal lineages of LA-MRSA circulating in livestock holdings were also detected in food, mainly in raw meat from pigs, poultry and veal calves (de Boer et al., 2009; Hartung and Käsbohrer, 2012). In Europe, the majority of MRSA in food of animal origin belonged to CC398 (de Boer et al., 2009; Buyukcangaz et al., 2013). As the majority of European CC398 MRSA lack the major virulence determinants described so far such as PVL and staphylococcal enterotoxins (Argudín et al., 2011), it was postulated that this clonal lineage has caused relatively little disease despite of its wide distribution in many European countries (Köck et al., 2011). However, CC398 MRSA can cause severe disease (Ekkelenkamp et al., 2006; Witte et al., 2007b; Pan et al., 2009; Köck et al., 2013).

Detection of non-CC398 MRSA in raw meat, mainly from poultry, was often described in reports from Asian countries (Lee et al., 2013; Boost et al., 2013) and the USA (Pu et al., 2009). Despite the frequent detection of MRSA in food, the risk to consumers is considered to be low, mainly because only small amounts of bacteria are expected in food (de Boer et al., 2009; Schilling et al., 2010). Moreover, no human clinical LA-MRSA infection could be linked to the consumption of food (ECDC, EFSA and EMEA, 2009).

In this paper, we report on the first detection of non-CC398 MRSA harbouring PVL-encoding genes (*lukS-PV* and *lukF-PV*) in wild boar meat.

2. Materials and methods

2.1. S. aureus isolates

In the course of a national monitoring programme in 2011, 28 MRSA isolates from wild boar meat were sent to the National Reference Laboratory for coagulase-positive staphylococci including *S. aureus* (NRL Staph) at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, for further analysis. They originated from 28 samples of fresh meat collected at retail in seven different Federal States of Germany. They had been independently isolated in various regional laboratories. The overall prevalence of MRSA in wild boar meat was 4.8% (Tenhagen et al., 2013).

2.2. Typing of the bacterial isolates

All isolates were examined by phenotypic tests (growth on Baird Parker agar (Merck, Darmstadt, Germany)), mannitol fermentation on mannitol salt agar (Mast Diagnostica, Reinfeld, Germany) and coagulase production at the NRL Staph. Subsequently DNA of the isolates was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) in order to confirm them as MRSA by means of multiplex-PCR (Poulsen et al., 2003). *spa*- (Shopsin et al., 1999; Harmsen et al., 2003), SCC*mec*- (Zhang et al., 2005) and Multi-Locus-Sequence-Typing (MLST) (Enright et al., 2000) of non-CC398 associated *spa* types were conducted as well.

2.3. DNA microarray

The PVL-positive isolates were further characterised using a commercially available microarray kit (Identibac *S. aureus* Genotyping, Alere Technologies GmbH, Jena, Germany) which covers 333 target sequences corresponding to approximately 185 distinct genes and their allelic variants. This included among others species-specific controls, genes encoding for relevant antibiotic resistance determinants and virulence factors as well as SCC*mec*-, *agr* group- and capsule typing markers. The array was performed according to the manufacturer's instructions. The analysis of the array profiles based on the presence or absence of the enquired genes was done using Bionumerics Software (version 6.6.4; Applied Maths, Sint-Martens-Latem, Belgium).

2.4. Macrorestriction PFGE analysis

Preparation of agarose plugs for pulsed-field gel electrophoresis (PFGE) was carried out as previously described (Mulvey et al., 2001). Genomic DNA in the plugs was digested with 30 U of restriction endonucleases *Sma*I and *Apa*I respectively. The generated fragments were separated using a CHEF-DRIII SYS220/240 system (Bio-Rad Laboratories, Munich, Germany).

2.5. Antimicrobial susceptibility testing

To determine the antimicrobial susceptibility, isolates were tested by broth microdilution according to the guidelines of CLSI (CLSI, 2006). The 19 antimicrobials tested were cefoxitin (FOX), chloramphenicol (CHL), ciprofloxacin (CIP), clindamycin (CLI), erythromycin (ERY), fusidate (FUS), gentamicin (GEN), kanamycin (KAN), linezolid (LIN), mupirocin (MUP), penicillin (PEN), quinupristin/dalfopristin (SYN), rifampicin (RIF), streptomycin (STR), sulphamethoxazole (SMX), tetracycline (TET), tiamulin (TIA), trimethoprim (TMP) and vancomycin (VAN). For interpretation of results, epidemiological cut-off values according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied (http://www.eucast.org).

