High prevalence of multidrug resistant *S. aureus*-CC398 and frequent detection of enterotoxin genes among non-CC398 *S. aureus* from pig-derived food in Spain.

Olouwafemi Mistourath Mama, Liliana Morales, Laura Ruiz-Ripa, Myriam Zarazaga, Carmen Torres*.

Departamento de Agricultura y Alimentación, Universidad de La Rioja, Logroño, Spain

***Corresponding author**

Professor Carmen Torres

Departamento de Agricultura y Alimentación

Universidad de La Rioja

Madre de Dios 53, 26006, Logroño, Spain

Tel.: +34 941299750; fax: +34 941299721

E-mail: carmen.torres@unirioja.es

Highlights

- *S. aureus* and MRSA were detected in 33.6% and 21.8% of pig-derived food samples.
- LA-MRSA CC398 (mostly *spa*-t011) found in 23% of pig-derived food, all MDR.
- *S. aureus* non-CC398 found in 16% of samples, with detection of toxigenic strains.
- Human-adapted clones (MSSA-CC398, MSSA-CC45) detected, suggesting human contamination.
- Pig-derived food carry MDR and toxigenic *S. aureus*, of public health relevance.

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) CC398 is a livestock-associated (LA) lineage, mainly detected in swine. Its dissemination via the food-chain could be a food-safety issue. This work aimed to study the diversity of *S. aureus* lineages in pork-products, to determine the prevalence of MRSA and methicillin-susceptible *S. aureus* (MSSA) of lineage CC398, and to study the antimicrobial resistance phenotype/genotype and the virulence traits of recovered isolates.

One hundred and one samples of pig-derived food were collected in Northern Spain for *S. aureus* isolation. Antibiotic resistance profile was analysed, and associated resistance genes were screened by PCR. Detection of CC398 lineage, *spa*-type, multilocus sequence-type (ST), virulence factors, immune evasion cluster (IEC) genes, and phage ΦSa3 integrase was performed by PCR/sequencing.

The prevalence of *S. aureus* and MRSA among pig-derived food was 33.6% and 21.8%, respectively. Thirty-nine *S. aureus* isolates were recovered and attributed to 19 *spa*-types and 12 STs, ST398 being the predominant lineage (n=25; 64 %). MRSA-CC398 isolates (n=23) were mainly *spa*-t011 (n=16) and 82.6% were multidrug-resistant (MDR). All

MRSA-CC398 were tetracycline-resistant and IEC-negative and four hosted either *eta*, *tst* or *sea* gene. The two MSSA-CC398 isolates detected were *spa*-t5452, IEC-positive, and were resistant to penicillin (*blaZ*) and erythromycin/clindamycin (inducible) (*ermT* with/without *ermC+msrA*). Among the 14 non-CC398 isolates, only two were MRSA (ST8, PVL-positive, enterotoxin-positive, IEC-negative). The 12 MSSA isolates included two of lineage CC45 and IEC-positive.

CC398 lineage is prevalent among *S. aureus* of pig-derived food (both MRSA and MSSA), LA-MRSA-CC398/t011 being the clone most represented. The presence of the IEC-positive MSSA-CC398 and MSSA-CC45 in food products highlights the potential implication of handlers in transmission of foodborne pathogens. Moreover, given the high frequency of MDR isolates and virulence genes detected, hygienic practices should be improved to limit the dissemination risk of *S. aureus* via the food chain.

Keywords: MRSA, MSSA, CC398, multidrug-resistance, enterotoxins, IEC

1. Introduction

Since the early 2000s, methicillin-resistant *Staphylococcus aureus* (MRSA) isolates of the clonal complex (CC) CC398 have been described as common colonizers of healthy farm animals, especially swine, and considered as livestock-associated MRSA (LA-MRSA). These isolates have also been found colonizing or causing infections in humans with livestock exposure (Benito et al., 2014b; Ceballos et al., 2019; Leibler et al., 2016). Whole genome analysis established that CC398 isolates cluster into distinct phylogenetic groups: a human-adapted ancestor clade (methicillin and tetracycline susceptible), and a livestock-adapted derived clade (methicillin and tetracycline resistant) (Price et al., 2012). According to Price et al., LA-MRSA-CC398 might have been evolved from the human-adapted clade, methicillin-susceptible *S. aureus* (MSSA) CC398. The jump from humans to animals would have been accompanied by the acquisition of methicillin and tetracycline resistance in one hand, and by the loss of the phage ΦSa3, that carries the immune evasion cluster (IEC) genes on the other hand (Price et al., 2012). The IEC system protects *S. aureus* against the human immune system; there are several IEC-types which all include the *scn* gene (encodes the staphylococcal complement inhibitor), then considered a marker for IEC detection (van Wamel et al., 2006).

S. aureus (SA) is known to be responsible for foodborne diseases and food poisoning. Staphylococcal food poisoning is due to the ingestion of sufficient amounts of staphylococcal enterotoxins (SEs) present in contaminated food (Argudín et al., 2010). Improper handling of food combined with uncontrolled storage conditions, may increase the risk of contamination by SA and the production of SEs which are resistant to heat and low pH (Argudín et al., 2010). MRSA has been isolated with different prevalence from diverse types of food, especially food of animal origin such as dairy products, or raw meat

(pork, beef, chicken, turkey etc.) (de Boer et al., 2009; Lozano et al., 2009; Papadopoulos et al., 2019).

