

1 **Local and transboundary transmission of methicillin-resistant *Staphylococcus aureus* sequence**
2 **type 398 through pig trading**

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22 **Running head:** Imported LA-MRSA ST398 in Southern Italy

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31 **ABSTRACT**

32 Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type (ST) 398 (LA-
33 MRSA ST398) is a genetic lineage for which pigs are regarded as the main reservoir. An increasing
34 prevalence of LA-MRSA ST398 has been reported in areas with high livestock density throughout
35 Europe. In this study, we have investigated the drivers contributing to the introduction and spread of
36 LA-MRSA ST398 along the pig farming system in Southern Italy. Whole-genome sequencing (WGS)
37 of LA-MRSA ST398 isolates collected in 2018 from pigs (n=53) and employees (n=14) from 10
38 farms in the Calabria region were comparatively analysed with previously published WGS data from
39 Italian ST398 isolates (n=45), an international ST398 reference collection (n=89) and isolates from
40 Danish pigs farms (n=283), which are the main suppliers of pigs imported to Italy. Single-nucleotide
41 polymorphisms (SNP) were used to infer isolates relatedness and, together with data from animal
42 trading, factors contributing to LA-MRSA ST398 dissemination were identified. The analyses
43 support the existence of two concurrent pathways for the spread of LA-MRSA ST398 in Southern
44 Italy: *i*) multiple introductions of LA-MRSA ST398 through the import of colonized pigs from other
45 European countries including Denmark and France and; *ii*) the spread of distinct clones dependent on
46 local trading of pigs between farms. Phylogenetically related Italian and Danish LA-MRSA ST398
47 isolates shared extensive similarities including carriage of antimicrobial resistance genes. Our
48 findings highlight the potential risk of transboundary transmission of antimicrobial-resistant bacterial
49 clones with a high zoonotic potential when importing pigs from countries with high LA-MRSA
50 prevalence.

51 **IMPORTANCE**

52 Over the past decade, livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type
53 398 (LA-MRSA ST398) has spread among pig holdings throughout Europe, in parallel with the
54 increased incidence of infections among humans, especially in intensive pig farming regions. Despite
55 the growing prevalence of LA-MRSA ST398 in Italian pig farms, the transmission dynamics of this
56 clone in Italy remains unclear. This work provides genome-based evidence to suggest transboundary
57 LA-MRSA ST398 transmission through trading of colonized pigs between European countries and
58 Italy, as well as between farms in the same Italian region. Our findings show that both international
59 and local trading of colonised pigs are important factors contributing to the global spread of LA-
60 MRSA ST398 and underscores the need for control measures on and off the farm to reduce the
61 dissemination of this zoonotic pathogen.

62 INTRODUCTION

63 *Staphylococcus aureus* is an opportunistic human pathogen that can cause a variety of diseases,
64 ranging from skin and soft tissue infections to life-threatening invasive infections. Some of these
65 infections are caused by drug-resistant strains, primarily methicillin-resistant *S. aureus* (MRSA).
66 Since the mid-2000s, MRSA clones colonizing livestock animals, the so-called livestock-associated
67 MRSA (LA-MRSA; 1), have emerged. The most common LA-MRSA lineage in the European Union
68 (EU) is sequence type (ST) 398.

69 Since the first EU baseline survey in 2008 (2), an increase in the prevalence of LA-MRSA ST398 has
70 been documented in several EU countries (3-6). Worryingly, this lineage has spread beyond the farm
71 setting, showing increasing prevalence among humans living in high-density livestock production
72 areas (7, 8).

73 The application of high-throughput whole-genome sequencing (WGS) has unveiled potential drivers
74 for LA-MRSA ST398 dissemination, and trading of colonised pigs, contaminated transport vehicles
75 and human carriers have been suggested as potential vectors for both local and transboundary
76 transmission of LA-MRSA (4, 5, 9).

77 Italy is the sixth largest pork producer in the EU (10), and in 2008 two nationwide surveys estimated
78 the prevalence of LA-MRSA ST398 among pig farms in Italy to be 14 to 28% (2, 11). Since then, the
79 prevalence of LA-MRSA ST398 in the Italian pig farming system has steadily increased, especially
80 in Southern Italy where the percentage of positive farms has been estimated to be ca. 60% (6, 12).

