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# A Livestock-Associated, Multidrug-Resistant, Methicillin-Resistant *Staphylococcus aureus* Clonal Complex 97 Lineage Spreading in Dairy Cattle and Pigs in Italy

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**Pandemic methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex 97 (CC97) lineages originated from livestock-to-human host jumps. In recent years, CC97 has become one of the major MRSA lineages detected in Italian farmed animals. The aim of this study was to characterize and analyze differences in MRSA and methicillin-susceptible *S. aureus* (MSSA) mainly of swine and bovine origins. Forty-seven CC97 isolates, 35 MRSA isolates, and 6 MSSA isolates from different Italian pig and cattle holdings; 5 pig MRSA isolates from Germany; and 1 human MSSA isolate from Spain were characterized by macrorestriction pulsed-field gel electrophoresis (PFGE) analysis, multilocus sequence typing (MLST), *spa* typing, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, and antimicrobial resistance pattern analysis. Virulence and resistance genes were investigated by PCR and microarray analysis. Most of the isolates were of SCC*mec* type V (SCC*mec* V), except for two German MRSA isolates (SCC*mec* III). Five main clusters were identified by PFGE, with the German isolates (clusters I and II) showing 60.5% similarity with the Italian isolates, most of which (68.1%) grouped into cluster V. All CC97 isolates were Panton-Valentine leukocidin (PVL) negative, and a few ($n = 7$) tested positive for *sak* or *scn*. All MRSA isolates were multidrug resistant (MDR), and the main features were *erm*(B)- or *erm*(C)-mediated ($n = 18$) macrolide-lincosamide-streptogramin B resistance, *vga*(A)-mediated ($n = 37$) pleuromutilin resistance, fluoroquinolone resistance ($n = 33$), *tet*(K) in 32/37 *tet*(M)-positive isolates, and *blaZ* in almost all MRSA isolates. Few host-associated differences were detected among CC97 MRSA isolates: their extensive MDR nature in both pigs and dairy cattle may be a consequence of a spillback from pigs of a MRSA lineage that originated in cattle as MSSA and needs further investigation. Measures should be implemented at the farm level to prevent spillover to humans in intensive farming areas.**

*Staphylococcus aureus* is a major pathogen causing considerable human morbidity and mortality worldwide, and it is a leading cause of infections of some economically important livestock species and, as a prominent bacterial cause of contagious bovine mastitis, a major economic burden for the dairy cattle industry (1). Methicillin-resistant *S. aureus* (MRSA) lineages are causes of health care- and community-associated infections, which are a major burden of disease on a global scale (2). In the last decade, MRSA sequence type 398 (ST398) has found an ecological niche in the pig, cattle, and poultry industries, although other MRSA lineages (e.g., ST1, ST5, ST9, ST97, ST130, and ST433) have been identified in farmed animals worldwide (3) All these lineages are currently termed "livestock-associated MRSA" (LA-MRSA).

The MRSA clonal complex 97 (CC97) lineage has been reported as the second most prevalent MRSA lineage in the Italian pig industry, where *spa* types t1730 and t4795, both belonging to ST97, were estimated to have colonized 11% of finishing holdings (4). In Italy, in the last 5 years, the CC97 MRSA lineage has also been increasingly detected in cattle primary production, either from mastitis in dairy cattle or from bulk tank milk (5–7). Occasionally, CC97 MRSA isolates have been detected in cattle in Germany and Spain.

CC97 is one of the major *S. aureus* clonal complexes in bovines (8), and recently, a livestock origin of the human pandemic CC97 MRSA strains has been demonstrated, resulting in two emergent human epidemic CC97 community-acquired/associated MRSA (CA-MRSA) clones (8).

The aim of this study was to determine the genetic relatedness of MRSA and methicillin-susceptible *S. aureus* (MSSA) CC97 isolates from pigs and dairy cattle in Italy in the last few years (2008 to 2012) for epidemiological and risk assessment purposes.