For the all above, we wondered whether the food chain could be a way of transmission of LA-MRSA-CC398 to humans, whether this specific clone could be a potential agent of food poisoning, and finally what the prevalence would be in pig-derived food in our region (La Rioja, Spain). A few years ago, our research group performed two studies about the prevalence of SA and MRSA in food samples of animal origin including pork, chicken, beef, lamb, and turkey, among others (Benito et al., 2014a; Lozano et al., 2009). These works revealed a low prevalence of 2.5% of MRSA-CC398 among pork samples and the absence of MSSA-CC398, but the number of samples analysed was low. The present work focussed on pig, as pig-derived products constitute an important sector of the region's food industry. The prevalence of CC398 (both MRSA and MSSA) and SA of other lineages was determined, and the antimicrobial resistance phenotypes/genotypes and the virulence gene profiles were studied.

2. Material and methods

2.1. S. aureus isolation and identification

A total of 101 samples of pig-derived food [chopped meat (n=54); fillet (n=30); and ear/pork snout (n=17)] were collected from 18 butcher retail shops of three market places in La Rioja, Spain, during the period March-October of 2018. Nine visits were performed to the butcher retail shops, and one or two samples were collected per visit (not all butcher retail shops were tested in each visit). All types of pig products included in this study are usually eaten in Spain.

Samples were enriched with 5 ml of Brain Hearth Infusion broth (BHI, +NaCl 6.5%) (Conda, Madrid, Spain), and incubated at 37ºC during 24h. Aliquots were then inoculated into mannitol-salt-agar (Conda, Madrid, Spain) and oxacillin-resistance-screening-agar-

base (Oxoid, Hampshire, England) for SA and MRSA recovery, respectively. Up to two colonies/plate, with different staphylococcal morphologies were chosen and further identified. The identification was performed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker, Massachusetts, USA), and SA isolates were characterized.

2.2. Antimicrobial resistance phenotype and resistance genes detection

For all the SA identified, susceptibility to penicillin, cefoxitin, gentamicin, tobramycin, tetracycline, chloramphenicol, erythromycin, clindamycin and linezolid was analysed by disk-diffusion method (CLSI, 2018). Cefoxitin resistance was considered the marker of methicillin resistance phenotype. The detection of the following antimicrobial resistance genes was performed by PCR, in accordance with resistance phenotype: beta-lactams (*mecA*, *mecC*, and *blaZ*), aminoglycosides [*aac*(6′)-Ie–*aph*(2″)-Ia, *ant*(4′)-Ia], tetracycline [*tetK*, *tetL*, and *tetM*], macrolides-lincosamides [*ermA*, *ermB*, *ermC*, *ermT*, *msrA*, *lnuA*, *lnuB*, and *vgaA*], and chloramphenicol (cat_{pC194}, cat_{pC221}, cat_{pC223}, *cfr*, *fexA* and *fexB*) (Benito et al., 2014b; Gómez-Sanz et al., 2010; Kehrenberg and Schwarz, 2006; Ruiz-Ripa et al., 2019).

2.3. Molecular typing and virulence genes study

For all the SA isolates, *spa*-typing was performed as previously described (Ruiz-Ripa et al., 2019). Multilocus sequence typing (MLST) was carried out for 19 representative strains (one of each *spa*-type detected, and all strains with a new *spa*-type). For this purpose, PCR and sequencing of seven housekeeping genes (www.pubmlst.org) were performed to determine the sequence type (ST) and the clonal complex (CC) (Ruiz-Ripa et al., 2019). In addition, a specific PCR was carried out for CC398 lineage determination (Stegger et al., 2011). Furthermore, the presence of the genes encoding the Panton Valentine Leukocidin (PVL) (*lukF/lukS-PV*), the exfoliative toxins (*eta* and *etb*), the toxic

151 shock syndrome toxin (*tst*), the haemolysins (*hla*, *hlb*, *hld*, *hlg* and *hlgv*) and the
152 staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*,
153 *seo*, *sep*, *seq*, *ser*, and *seu*) was screened by PCR (Benito et al., 2014a). The presence of
154 *scn* gene was studied and the IEC type was determined for *scn*-positive isolates (van
155 Wamel et al., 2006). Moreover the presence of the Phage ΦSa3 integrase (Sa3int) was
156 studied for the IEC-positive isolates, as previously described (Goerke et al., 2009).
157 Positive and negative control strains of the University of La Rioja were included in all
158 PCR reactions.

159 ## 3. Results

160 ### *3.1. Prevalence of S. aureus and MRSA in* pig-derived food

161 *Staphylococcus aureus* was detected in 33.6% of the samples analysed (34/101).
162 Considering the different types of samples, the prevalence was of 24.1%, 26.6%, and
163 76.5% in chopped meat, fillet and ear/snout samples, respectively. The prevalence of
164 MRSA was of 21.8% (22/101) with different rates depending on food types: 3.3% (fillet),
165 14.8% (chopped meat) and 76.5% (ear/snout).