3. Results and discussion

3.1. Typing of isolates

In total, 28 putative MRSA isolated from wild boar meat in 2011 were sent to the NRL Staph. *spa* typing revealed that 20 isolates comprised *spa* types related to the clonal complex CC398 (t011, t034, t1456 and t1250) which is the predominant clone in livestock (Smith and Pearson, 2010) and meat (de Boer et al., 2009; Weese et al., 2010).

However, eight isolates belonged to *spa* types of other CCs: t015 (CC45; one isolate), t202 (CC93; one isolate) and t008 (CC8; six isolates). These non-CC398 MRSA can regularly be found in meat and meat preparations. O'Brien et al. (2012) assigned 46.2% of 26 MRSA isolated from retail pork products to *spa* type t002 and also to *spa* type t008; the majority of 127 MRSA isolated from pork, beef and chicken meat belonged to *spa* type t899 (CC9), with chicken meat displaying the widest range of different *spa* types (Boost et al., 2013).

However, data on the occurrence of *S. aureus* and MRSA in the wild animal population and game meat are scarce up to the present (de Boer et al., 2009; Membré et al., 2011; Wendlandt et al., 2012). Cuny et al. (2012) did not detect *S. aureus* or MRSA in nasal swabs taken from 120 of wild boars immediately after shooting them. In contrast, methicillin-susceptible *S. aureus* (MSSA) were found in 6.8% of 117 nasal swab samples from wild boars in several geographic regions in Germany (Meemken et al., 2013). MSSA were also detected in game meat samples in Brazil: 11% and 22% of 27 capybara and collared pekari samples, resp. were MSSA positive (Sarkis et al., 2003), while in *Sus scrofa scrofa* meat samples no MSSA were found. Although MRSA were not detected in wild boars in central Europe, de Boer et al. (2009) found MRSA in 2.2% of 178 game meat samples in The Netherlands. All of them were non-CC398 MRSA but differed from the isolates detected in our study (*spa* types t001, t003 and t311).

3.2. Microarray based genotyping and pulsed-field gel electrophoresis

The eight non-CC398 isolates were selected for further analyses. To simultaneously screen a large number of significant molecular targets a DNA microarray was used. Panton–Valentine leukocidin encoding genes (*lukS-PV* and *lukF-PV*) were detected in seven of the eight analysed isolates. PVL-positive MRSA have been reported with varying prevalence in retail meat and livestock, resp. in various regions of the world previously. Researchers in the USA detected one PVL-positive MRSA in pork when analysing 165 different fresh meat samples

(Hanson et al., 2011). As in the present study, the PVL-positive MRSA was of *spa* type t008. Hanson et al. (2011) postulated contamination of the meat subsequent to slaughter by humans. One PVL-positive MRSA of *spa* type t008 was also detected in 100 retail beef meat samples in Georgia (USA), and again, the authors suggested a contamination of retail beef by a human source (Jackson and Davis, 2013). A remarkably high proportion (58.9%) of MRSA harbouring *luk-PV* genes compared to industrialized countries was described by Fall et al. (2012) who conducted a study among pigs and farmers in Dakar, Senegal. They isolated 73 MRSA from 464 pigs and 52 farmers, resp. The 43 PVL-positive MRSA in this study were of 10 different *spa* types, and included all major CCs (Fall et al., 2012). While *luk-PV* positive MRSA were detected in retail meat and pigs before, the present study is to our knowledge the first report of PVL-positive MRSA in wild boar meat. We found a surprisingly high proportion of isolates harbouring *luk-PV* genes (7 of 28 isolates; 25.0%).

All but the CC45 isolate that did neither exhibit Panton–Valentine leukocidin encoding genes, nor show any other uncommon features in terms of virulence and resistance determinant content yet described for this *S. aureus* lineage were further characterised. Table 1 displays a simplified summary of the array results of the PVL-positive isolates which could be divided into two different virulence profiles. The isolates of *spa* type t008 (CC8) were assigned to profile V1 whereas the single isolate of *spa* type t202 (CC93) belonged to profile V2, a lineage (also know as "Queensland CA-MRSA") that is restricted to Australia (Coombs et al., 2012) and can be found in Europe in travel-associated cases only (Ellington et al., 2006). All seven isolates were negative for toxic shock syndrome toxin (*tst*), exfoliatins (*etA/B/D*), epidermal cell differentiation inhibitors (*edinA/B/C*) and the *egc*-cluster (*seg*, *sei*, *sem*, *sen*, *seo* and *seu*). The beta-hemolysin converting phage carrying the immune evasion cluster (*sak*, *chp* and *scn*) could be detected in all PVL-positive isolates but the content of enterotoxins and leukocidins, the presence of the arginine catabolic mobile element (ACME locus) as well as the capsule type and accessory gene regulator allele differed between the two virulence profiles. Moreover, the array data confirmed the PCR results of the SCC*mec* typing. All seven PVL-positive isolates harboured SCC*mec* type IVa. A dendrogram based on all hybridization results obtained with the array is shown in Fig. 1. It illustrates the high similarity of the CC8 isolates. Main differences were identified in their resistance pattern, only.