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## MATERIALS AND METHODS

**Collection of bacteria.** A total 47 CC97 *S. aureus* isolates were studied: 35 MRSA isolates from pigs ($n = 15$) and dairy cattle ($n = 20$) and 6 MSSA isolates (1 from veal calf, 1 from wild boar, and 4 from dairy cattle) from Italy along with 5 MRSA isolates of swine origin from Germany and 1 CC97 MSSA isolate of human origin from Spain, isolated in 2002. Isolates from animals were collected from population-based studies (surveys of pigs/wild boar and cattle) and passive laboratory surveillance programs (mastitis in dairy cattle). Isolates from swine holdings were obtained from nostril swabs or from dust swabs collected in colonized holdings, those from dairy cattle were obtained from intramammary infections or bulk tank milk, and those from veal calf and wild boar were obtained from nasal swabs of healthy animals. Only one isolate per holding of origin was included in the study. The human MSSA isolate was obtained from a healthy individual (9). Metadata for the isolates under study are included in Table S1 in the supplemental material.

Most of the isolates of animal origin (e.g., nasal swabs and milk) were cultured as previously described (4), with slight modifications. Briefly, after selective enrichment, a loopful of broth was plated onto oxacillin resistance screen agar (ORSA; Oxoid, Basingstoke, United Kingdom). Suspect *S. aureus* colonies (denim blue colonies on ORSA) were subcultured on 5% sheep blood agar and incubated at 37°C for 24 h. In some cases, animal isolates from passive surveillance were detected by direct plating onto 5% sheep blood agar or the selective agar ORSA. *S. aureus* isolates were identified by means of standard techniques, including colony morphology, Gram staining, catalase, and coagulase tube tests, and further confirmed by PCR assays of the *nuc* gene, as previously described (10).

**Antimicrobial susceptibility testing.** Isolates were tested for their antimicrobial susceptibility by broth microdilution (Trek Diagnostic Systems, Westlake, OH, USA). The drugs tested were those proposed in recommendations by the European Food Safety Authority (11), including penicillin (PEN), cefoxitin (FOX), ciprofloxacin (CIP), chloramphenicol (CHL), clindamycin (CLI), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), linezolid (LZD), quinupristin-dalfopristin (SYN), fusidic acid (FUS), mupirocin (MUP), rifampin (RIF), tetracycline (TET), tiamulin (TML), sulfamethoxazole (SMX), trimethoprim (TRI), and vancomycin (VAN).

MICs were determined, and results were interpreted according to guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/), using epidemiological cutoffs (see Table S1 in the supplemental material) for the categorization of "microbiological resistance." The MIC values obtained for the drugs tested were also assessed according to EUCAST clinical breakpoints (CBs) or, in the absence of EUCAST CBs, according to Clinical and Laboratory Standards Institute (CLSI) clinical breakpoints (i.e., for kanamycin and sulfamethoxazole), for the categorization of "clinical resistance." Epidemiological cutoffs only were used for tiamulin and streptomycin, since no CBs are currently available in both EUCAST and CLSI international standards.

Multidrug resistance was defined as resistance to at least any three antimicrobial drugs considered (3+R) (12, 13).

**Methicillin resistance.** The methicillin resistance phenotype was confirmed by a PCR assay for the *mecA* gene (14) or by means of a multiplex PCR for the *mecA*, *mecC*, *spa*, and Panton-Valentine leukocidin (PVL) genes (15).

**Genotyping.** All isolates were submitted for molecular characterization using *spa* typing and multilocus sequence typing (MLST) (16, 17). Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of MRSA isolates was performed by using gene-specific PCRs. Briefly, all the isolates were tested for cassette types I to VI, VIII, and IX by using two multiplex PCRs (18). In the case of a negative result for the *mec* gene complex, PCR for the *mec* gene C1 complex (present in SCC*mec* type VII [SCC*mec* VII] and SCC*mec* X) was performed (19). The SCC*mec* type IV variants were further subtyped as previously described (20).

**Microarray testing.** Microarray testing for the detection of a variety of pathogenicity- and virulence-associated genes, antimicrobial resistance (AMR) genes, and strain- or host-specific markers of *S. aureus*, including the accessory gene regulator (*agr*) genes and superantigenic toxin-encoding genes, was performed with a genotyping kit (Alere GmbH, Germany), as previously described (21). The results were interpreted according to the manufacturer's specifications.