166 A total of 39 isolates were recovered (25 MRSA and 14 MSSA) (Table I), corresponding
167 to one isolate/positive-sample, except for five samples which harboured two SA isolates
168 with different *spa*-types and/or antimicrobial resistance phenotypes (included in Table
169 II). The antimicrobial resistance rates and genes detected among all the isolates recovered
170 in this study are shown in Table III. The isolates were assigned to 19 different *spa*-types
171 and 12 STs. The CC398 was the predominant lineage, being assigned to 25 isolates, all
172 of them of sequence type ST398 (64.1%).

173 ### *3.2. S. aureus-CC398 and characteristics of recovered isolates*

174 The 25 isolates of lineage CC398 were recovered from 21 samples (global prevalence of
175 20.8%), with the presence in four samples of two distinct CC398 isolates (Table II): a)

one chopped meat sample carrying both MSSA-CC398 and MRSA-CC398 isolates; b) three snout/ear samples with two MRSA-CC398 isolates (with different *spa*-type or antimicrobial resistance profile). The 25 CC398 isolates were divided into two groups: MRSA-CC398 (n=23; detected in 20 samples) and MSSA-CC398 (n=2; detected in 2 samples).

The 23 MRSA-CC398 isolates belonged to six *spa*-types (t011, t1451, t1606, t4030, t108 and t779), and most of them were t011 (n=16) (Table I). They all carried the *blaZ* gene and showed resistance to tetracycline (always mediated by *tetM* and *tetK* genes, combined with *tetL* in nine isolates). The frequent resistance to macrolides and lincosamides among MRSA-CC398 strains was also noted, with the following phenotypes/genotypes: erythromycin+clindamycin (n=11; *ermB*, *ermC*, *ermT*, *msrA*, and/or *vgaA*), erythromycin (n=1; *msrA*) and clindamycin (n=5; *lnuA* and/or *vgaA*) (Table I). Moreover, 82.6% of the isolates showed a multidrug resistance (MDR) phenotype, and resistance to beta-lactams, tetracycline, macrolides and/or lincosamides was the most common profile.

Regarding the virulence, two MRSA-CC398 isolates hosted the *eta* gene, one harboured the *sea* gene and another one the *tst* gene. All MRSA-CC398 isolates were IEC-negative. Concerning the haemolysin encoding genes, *hla* was present in all these isolates. The genes *hld* and *hlg* were found in all but three isolates, while the gene *hlb* was undetected in six. All the isolates lacked the *hlgv* gene.

On the other hand, the two MSSA-CC398 isolates were assigned to the *spa*-type t5452. They were resistant to penicillin (*blaZ*), and both showed an erythromycin/clindamycin-inducible resistance phenotype mediated by the *ermT* gene, alone or associated with *ermC* and *msrA* genes (Table I). One isolate was additionally resistant to gentamicin. Both isolates were typed as IEC-C and carried the phage integrase Sa3int. It is also notable that

both MSSA-CC398 isolates showed a MDR phenotype, and had the same haemolysin genes profile (*hla*, *hld* and *hlg*).

3.3. S. aureus of non-CC398 lineages and characteristics of recovered isolates

Among the 14 non-CC398 isolates, two were MRSA (ST8/CC8-t8151) and 12 MSSA (10 ST/CC and 11 different *spa*-types detected, including two new ones: t18358 and t18461) (Table I). The MRSA isolates showed only resistance for beta-lactams, carrying the *mecA* and *blaZ* genes. Both harboured the PVL encoding genes, as well as one SE gene (*see* or *seq*); moreover, these MRSA isolates were *scn*-negative, and consequently, IEC negative. Additionally, they carried the same haemolysin genes (*hla*, *hlb*, *hld* and *hlgv*).

The MSSA isolates were mainly resistant to penicillin (n=7; *blaZ*), tetracycline (n=4; *tetM*, *tetK*, *tetL*) and clindamycin (n=4; *vgaA*), although resistance to antibiotics of the macrolide and aminoglycoside families was also observed (Table I). Eight of the 12 MSSA non-CC398 isolates (of lineages ST45, ST9, ST1, ST22, ST133 and ST581) harboured enterotoxin genes, being five of them carriers of the *egc*-like cluster (Table I). Furthermore, the *tst* gene was detected in one isolate (ST133). All the isolates were *scn*-negative, except the two ST45 (typed as IEC-B and containing the phage integrase Sa3int). Moreover, they all carried the genes *hla* and *hld*, and only one lacked the gene *hlb*. Interestingly, all the isolates carried the *hlgv* gene, except three which were positive for *hlg*. None of the isolates hosted both *hlg* and *hlgv*; most of the isolates CC398 were *hlg*-positive while the non-CC398 were mostly *hlgv*-positive.

4. Discussion

Livestock, especially swine, constitutes a reservoir for SA and MRSA belonging to different clonal lineages, including mostly CC398. The contamination of animal-derived food by SA isolates would be a concern for human health and transmission could occur through the food chain by eating or manipulating.

The present work revealed a prevalence of SA among pig products of 33.6%. Previous studies found the presence of SA in pig-derived food with a higher frequency ranging from 45 to 60% (Jackson et al., 2013; Tang et al., 2017). The rate of MRSA isolates in pig-derived food in our work is high (21.8%) compared to results obtained few years ago in different countries, including Spain (2-10%) (Benito et al., 2014a; Lozano et al., 2009; O'Brien et al., 2012). Comparing the different types of food analysed in our study, it appears that samples with skin (ears and snout: 76.5%) carry more frequently MRSA than those without skin (chopped meat and fillet: 10.7%), which could explain the high rate observed in our study. Previous studies also found higher rates of SA and/or MRSA in samples with skin (snout, nares, carcasses, etc.) compared to samples without skin (minced meat, bacon, etc.) (Verhegghe et al., 2016). This was expected since this bacterium is described as commensal of animals and humans' skin and nares.