Using pulsed-field gel electrophoresis six of the seven PVL-positive MRSA belonging to the same MLST/*spa* type (ST8, t008) were highly similar and exhibited the same *Sma*I and *Apa*I macrorestriction pattern (Fig. 2). This pattern could be assigned to the well-known CA-MRSA clone USA300 (McDougal et al., 2003; Patel et al., 2008). This clone is among the most frequently described MRSA globally. It has mainly been associated with infections of the skin and soft tissues but can also cause severe life-threatening conditions such as necrotizing pneumonia, osteomyelitis, and septic arthritis in the community and in healthcare settings (Nimmo, 2012). The presence of the ACME locus in all of the t008 isolates of the present study is a striking feature of USA300 that probably contributes to its pronounced survival (Diep et al., 2006; Otto, 2013).

3.3. Phenotypic and genotypic characterisation of antimicrobial resistance

The susceptibility of the seven PVL-positive isolates to antimicrobials was tested by broth microdilution. The microarray was used to determine the presence of designated resistance determinants. The seven isolates showed phenotypic resistance to kanamycin (three isolates), ciprofloxacin (six isolates), erythromycin (three isolates), cefoxitin, and penicillin (all seven isolates). They were assigned to three different phenotypic resistance patterns.

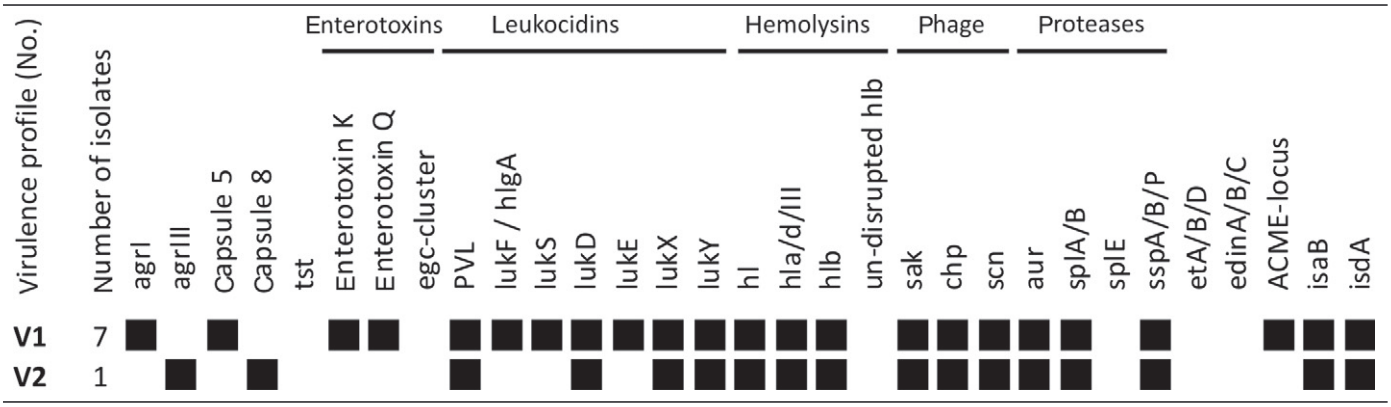
All isolates contained the *mecA* gene and *sdrM*, the gene for a putative multidrug efflux pump. The *blaZ* gene conferring resistance to ampicillin–penicillin was found in four isolates. The macrolide resistance genes *msrA* and *mphC*, the neomycin/kanamycin resistance gene *aphA3* and the streptothricin resistance gene *sat* were detected in three isolates, respectively. All of the six isolates of *spa* type t008 harboured the bacillithiol transferase gene *fosB* that probably confers fosfomycin resistance on *S. aureus* in interaction with bacillithiol (Roberts et al., 2013; Thompson et al., 2014). This gene is usually present in CA-MRSA USA300 (Kazakova et al., 2005) although USA300 is phenotypically susceptible to fosfomycin, as shown with the isolates of the present study (data not shown). *qacC*, the gene for another putative multidrug efflux pump conferring resistance to biocides like quaternary ammonium compounds (Smith et al., 2008), was only present in the CC93 isolate.