81 However, the transmission routes that have caused such a major increase of LA-MRSA ST398
82 prevalence have not been investigated so far.

83 In this study, we have integrated WGS data of LA-MRSA ST398 isolated from pigs farmed in
84 Southern Italy (6, 13) with genome data available in international sequence repositories in order to
85 trace the local and transboundary dissemination dynamics of LA-MRSA ST398.

86 RESULTS

87 **Selection of LA-MRSA ST398 isolates for WGS.** WGS was performed on 67 recently isolated LA-
88 MRSA ST398 strains representative of the major clones, as defined by *spa* typing, circulating among
89 pigs and farm workers in a large area of Southern Italy (Calabria region; 15,222 km²). Fifty-three
90 strains originated from pigs and 14 from farm workers. All of them were isolated in intensive farms
91 during a survey conducted in 2018 (6, 13). Antibiotic-resistance profiles, *spa*- and *SCCmec*-types
92 were previously determined (Table S1 in supplemental material). Isolates were selected according to
93 the criteria described in Materials and Methods.

94 **Phylogeographic context and comparative genomics of LA-MRSA ST398.** Animal movement is
95 considered as a driver for LA-MRSA ST398 spread amongst pig farms (5, 9, 14). To trace the source
96 of recent Italian LA-MRSA ST398 to potential country of origin, the genomes of the 67 LA-MRSA
97 ST398 isolates from Southern Italy were compared with 45 genomes from previously sequenced
98 Italian *spa* type t899-related ST398 isolates (15) and an international reference collection of 89
99 genomes of methicillin-resistant and -susceptible *S. aureus* ST398 (16) including the *S. aureus* ST398
100 reference strain S0385. In total, 201 genomes were investigated to reconstruct phylogenetic
101 relationships based on single-nucleotide polymorphisms (SNPs).

102 After removal of 327 sites in recombinant regions, 6,400 core genome SNPs in the 201 isolates were
103 used to construct a rooted maximum likelihood tree (Fig 1 and Fig. S1 in supplemental material). The
104 analysis revealed a non-uniform distribution of the isolates from Southern Italy, which appeared
105 intermingled throughout the phylogeny and did not cluster according to geographic origin (Fig. 1).
106 Seven groups (A-G in Fig. 1) comprising the recent isolates from Southern Italy with their closest
107 neighbour from the international reference collection (16) were arbitrarily defined. All groups were
108 supported with bootstrap values >90%. Groups A, B, C and F were composed solely of isolates
109 originating from single farms (IDs 01CZ, 05CS, 07KR and 29RC, respectively). Six isolates from
110 farm ID 07KR clustered with isolates from farm ID 18CS (Group D). Groups E and G were composed

111 of isolates originating from three farms (IDs 03CZ, 19RC, 32RC) and two farms (IDs 02CZ and
112 11RC), respectively.

113 The relatedness of isolates from Southern Italy and other countries were examined using the
114 phylogeny (Fig 1). Groups A, B and G did not exhibit any close relationship with isolates from
115 countries other than Italy. Conversely, the closest neighbour(s) of Italian isolates clustering in Groups
116 C, D, E and F originated from different EU countries (16). Group C was related to isolates from
117 Austria and Slovenia, whereas Group D was related to one French isolate. Both groups E and F were
118 closely related to isolates from Denmark.

119 **Evidence of transboundary and local transmission of LA-MRSA ST398.** To investigate any
120 possible transboundary and/or local dissemination of LA-MRSA ST398 via pig movement, farms
121 from which the 67 isolates originated from were inquired about the source of their pigs (Table 1).
122 Three farms (IDs 03CZ, 19RC and 29RC) reported they had purchased animals from Denmark (Table
123 1), which in 2015 was the country with the highest number of pigs imported into Italy (17; Fig. 2),
124 and has experienced a remarkable increase in LA-MRSA ST398 prevalence in pig farming (18). Since
125 the structure of the LA-MRSA ST398 population in Danish pigs has recently been characterized by
126 WGS analysis (5), Denmark was selected as study case to uncover potential LA-MRSA ST398
127 transmission through pig trading to Southern Italy. To this purpose, additional genome data of isolates
128 from Danish pig farms (n=283; ref. 5) were incorporated in the analyses.