**Macrorestriction PFGE.** All the CC97 *S. aureus* isolates were subclassified by using macrorestriction pulsed-field gel electrophoresis (PFGE) (22). After 4 h of incubation with 30 U of SmaI (Thermo Fisher Scientific, Waltham, MA, USA) at 30°C, the digested plugs were placed into agarose gel. A run was performed with the Chef-DRII system (Bio-Rad Laboratories GmbH, Munich, Germany) according to a previously harmonized protocol (23). Cluster analysis was performed by using BioNumerics 7 software (Applied Maths, Sint-Martens-Latem, Belgium), and a dendrogram was built by applying the Dice similarity coefficient with optimization and tolerance of 1.5% each and clustering using the unweighted pair group method with arithmetic means (UPGMA). *S. aureus* NCTC 8325 was the reference standard strain used.

**Statistical analysis.** In order to determine whether the difference between two proportions was significant (e.g., presence of characteristics in isolates from pigs versus isolates from cattle), the Fisher exact test was performed by using the StatCalc utility of Epi Info version 7.1.5 software (http://wwwn.cdc.gov/Epiinfo/7/index.htm).

## RESULTS

**Genotyping and array typing.** The CC97 isolates belonged to ST97 ($n = 41$; *spa* types t4795, t1730, t1236, t2112, t267, t345, t3992, t5487, and t426) or to the single-locus variants (SLVs) ST71 ($n = 3$; *spa* type t524) and ST352 ($n = 3$; *spa* types t359 and t267). Figure 1 summarizes the results for selected virulence and antimicrobial resistance marker genes and elements detected in the isolates under study along with the macrorestriction PFGE dendrogram and AMR pattern. The complete set of genes and genetic elements determined, including AMR genes and elements, along with AMR resistance phenotypic patterns are available in Table S1 in the supplemental material.

All MRSA isolates carried SCC*mec* type V (5C2), except for two German isolates that carried SCC*mec* type III (3A). Thirty-seven out of forty CC97 MRSA SCC*mec* type V isolates were positive for *mecA*, *ugpQ*, *ccrAA* (or *ccrD*, homologue to the cassette chromosome recombinase A gene), and *ccrC* (85-2082). The human MSSA isolate harbored the *xylR* and *ccrA3* genes only.

All isolates were PVL negative. Conversely, all isolates (MRSA and MSSA) were positive for genes encoding components of other leukotoxin families: LukF-LukS-HlgA, LukD-LukE, and LukX-LukY. One MSSA isolate (ST97) from dairy cattle carried the LukM and LukF-PV(P83) genes encoding the highly active bicomponent leukocidin typical of *S. aureus* strains from cattle and ruminants (24). All isolates carried the hemolysin genes *hlb*, *hl*, and *hlIII* (*hl* and *hlIII* coding for a putative membrane protein [33]), and all but three cattle isolates were also positive for *hla*.

The determination of the quorum sensing system *agr* groups showed that all isolates belong to the *agr* type I group. One MSSA isolate and one MRSA isolate from cattle and the human isolate were positive for both the *sak* and *scn* genes, within the immune evasion cluster (IEC), and were usually detected in isolates with human adaptation. Moreover, four MRSA isolates from pigs were positive only for the *sak* gene.

All isolates were positive for several superantigens/enterotoxin-like genes, with few isolates (6/47; 12.8%) from cattle testing positive only for staphylococcal enterotoxin genes: *seg* (1 MRSA