Apart from the differences seen between the two *spa* types and those within the CC8 group of isolates (Fig. 1), all were phenotypically resistant to at least two antimicrobials. However, they harboured up to eight different resistance genes. Clearly, the isolates differed in their antimicrobial resistance from the LA-MRSA clone CC398 (Argudín et al., 2011). The phenotypic resistance of the t008 isolates to ciprofloxacin and erythromycin is in line with recent findings in other USA300 strains (Marchese et al., 2009).

3.4. Trace back investigation

The PVL-positive MRSA found in wild boar meat were assigned to CCs that are usually considered as HA-MRSA or CA-MRSA (CC8, CC45, CC93). All of them displayed typical characteristics of human-adapted

Table 1
Virulence profiles of the PVL-positive MRSA on the basis of selective virulence determinants. Black squares indicate a positive hybridization result.



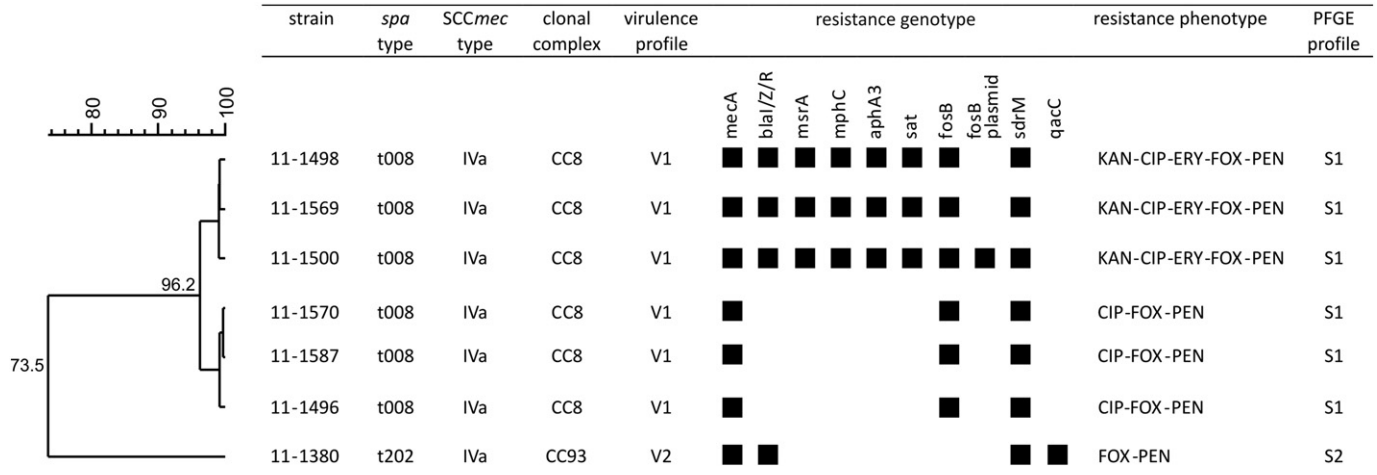


Fig. 1. Dendrogram showing the similarity between the array hybridization profiles of the PVL-positive MRSA by using the Jaccard's coefficient and unweighted-pair group method with arithmetic averages (UPMGA). On the right side of the dendrogram the resistance phenotype is shown in comparison to the resistance genotype.

clones and differed substantially from LA-MRSA. Therefore, colonised food handlers are the most likely source of contamination. However, it cannot be excluded that the wild boars were colonised earlier, for example while scavenging garbage. In Spain, the only isolate (ST217-SCC*mec*IVa-t032) found in raw wild boar meat in 2009 was also considered to be of human origin, as this type is associated with human infections. However, this isolate lacked the PVL encoding genes (Lozano et al., 2009). Likewise, the MRSA isolated from game meat in The Netherlands did not harbour the *lukS-PV* and *lukF-PV* genes (de Boer et al., 2009), although humans were the likely source.