Our SA isolates belonged to a great diversity of genetic lineages with predominance of CC398 (64.1%). Many studies consider the MRSA-CC398 clone as the most frequent among livestock in Europe, particularly in pig and pig-derived food (Lozano et al., 2011; Sharma et al., 2016). It is worth noting that t011 was the *spa*-type mostly associated with cases of LA-MRSA-CC398, as was the case in our study. Moreover, t011 was the predominant *spa*-type detected among MRSA-CC398 of Spanish hospitals located in areas with high pig density (Ceballos et al., 2019). This highlights the easy ability of adaptation of MRSA-CC398, especially t011 to distinct ecosystems, its potential ways of dissemination and the important role of swine as MRSA-CC398 carrier.

MSSA-CC398 is considered of human-adapted origin and the ancestor of the LA-MRSA-CC398 of specific *spa*-types (Price et al., 2012). MSSA-CC398 has been increasingly reported in human infection cases mainly in Europe, and the *spa*-type t571 was associated with cases of bacteremia in French patients, living in animal free environments (Bonnet

et al., 2018). Both MSSA-CC398 (t5452) isolated obtained in our study were susceptible to tetracycline and interestingly, were typed as IEC-C. The presence of IEC in these MSSA-CC398 isolates suggests an human origin (Cuny et al., 2019). To the best of our knowledge, the detection of MSSA-CC398/IEC-positive, out of the human niche, is scarce. However, MSSA-CC398 isolates (t571, IEC-C, tetracycline-susceptible, erythromycin-resistant) were detected from white storks nestlings exposed to human residues in Spain (Gómez et al., 2016). These results highlight the spread of MSSA-CC398 of human clade in animals and animal-derived food.

Comparing the MRSA-CC398 and MSSA-CC398 isolates of this study, it appears that resistance to tetracycline is a constant phenotype among MRSA-CC398 isolates, while absent among MSSA-CC398 isolates. Resistance to macrolides and lincosamides was also frequently observed among both groups, but the phenotype erythromycin-clindamycin-inducible was only notable among MSSA isolates. Based on these results, it should be further investigated the erythromycin-clindamycin inducible resistance phenotype as a potential marker for MSSA-CC398 detection. The gene *ermT*, present in both MSSA-CC398, has been previously suggested as marker of the MSSA-CC398 human clade (Bonnet et al., 2018). Otherwise, the carriage of both MRSA and MSSA-CC398 by the same sample highlights the coexistence of isolates of different phylogenetic clades, and the presence of IEC genes in MSSA-CC398 suggests a human contamination. The detection of toxin encoding genes (*sea*, *eta* or *tst*) among MRSA-CC398, though infrequent, is worrisome especially in food products (Argudín et al., 2010). Globally, it was observed a high frequency of MDR among CC398 isolates and the presence of relevant virulence genes, which is a concern for public health, knowing the easiness of adaptation of this lineage to distinct environments.

A great diversity of genetic lineages was observed among the non-CC398 isolates. MRSA isolates were ascribed to CC8, while MSSA were assigned to ten other CCs being especially relevant CC45 and CC1. Both CC8 isolates harbored the PVL encoding genes, which is a marker of Community-associated (CA) MRSA (Planet et al., 2015), but interestingly lacked the *scn* gene, suggesting an animal origin. Three MRSA-CC8/PVL-positive/*scn*-positive isolates were previously detected in pork samples in Denmark (Tang et al., 2017), and two MRSA-CC8-USA300/*scn*-positive isolates in lamb and goat samples in Spain (Mama et al., 2019). Consequently, the CA-MRSA-CC8 should be considered a potential contaminant of animal-derived food, and its dissemination and adaptation in livestock should be further investigated. MSSA-ST45 has been found in chicken meat samples (Benito et al., 2014a) and in calve nasal samples in Spain (Mama et al., 2019). In both cases, the isolates carried the *egc*-like cluster genes, and were thought of human origin (IEC-B), as noted in the present study. MRSA-CC1/t127 is well-known as an important human pathogen, although it is increasingly being recognized as an LA-MRSA lineage (Elstrøm et al., 2019). The high presence of pyrogenic toxin superantigens in our isolates is worrisome, knowing their role in food poisoning (Argudín et al., 2010). Overall, transmission of SA CC398 and of other genetic lineages from food of animal origin to the handlers could occur during manipulations at the butchery level, and vice-versa (Verhegghe et al., 2016). Hence the importance to respect the good hygiene and manufacturing practices and to instore surveillance measures in pork production chains.

5. Conclusion

Pig-derived food frequently contain the LA-MRSA-CC398 clone, which is characterized by a tetracycline resistance phenotype. Moreover, the detection of MSSA-CC398 isolates carrying the IEC system highlights the risk of meat contamination by isolates of possible human origin through manipulation. Finally, high rates of MDR CC398 isolates and high

300 frequency of toxigenic isolates among non-CC398 isolates, including those of
301 community-associated lineages (ST8 and ST45) in food products, is worrisome for human
302 health. Therefore, hygienic practices of the food production chain should be improved in
303 order to limit the presence of food-borne pathogens, as well as their resistance and
304 virulence genes.