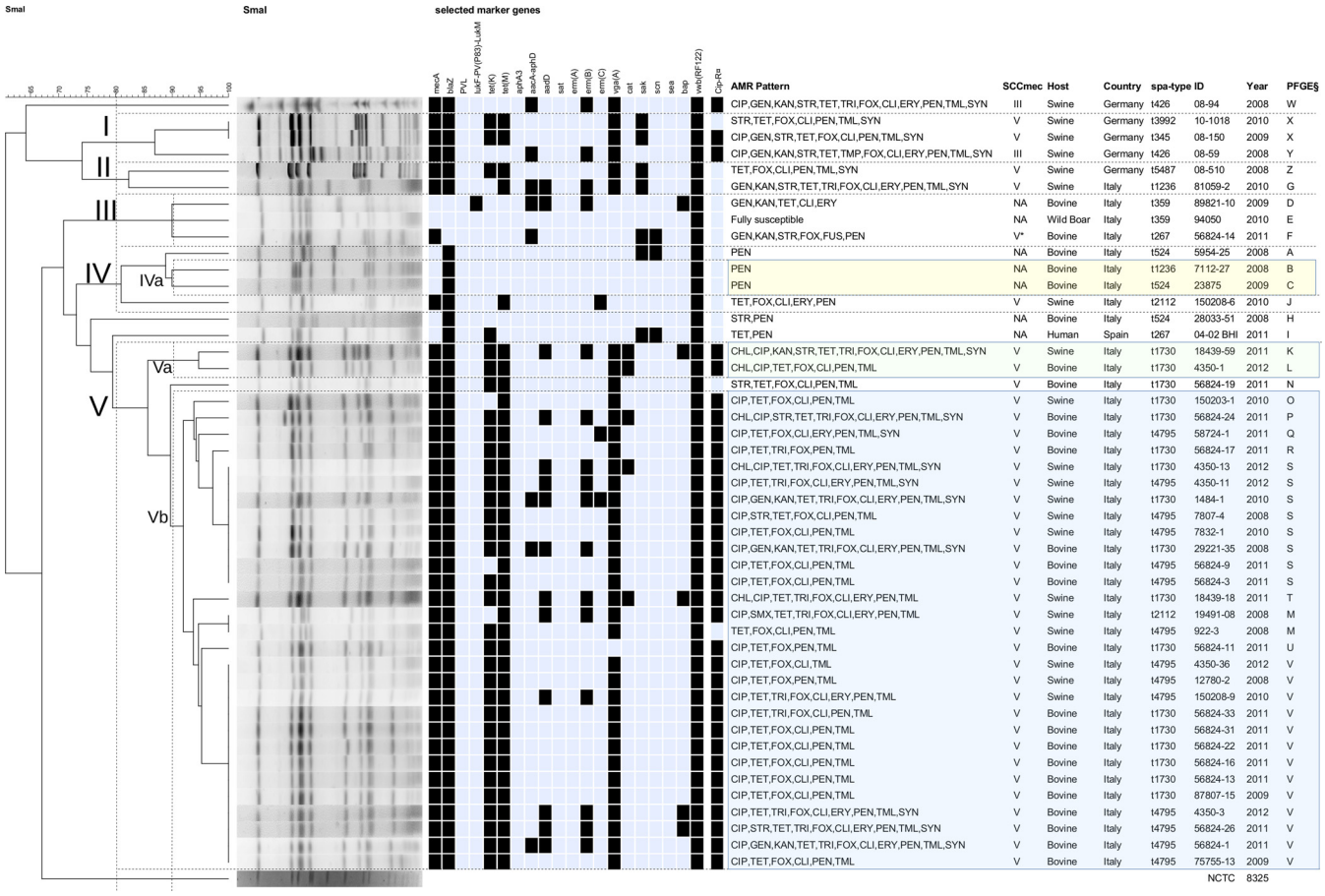


**FIG 1** Dendrogram of SmaI PFGE macrorestriction patterns with selected virulence and antimicrobial resistance marker genes and elements and antimicrobial resistance pattern of clonal complex 97 MSSA and MRSA isolates from animals and humans. ✧, ciprofloxacin resistance; §, PFGE pulsotype; *, SCC*mec* V and SCC*fus*.

isolate) within the enterotoxin gene cluster; *sed*, *sej*, and *ser* (1 MSSA isolate); *sed* (1 MRSA isolate); or the open reading frame (ORF) CM14, an enterotoxin-like protein (3 MSSA isolates). None of the isolates were positive for the enterotoxin *sea*, and none were positive for the toxin shock syndrome toxin gene *tst-1* or the human or the bovine allele. All isolates were negative for the arginine catabolic mobile element (ACME) locus genes.