Trace back investigations were carried-out to verify, whether the six, almost identical PVL-positive MRSA isolates in different wild boar meat samples resulted from a common source of contamination. Epidemiological investigation revealed that the six MRSA were isolated from six different raw wild boar meat products. Four of them originated from the USA while the other two were from Germany (strains 11-1496 and 11-1498; see Fig. 1). The raw material was further processed and packaged in at least three different batches by two German companies selling game meat and products thereof. These two companies, one located in the North of Germany, the other in the South, belong to one major holding with various branches. However, a common source or route of contamination with USA300 MRSA could not be identified. Therefore, multiple introductions by several colonised food handlers cannot be excluded, particularly as slight differences in the microarray profiles were seen. USA300 is well-known but rarely detected in Germany (Witte et al., 2007a; Robert Koch-Institute, 2013).

Unfortunately, no further epidemiological information about the food handlers such as previous hospitalization or travel to countries where the epidemic clones described in this study are more common could be obtained. The isolation of other human adapted non-CC398

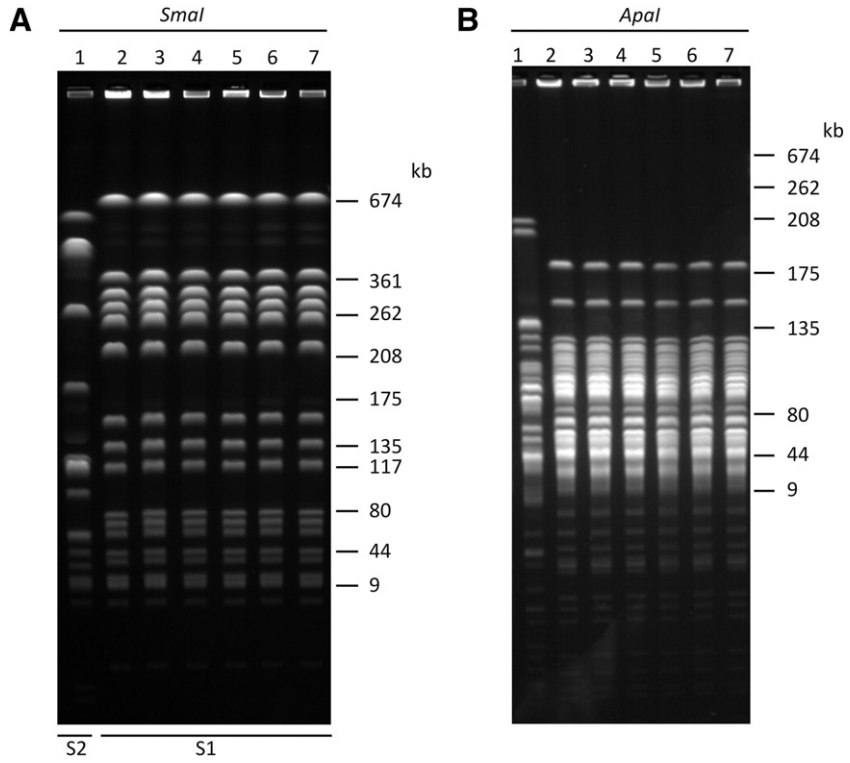


Fig. 2. PFGE profiles of the seven PVL-positive MRSA. (A) *Sma*I profiles; (B) *Apa*I profiles; lane 1, strain 11-1380; lane 2, strain 11-1496; lane 3, strain 11-1498; lane 4, strain 11-1500; lane 5, strain 11-1569; lane 6, strain 11-1570; lane 7, strain 11-1587; S1–S2, *Sma*I profiles.

MRSA (t015, t202) from wild boar meat underlines the possibility of multiple entry pathways along the food chain. The results emphasise that correct handling of game meat at all stages of the processing and marketing chain from hunting to marketing at retail is a prerequisite for a high food quality (Atanassova et al., 2008). It can be assumed that the processing of game meat which involves a considerable amount of handling by different people easily allows for the introduction of human-adapted pathogens.

In conclusion, to our knowledge this is the first description of PVL-positive MRSA in wild-boar meat which were most likely introduced by humans handling the food. Multiresistant MRSA that are positive for PVL/ACME in food have to be considered as a more severe hazard to public health than the typical LA-MRSA. No definite knowledge on their transmissibility to humans via food either during handling/preparation or via ingestion of cross-contaminated raw food items (e.g. salad) is available so far. This warrants further investigation. Suitable hygienic measures, including personal hygiene when handling food are needed at all processing steps to reduce the likelihood of introducing pathogens into the food chain. Regular screening of food handlers for MRSA should be considered, in particular after hospitalization or travelling abroad. The results of the study emphasise that monitoring from farm to fork is essential to accurately characterise the total exposure of the public to MRSA via food. Subsequent in-depth analyses of isolates are needed to allow for the identification of their epidemiological relatedness.

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