305 **Acknowledgments**

306 This work was supported by project SAF2016-76571-R from the Agencia Estatal de
307 Investigación (AEI) of Spain and the Fondo Europeo de Desarrollo Regional (FEDER)
308 of EU. OMM has a predoctoral fellowship of Mujeres por África-Universidad de La Rioja
309 (Spain). LRR has a predoctoral FPI fellowship of the Universidad de La Rioja (Spain).

310 **References**

311 Argudín, M.Á., Mendoza, M.C., Rodicio, M.R., 2010. Food poisoning and
312 *Staphylococcus aureus* enterotoxins. Toxins (Basel). 2, 1751–73.
313 https://doi.org/10.3390/toxins2071751

314 Benito, D., Gómez, P., Lozano, C., Estepa, V., Gómez-Sanz, E., Zarazaga, M., Torres,
315 C., 2014a. Genetic lineages antimicrobial resistance and virulence in
316 *Staphylococcus aureus* of meat samples in Spain: analysis of immune evasion cluster
317 (IEC) genes. Foodborne Pathog. Dis. 11, 354–356.
318 https://doi.org/10.1089/fpd.2013.1689

319 Benito, D., Lozano, C., Rezusta, A., Ferrer, I., Vasquez, M.A., Ceballos, S., Zarazaga,
320 M., Revillo, M.J., Torres, C., 2014b. Characterization of tetracycline and methicillin
321 resistant *Staphylococcus aureus* strains in a Spanish hospital: Is livestock-contact a
322 risk factor in infections caused by MRSA CC398? Int. J. Med. Microbiol. 304, 1226–
323 1232. https://doi.org/10.1016/j.ijmm.2014.09.004

324 Bonnet, I., Millon, B., Meugnier, H., Vandenesch, F., Maurin, M., Pavese, P., Boisset, S.,

2018. High prevalence of *spa* type t571 among methicillin-susceptible *Staphylococcus aureus* from bacteremic patients in a French University Hospital. PLoS One 13, e0204977. https://doi.org/10.1371/journal.pone.0204977

Ceballos, S., Aspiroz, C., Ruiz-Ripa, L., Reynaga, E., Azcona-Gutiérrez, J.M., Rezusta, A., Seral, C., Antoñanzas, F., Torres, L., López, C., López-Cerero, L., Cercenado, E., Zarazaga, M., Torres, C., 2019. Epidemiology of MRSA CC398 in hospitals located in Spanish regions with different pig-farming densities: a multicentre study. J Antimicrob.Chemother. 74, 2157–2161. https://doi.org/10.1093/jac/dkz180.

Clinical Laboratory Standards Institute, Wayne, PA, 2018. Performance standards for antimicrobial susceptibility testing: 28th ed. CLSI supplement M100;

Cuny, C., Layer, F., Hansen, S., Werner, G., Witte, W., 2019. Nasal colonization of humans with occupational exposure to raw meat and to raw meat products with methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. Toxins (Basel). 11, 1–10. https://doi.org/10.3390/toxins11040190

de Boer, E., Zwartkruis-Nahuis, J.T.M., Wit, B., Huijsdens, X.W., de Neeling, A.J., Bosch, T., van Oosterom, R.A.A., Vila, A., Heuvelink, A.E., 2009. Prevalence of methicillin-resistant *Staphylococcus aureus* in meat. Int. J. Food Microbiol. 134, 52–56. https://doi.org/10.1016/j.ijfoodmicro.2008.12.007

Elstrøm, P., Grøntvedt, C.A., Gabrielsen, C., Stegger, M., Angen, Ø., Åmdal, S., Enger, H., Urdahl, A.M., Jore, S., Steinbakk, M., Sunde, M., 2019. Livestock-Associated MRSA CC1 in Norway; introduction to pig farms, zoonotic transmission, and eradication. Front. Microbiol. 10, 139. https://doi.org/10.3389/fmicb.2019.00139

Goerke, C., Pantucek, R., Holtfreter, S., Schulte, B., Zink, M., Grumann, D., Bröker, B.M., Doskar, J., Wolz, C., 2009. Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages. J. Bacteriol. 191, 3462–3468.

https://doi.org/10.1128/JB.01804-08

Gómez-Sanz, E., Torres, C., Lozano, C., Fernández-Pérez, R., Aspiroz, C., Ruiz-Larrea, F., Zarazaga, M., 2010. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in spanish slaughter pigs of different age groups. Foodborne Pathog. Dis. 7, 1269–1277. https://doi.org/10.1089/fpd.2010.0610

Gómez, P., Lozano, C., Camacho, M.C., Lima-Barbero, J.F., Hernández, J.M., Zarazaga, M., Höfle, Ú., Torres, C., 2016. Detection of MRSA ST3061-t843-*mecC* and ST398-t011-*mecA* in white stork nestlings exposed to human residues. J. Antimicrob. Chemother. 71, 53–57. https://doi.org/10.1093/jac/dkv314