In all the MRSA and the MSSA isolates tested, the MICs that were above the epidemiological cutoff (microbiological resistance) were also above the MIC breakpoint of clinical resistance for the following antimicrobials: PEN, FOX, CIP, CHL, ERY, GEN, KAN, FUS, TET, SMX, and TRI (see Table S1 in the supplemental material). As for CLI and SYN, 56.8% (21/37) and 18.7% (3/16) of microbiologically resistant isolates, respectively, were also clinically resistant. Additionally, all MRSA isolates proved to be multidrug resistant (i.e., 3+R) when the MIC results were interpreted according to either the epidemiological cutoffs or the CBs. All but one of the MRSA isolates from animals were tetracycline resistant. This resistance was mediated by the *tet*(M) gene in 37/40 (92.5%) isolates and was also mediated by the *tet*(K) gene in 32/40 isolates (82.5%). All but one of the MRSA isolates also carried the *blaZ* penicillinase gene. The aminoglycoside (GEN-KAN) resistance gene *aacA-aphD* was present in 7/40 MRSA isolates, often in the copresence of *aadD*, and no isolate carried the *aphA3* (kanamycin) or *sat* (streptothricin) resistance gene. Macrolide-lincosamide resistance was mediated by *erm*(B) ($n = 15$), *erm*(C) ($n = 2$), or both genes ($n = 1$). The vast majority (38/40; 95.0%) of MRSA isolates showed microbiological pleuromutilin resistance (tiamulin MIC of >4 mg/liter), attributed to the *vga*(A) gene, which also contributes to resistance to streptogramins A and lincosamides. All isolates carried *sdrM*, a chromosomal multidrug-resistant (MDR) efflux pump, and two porcine isolates harbored the plasmid-borne gene *qacC*. A total of 33 isolates (70.2%) showed fluoroquinolone resistance (MIC range of 2 to >8 mg/liter; mode, 2 mg/liter), and these strains belonged mainly to PFGE cluster V (30/32; 93.7%). Chloramphenicol resistance was detected in 5 MRSA isolates (10.6%), all from Italian pigs and cattle, and was mediated by the *cat* gene.

All MRSA isolates had the same pattern of capsule- and biofilm-associated "core" genes (see Table S1 in the supplemental material). Five isolates, (2 bovine and 2 swine MRSA isolates and 1 bovine MSSA isolate) also carried the *bap* gene encoding the "biofilm-associated protein" Bap. All isolates carried several genes encoding microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), including *bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, and *vwb*.

All isolates, irrespective of animal species, tested positive for

the *Staphylococcus aureus* pathogenicity island (SaPI)-carried *vwb* (RF122) allelic variant of the Von Willebrand binding protein.

The two *S. aureus* isolates of *spa* type t267 (one cattle MRSA isolate and one human MSSA isolate) that had the same genetic "core profile" (including *sak* and *scn*) differed in acquired resistance genes/elements (e.g., SCC*mec*, *blaZ*, *aacA-aphD*, and the fusidic acid resistance gene Q6GD50 [or *fusC*]), were distinguishable by PFGE, and grouped into two different sequence types (ST352 and ST97).

**Analysis of PFGE macrorestriction patterns.** PFGE macrorestriction analysis separated the strains into 26 PFGE profiles (profiles A to Z) within two main branches and identified five main clusters (clusters I to V) at an 80% similarity cutoff. Clusters IV and V were subdivided into different subclusters (e.g., cluster Va) based on 90% similarity (Fig. 1).

All three ST352 isolates (*spa* types t359 and t267) shared 90% homology (Dice coefficient) and were placed into the same cluster (cluster III). Other less represented *spa* type-ST combinations (e.g., t267-ST97, t426-ST97, t524-ST71, and t1236-ST97) were grouped into different small clusters (clusters I, II, and IV) or not grouped because the level of homology was <80%. The most represented *spa* types, t1730 and t4795 ($n = 31$; 65.9% of all isolates), showed high similarity (>85%) and were all grouped into cluster V (Fig. 1). This cluster was composed of 12 profiles (profiles K to V), with subcluster Vb being the dominant one. Subcluster Vb included two groups of indistinguishable isolates: profile "S" with 8 strains and profile "V" with 13 strains. These were all Italian isolates and originated from either swine or bovine samples (Fig. 1).