Jackson, C.R., Davis, J.A., Barrett, J.B., 2013. Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* isolates from retail meat and humans in Georgia. J. Clin. Microbiol. 51, 1199–1207. https://doi.org/10.1128/JCM.03166-12

Kehrenberg, C., Schwarz, S., 2006. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. Antimicrob. Agents Chemother. 50, 1156–1163. https://doi.org/10.1128/AAC.50.4.1156-1163.2006

Leibler, J.H., Jordan, J.A., Brownstein, K., Lander, L., Price, L.B., Perry, M.J., 2016. *Staphylococcus aureus* nasal carriage among beefpacking workers in a midwestern United States slaughterhouse. PLoS One 11, e0148789. https://doi.org/10.1371/journal.pone.0148789

Lozano, C., Aspiroz, C., Ara, M., Gómez-Sanz, E., Zarazaga, M., Torres, C., 2011. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 in a farmer with skin lesions and in pigs of his farm: Clonal relationship and detection of *lnu*(A) gene. Clin. Microbiol. Infect. 17, 923–927. https://doi.org/10.1111/j.1469-0691.2010.03437.x

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, 1325–1346. https://doi.org/10.1093/JAC/DKP378

Mama, O.M., Gómez-Sanz, E., Ruiz-Ripa, L., Gómez, P., Torres, C., 2019. Diversity of staphylococcal species in food producing animals in Spain, with detection of PVL-positive MRSA ST8 (USA300). Vet. Microbiol. 233, 5–10. https://doi.org/10.1016/j.vetmic.2019.04.013

O'Brien, A.M., Hanson, B.M., Farina, S.A., Wu, J.Y., Simmering, J.E., Wardyn, S.E., Forshey, B.M., Kulick, M.E., Wallinga, D.B., Smith, T.C., 2012. MRSA in conventional and alternative retail pork products. PLoS One 7, e30092. https://doi.org/10.1371/journal.pone.0030092

Papadopoulos, P., Papadopoulos, L., Angelidis, A.S., Kotzamanidis, C., Zdragas, A., Papa, A., Filioussis, G., Sergelidis, D., 2019. Prevalence , antimicrobial susceptibility and characterization of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolated from dairy industries in north-central and north-eastern Greece. Int. J. Food Microbiol. 291, 35–41. https://doi.org/10.1016/j.ijfoodmicro.2018.11.007

Planet, P.J., Diaz, L., Kolokotronis, S., Narechania, A., Reyes, J., Xing, G., Rincon, S., Smith, H., Panesso, D., Ryan, C., Smith, D.P., Guzman, M., Zurita, J., Sebra, R., Deikus, G., Nolan, R.L., Tenover, F.C., Weinstock, G.M., Robinson, D.A., Arias, C.A., 2015. Parallel epidemics of community-associated methicillin-resistant *Staphylococcus aureus* USA300 infection in North and South America. J. Infect. Dis. 212, 1874–1882. https://doi.org/10.1093/infdis/jiv320

Price, L.B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, S., Pearson, T.,

Waters, A.E., Foster, J.T., Schupp, J., Gillece, J., Driebe, E., Liu, C.M., Springer, B., Zdovc, I., Battisti, A., Franco, A., Zmudzki, J., Schwarz, S., Butaye, P., Jouy, E., Pomba, C., Porrero, M.C., Ruimy, R., Smith, T.C., Robinson, D.A., Weese, J.S., Arriola, C.S., Yu, F., Laurent, F., Keim, P., Skov, R., Aarestrup, F.M., 2012. Adaptation and emergence of *Staphylococcus aureus* CC398: Host adaptation and emergence of methicillin resistance in livestock. MBio 3, e00305. https://doi.org/10.1128/mBio.00305-11.Editor

Ruiz-Ripa, L., Gómez, P., Alonso, C.A., Camacho, M.C., de la Puente, J., Fernández-Fernández, R., Ramiro, Y., Quevedo, M.A., Blanco, J.M., Zarazaga, M., Höfle, U., Torres, C., 2019. Detection of MRSA of lineages CC130-*mecC* and CC398-*mecA* and *Staphylococcus delphini-lnu*(A) in magpies and cinereous Vultures in Spain. Microb. Ecol. 78, 409–415. https://doi.org/10.1007/s00248-019-01328-4

Sharma, M., Nunez-Garcia, J., Kearns, A.M., Doumith, M., Butaye, P.R., Angeles Argudín, M., Lahuerta-Marin, A., Pichon, B., AbuOun, M., Rogers, J., Ellis, R.J., Teale, C., Anjum, M.F., 2016. Livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) clonal complex (CC) 398 isolated from UK animals belong to European lineages. Front. Microbiol. 7, 1741. https://doi.org/10.3389/fmicb.2016.01741

Stegger, M., Lindsay, J.A., Moodley, A., Skov, R., Broens, E.M., Guardabassi, L., 2011. Rapid PCR detection of *Staphylococcus aureus* clonal complex 398 by targeting the restriction-modification system carrying sau1-hsdS1. J. Clin. Microbiol. 49, 732–734. https://doi.org/10.1128/JCM.01970-10

Tang, Y., Larsen, J., Kjeldgaard, J., Andersen, P.S., Skov, R., Ingmer, H., 2017. Methicillin-resistant and -susceptible *Staphylococcus aureus* from retail meat in Denmark. Int. J. Food Microbiol. 249, 72–76.

https://doi.org/10.1016/j.ijfoodmicro.2017.03.001

Verhegghe, M., Crombé, F., Luyckx, K., Haesebrouck, F., Butaye, P., Herman, L., Heyndrickx, M., Rasschaert, G., 2016. Prevalence and genetic diversity of livestock-associated methicillin-resistant *Staphylococcus aureus* on Belgian pork. J. Food Prot. 79, 82–89. https://doi.org/10.4315/0362-028x.jfp-15-266

van Wamel, W.J.B., Rooijakkers, S.H.M., van Kessel, K.P.M., van Strijp, J.A.G., Ruyken, M., 2006. The innate immune modulators Staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β-Hemolysin-converting bacteriophages. J. Bacteriol. 188, 1310–1315. https://doi.org/10.1128/JB.188.4.1310

Table I: Molecular characteristics, antimicrobial resistance phenotype/genotype and virulence factors of MRSA and MSSA isolated from 101 samples of pig-derived food

ST/CC (Nº isolates)	***spa*-type** (Nº isolates)	**Antimicrobial resistance phenotype**[a] (Nº isolates) **Beta lactams / Others**	**Antimicrobial resistance genes** (Nº isolates)	**Virulence genes** (Nº isolates)	**IEC type** (Nº isolates)
ST398/CC398 (25)	t011 (16)	PEN, **FOX** / TET, ERY, CLI (6)	*blaZ*(6), *mecA*(6), *tetM*(6), *tetK*(6), *tetL*(5), *ermB*(2), *ermC*(4), *ermT*(4), *msrA*(1), *vgaA*(1)	***eta***(1), *hla*(6), *hlb*(5), *hld*(5), *hlg*(4)	-
		PEN, **FOX** / TET, CLI (4)	*blaZ*(4), *mecA*(4), *tetM*(4), *tetK*(4), *tetL*(1), *lnuA*(1), *vgaA*(2)	***sea***(1), *hla*(4), *hlb*(4), *hld*(2), *hlg*(4)	-
		PEN, **FOX** / TET (3)	*blaZ*(3), *mecA*(3) *tetM*(3), *tetK*(3), *tetL*(1)	*hla*(3), *hlb*(2), *hld*(3), *hlg*(3)	-
		PEN, **FOX** / TET, GEN, TOB, CLI (1)	*blaZ*, *mecA*, *tetM*, *tetK*, *aac(6')-Ie-aph(2")-Ia*, *vgaA*	*hla*, *hlb*, *hld*, *hlg*	-
		PEN, **FOX** / TET, ERY, CLI, CHL (1)	*blaZ*, *mecA*, *tetM*, *tetK*, *ermT*, *vgaA*, *fexA*	*hla*, *hlb*, *hld*, *hlg*	-
		PEN, **FOX** / TET, CHL (1)	*blaZ*, *mecA*, *tetM*, *tetK*	***eta***, *hla*, *hlb*, *hld*, *hlg*	-
	t1451 (2)	PEN, **FOX** / TET, ERY, CLI (1)	*blaZ*, *mecA*, *tetM*, *tetK*, *ermC*, *msrA*	***tst***, *hla*, *hlb*, *hld*, *hlg*	-
		PEN, **FOX** / TET (1)	*blaZ* ,*mecA*, *tetM*, *tetK*	*hla*, *hld*, *hlg*	-
	t1606 (2)	PEN, **FOX** / TET, ERY, CLI, TOB, CHL (1)	*blaZ*, *mecA*, *tetM*, *tetK*, *tetL*, *ermC*, *msrA*, *ant(4')-Ia*, *fexA*	*hla*, *hld*, *hlg*	-
		PEN, **FOX** / TET, ERY, CLI, CHL (1)	*blaZ*, *mecA*, *tetM*, *tetK*, *ermC*,*ermT*, *msrA*, *fexA*	*hla*, *hld*, *hlg*	-
	t4030 (1)	PEN, **FOX** / TET, ERY, CLI	*blaZ*, *mecA*, *tetM*, *tetK*, *tetL*, *ermC*, *msrA*, *vgaA*	*hla*, *hlb*, *hld*, *hlg*	-
	t108 (1)	PEN, **FOX** / TET, ERY, CHL	*blaZ*, *mecA*, *tetM*, *tetK*, *msrA*, *fexA*	*hla*, *hlb*, *hld*, *hlg*	-
	t779 (1)	PEN, **FOX** / TET, GEN, TOB	*blaZ*, *mecA*, *tetM*, *tetK*, *aac(6')-Ie-aph(2")-Ia*	*hla*, *hld*	-
	t5452 (2)	PEN / ERY, CLI[c] (1)	*blaZ*, *ermT*, *ermC*, *msrA*	*hla*, *hld*, *hlg*	**C**
		PEN / ERY, CLI[c], GEN (1)	*blaZ*, *ermT*, *aac(6')-Ie-aph(2")-Ia*	*hla*, *hld*, *hlg*	**C**
Other lineages (14)					
ST8/CC8 (2)	t8151(2)	PEN, **FOX** (2)	*blaZ*(2), *mecA*(2)	***lukF/lukS-PV***(2), ***seq***(1), ***see***(1), *hla*(2), *hlb*(2), *hld*(2), *hlgv*(2)	-
ST45/CC45 (2)	t230 (2)	PEN (2)	*blaZ*(2)	***egc*-like**[d](2), ***sec***(2), *hla*(2), *hlb*(2), *hld*(2), *hlg*(2)	**B** (2)
ST9/CC9 (2)	t1939 (1)	PEN / TET, ERY, CLI	*blaZ*, *tetM*, *tetK*, *ermB*, *vgaA*	***egc*-like**[d], *hla*, *hlb*, *hld*, *hlgv*	-
	t4644 (1)	PEN	*blaZ*	***egc*-like**[d], *hla*, *hlb*, *hld*, *hlgv*	-
ST1/CC1(1)	t127	/ ERY, CLI	*ermA*	***seh***, *hla*, *hlb*, *hld*, *hlgv*	-