## DISCUSSION

This study provides a broad molecular characterization of CC97 MRSA isolates from farm animals and offers useful information for further insight into the relatedness and similarities with MRSA isolates of different origins, including humans.

The PFGE results showed a good concordance with origin and a series of genetic markers, often carried by mobile genetic elements (MGEs), bacteriophages, and subject to loss or acquisition in relation to the host adaptation process. This may prove useful for further molecular epidemiology studies. For instance, the few IEC-positive MRSA and MSSA isolates are interspersed in the dendrogram (e.g., in clusters I, II, III, and IV) and are absent in the most represented IEC-negative group from dairy cattle and pigs (cluster V). Moreover, these IEC-positive isolates lack the macrolide-lincosamide resistance gene *erm*(B) or *erm*(C), found in 45% of MRSA isolates from farm animals. These features suggest that a human-to-animal pattern of transmission of the *sak*- and/or *scn*-positive isolates or acquisition by the animal isolates of the beta-toxin-converting phages (25) and of the fusidic acid resistance determinant Q6GD50 (*fusC*) with SCC*mec fus* (*spa* type t267-ST352 from dairy cattle) occurred at the human-animal interface in farm settings.

MRSA isolates from Germany were grouped separately from all other Italian isolates (clusters I and II), except for one single Italian MRSA isolate of swine origin, which also grouped in cluster II.

The two MSSA isolates not clustered by PFGE (PFGE profiles H and I), one of human origin from Spain and one from an Italian dairy cattle herd, represented unique profiles and shared ~75% homology with cluster V.

Interestingly, ~10% of isolates carried the *bap* gene encoding the biofilm-associated protein Bap, a surface protein which is carried in a putative transposon inserted into SaPIbov2, (26), described in a minority of cases of bovine mastitis (27), and which has been implicated in biofilm formation in chronic mastitis cases (28). Among these five *bap*-positive isolates, two from dairy cattle were within the most represented PFGE profile, cluster V, and two were from pigs (profiles K and T), all of which grouped within PFGE cluster V. The remaining isolate, a LukM-LukF(P83)-positive MSSA isolate from a veal calf, was clearly separated far from these isolates in cluster III (Fig. 1). This suggests that the transposon carrying the *bap* gene may be capable of moving from one *S. aureus* genome to another across different production systems.

The presence/absence of selected marker genes often allowed further differentiation within clusters/subclusters, even within patterns showing 100% PFGE similarity.

All isolates lacked the ACME locus genes, which is rarely reported in CC97-SCC*mec* V isolates from humans (29). The strains were also negative for exfoliative toxin genes and toxic shock syndrome toxin genes, similarly to isolates of the other major LA-MRSA clonal complexes (i.e., CC398 and CC1) in Italy.

The CC97 MRSA isolates from pigs and cattle studied proved to be multidrug resistant to several classes of antimicrobials other than tetracyclines and beta-lactams, such as macrolide-lincosamide-streptogramin B (MLSb) antimicrobials, aminoglycosides, diaminopyrimidines, and pleuromutilins. The genetic basis (see Table S1 in the supplemental material) for these resistance traits is attributed to genes acquired through MGEs (30, 31). Additionally, the dominant cluster of isolates (PFGE cluster V) presents a high prevalence of fluoroquinolone resistance, which, although not genetically determined in this study, has been attributed to various combinations of mutations at different chromosomal targets (32). This trait among isolates of cluster V (Fig. 1), which is not significantly different ($P > 0.05$ by a Fisher exact test) in the swine or bovine host, may be an evolutionary feature of this cluster.