ST6/CC6 (1)	t701	PEN	/ TET	*blaZ*	*hla*, *hlb*, *hld*, *hlgv*	-
ST7/CC7 (1)	t091	Susceptible		-	*hla*, *hlb*, *hld*, *hlgv*	-
ST12/CC12 (1)	t156	Susceptible		-	*hla*, *hlb*, *hld*, *hlgv*	-
ST22/CC22 (1)	t1862	Susceptible		-	***egc*-like**[d], *hla*, *hlb*, *hld*, *hlg*	-
ST133/CC133 (1)	t18461[b]	Susceptible		-	***tst***, ***sec***, *hla*, *hlb*, *hld*, *hlgv*	-
ST581 (1)	t18358[b]	PEN	/ TET, ERY, CLI	*blaZ*, *tetM*, *ermB*, *msrA*	***see***, *hla*, *hlb*, *hld*, *hlgv*	-
ST692 (1)	t2247	PEN	/ TET, CLI, GEN, TOB	*blaZ*, *tetL*, *aac(6')-Ie-aph(2")-Ia*, *ant(4')-Ia*	*hla*, *hld*, *hlgv*	-

[a] PEN: penicillin; FOX: cefoxitin; TET: tetracycline; ERY: erythromycin; CLI: clindamycin; GEN: gentamicin; TOB: tobramycin; CHL: chloramphenicol

[b] new *spa*-type

[c] inducible resistance phenotype

[d] *egc*-like cluster (*seg*, *sei*, *sem*, *sen*, *seo* and *seu*)

Resistance to cefoxitin (FOX), and *tst*, *eta*, enterotoxins, and PVL encoding genes are marked in bold

Table II: Pattern of coexistence of S. aureus isolates in a same pig-derived food sample

Sample	Origin	MRSA/MSSA	Isolate	*spa*-type	ST[a]	PCR CC398	IEC-type	Antimicrobial resistance phenotype[b]
1	Ear	MRSA	X74	t1451	(ST398)	+	-	PEN, FOX, TET, ERY, CLI
		MRSA	X42	t1606	(ST398)	+	-	PEN, FOX, TET, ERY, CLI, CHL
30	Ear	MRSA	X358	t011	(ST398)	+	-	PEN, FOX, TET
		MRSA	X352	t011	(ST398)	+	-	PEN, FOX, TET, CHL
6	Snout	MRSA	X65	t011	(ST398)	+	-	PEN, FOX, TET, CLI
		MRSA	X48	t4030	ST398	+	-	PEN, FOX, TET, ERY, CLI
CP17	Chopped meat	MSSA	X504	t5452	ST398	+	C	PEN, ERY, CLI[c], GEN
		MRSA	X488	t011	(ST398)	+	-	PEN, FOX, TET, CLI
F15	Fillet	MSSA	X699	t127	(ST1/CC1)	-	-	ERY, CLI
		MRSA	X664	t011	(ST398)	+	-	PEN, FOX, TET, ERY, CLI

[a] the STs in brackets are assumed according to the *spa*-type detected. The MLST has been determined by PCR/sequencing for the STs without brackets

[b] PEN: penicillin; FOX: cefoxitin; TET: tetracycline; ERY: erythromycin; CLI: clindamycin; GEN: gentamicin; TOB: tobramycin; CHL: chloramphenicol

[c] inducible resistance phenotype

Table III: Antibiotic resistance rate and resistance genes detected among 39 S. aureus isolates obtained from 101 samples of pig-derived food

Antibiotics tested	**Nº of resistant isolates (%)**	**Resistance genes detected (Nº of positive isolates)**
Penicillin	35 (89.7%)	*blaZ* (33)
Cefoxitin	25 (64.1%)	*mecA* (25)
Tetracycline	27 (69.2%)	*tet*(M) (26), *tet*(K) (25), *tet*(L) (9)
Clindamycin	23 (58.9%)	*lnu*(A) (1), *vgaA* (7)
Erythromycin	17 (43.6%)	*erm*(A) (1), *erm*(B) (4), *erm*(C) (10), *erm*(T) (9), *msrA* (9)
Tobramycin	5 (12.8%)	*ant*(4')-*Ia* (2)
Gentamicin	4 (10.3%)	*aac(6')-Ie-aph(2")-Ia* (4)
Tobramycin + gentamicin	3 (7.7%)	*aac(6')-Ie-aph(2")-Ia* (3), *ant*(4')-*Ia* (1)
Chloramphenicol	4 (10.3%)	*fexA* (4)
Linezolid	0	-