Interestingly, among the CC97 strains, aminoglycoside resistance was never mediated by the human-associated, transposon-borne Tn*5405 aphA3* and *sat* genes. This differs from what has been observed for CC1 LA-MRSA isolates from Italian dairy cattle, which are closely related and most likely derived from a human CC1 *SCCmec* IVa lineage (33). This may also indicate that the CC97 MRSA strains are unlikely to have originated from human-to-cattle spillback. A remarkable feature is the concurrent presence of the *blaZ* gene in almost all MRSA isolates and the high frequency of the plasmid-borne *tet*(K) gene in *tet*(M)-positive isolates from either pigs or dairy cattle, thus suggesting that the redundancy of beta-lactam and tetracycline resistance may be the result of the acquisition of genes and elements that occurred on different occasions over time. Additionally, recent work suggests that the role of the BlaZ-BlaR1-BlaI gene cluster system in *S. aureus* regulation extends beyond the scope of beta-lactamase expression, contributing to the pathogen's immune evasion and virulence through the BlaI gene, which has been demonstrated to be a novel cathelicidin resistance factor (34).

Molecular characterization of CC97 isolates detected through population-based studies in Italian farmed animals demonstrates that more than half of the isolates, although detected in different holdings, share very similar molecular features; are mostly grouped into PFGE cluster V, with dominant subcluster Vb being composed mainly of indistinguishable isolates (ST97 and *spa*

types t1730 and t4795); and show a specific genetic profile. All these features indicate that a particular MRSA CC97 clone has successfully spread among Italian pig and dairy cattle holdings, probably in recent years.

It is still to be clarified whether this lineage originated as an MDR MRSA isolate in dairy cattle or was transmitted as an MSSA isolate from cattle to pigs, acquired SCC*mec* and its MDR nature in the intensive pig industry environment, and then spilled back among cattle holdings in areas with high densities of both dairy and pig farms. Ideally, further insight into these aspects could be achieved through whole-genome sequencing and a phylogenetic approach, which could also clarify the relationships between the Italian lineage in livestock and CC97 clones that have been causing human disease worldwide. Intriguingly, in the CC97 MRSA isolates studied, there is no evident clear separation of genetic characteristics (e.g., PFGE-based cluster, AMR gene, and SCC*mec* analyses and determination of the presence/absence of IEC genes) between isolates of swine and bovine origins. This differs from what has been demonstrated for MRSA CC1, another major LA-MRSA clonal complex in Italy, in which the swine lineage is clearly separated from the bovine-human lineage (33) and most likely underwent a different microevolution process. In the case of the dominant PFGE cluster V CC97 MRSA lineage, other factors must have played a role in its selection in both pigs and cattle. Further studies are necessary to elucidate what drives its epidemiology.

In conclusion, few differences were found among the MRSA and MSSA CC97 isolates studied. Only a minority of isolates harbor genes overtly associated with human adaptation (the IEC genes *sak*, *scn*, and *sea*), while other marker genes clearly point to a bovine origin. In this respect, the SaPI-carried *vwb* (RF122) allelic variant of the Von Willebrand binding protein is considered to be among the mechanisms for *S. aureus* pathogenicity associated with cattle and a specific marker of host adaptation (35).

Conversely, the MDR profiles and the resistance gene patterns of both bovine and swine MRSA isolates in the most represented PFGE cluster, cluster V, have some features [e.g., CIP resistance and *vga*(A)-mediated pleuromutilin resistance] in common with those observed in the CC1 MRSA lineage spreading in Italian pig holdings (33). These traits may be suggestive of a swine-to-bovine spillback of a few MDR *spa* types (t1730 and t4795) of this CC97 MRSA lineage, which are prevalent in Italian pig farms. This hypothesis is also supported by the very low rates of antimicrobial resistance and the absence of multidrug resistance in CC97 MSSA isolates from dairy cattle, as already noticed by other authors (8).

As a recent international study demonstrated (8), the LA-MRSA CC97 lineage has the capability of making the host jumps and is able to colonize and infect humans. Indeed, the spillover from food-producing animals of the CC97 MRSA lineage described in our study should be considered a possible public health threat. Since intensive animal production can be a novel source of human infections, strict biosecurity and management measures should be implemented at the farm level, with actions aimed at minimizing the risk of animal colonization, infection (e.g., mastitis in dairy cattle), and within-herd transmission. Overall, these measures are also likely to reduce the risk of occupational exposure (farmers, veterinarians, and slaughterhouse workers) and further spread in the community.

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