129 A total of 484 isolates were included in this analysis, and differed in 6,059 core genome SNPs, after
130 the removal of 591 sites falling into recombination regions. The rooted SNPs-based maximum
131 likelihood tree is shown in Fig. 3 (see also Fig. S2 in supplemental material for additional details).
132 The isolates from the three Italian farms importing animals from Denmark (IDs 03CZ, 19RC and
133 29RC; Table 1) clustered with the prevalent L1 and L3 Danish lineages (5) (Fig 3). Two farms (IDs
134 07KR and 32RC) reported importing pigs from France despite exhibiting a close relatedness with the
135 Danish L2 and L3 lineages.

136 Three unique Italian clusters were defined in Fig. 3; two of them were composed of isolates from
137 farms IDs 01CZ and 05CS (corresponding to Groups A and B in Fig. 1, respectively). Farm ID 01CZ
138 imported animals from Spain, while farm ID 05CS had an autonomous breeding system (Table 1).
139 The third Italian cluster (corresponding to Group D in Fig. 1) was composed of isolates from farms
140 reporting inter-farm trading of pigs (farm ID 07KR sold pigs to farm ID 18CS; ref. 6) (Table 1).
141 Interestingly, farm ID 07KR imported animals from France (Table 1) and the isolate from the
142 international reference collection most closely related to this cluster also originated from France (Fig.
143 3). Lastly, all the t899-related isolates clustered together (t899 cluster, Fig 3), supporting the notion
144 that *spa* type t899 represents a monophyletic entity (15, 16). Within this cluster, all isolates from
145 Southern Italy appeared to be closely related (group G in Fig. 1), originating from two farms importing
146 animals from Northern Italy (IDs 02CZ and 11RC; Table 1).

147 **Distribution of antimicrobial resistance genes.** The distribution of antimicrobial resistance genes
148 among the 67 LA-MRSA ST398 isolates from Southern Italy is reported in Table 2 and Fig. S3 in
149 supplemental material. The previously documented presence of *mecA* and *tet(M)* (6, 13) was
150 confirmed in all isolates. Aminoglycoside resistance genes were present in >80% of the isolates, with
151 *ant(9)-Ia* (syn. *spc*) being the most prevalent, followed by *aac(6')-aph(2'')* and *aadD* (Table 2).
152 Interestingly, the zinc/cadmium resistance gene *cztC* was exclusively detected in isolates carrying
153 SCC*mec* type V (85% of the isolates; Fig. S3 in supplemental material). The *erm(B)* and *erm(C)*
154 genes, conferring resistance to macrolide-lincosamide-streptogramin B (MLS_B), were present in 18%
155 and 19% of the isolates, respectively. Lincosamide resistance was encoded by *lnu(B)* and *lnu(A)* in
156 55.2% and 7.5% of the isolates, respectively. Similarly, trimethoprim resistance was encoded by *dfrG*
157 and *dfrK* in 34.3% and 7.5% of the isolates, respectively.

158 The expansion of the L1, L2 and L3 lineages among Danish LA-MRSA ST398 was suggested to be
159 driven by the use of antimicrobials (5). To investigate the transmission of antimicrobial resistance
160 genes along with pig movement, the antibiotic resistome in Italian and Danish isolates of lineages L1,
161 L2 and L3 was compared (Table 2). Italian isolates clustering within L1, L2 and L3 lineages showed

162 very similar resistance gene patterns as the Danish isolates from the same lineages. When data for all
163 three lineages were combined, significant differences between Italian and Danish isolates were
164 observed for only three genes, namely *ant(9)-Ia* (spectinomycin resistance) was significantly more
165 frequent amongst Italian isolates whereas *aph(6)-Ic* (syn. *str*, streptomycin resistance) and *erm(B)*
166 (macrolide resistance) were more frequent amongst Danish isolates (Table 2).

167 **DISCUSSION**

168 WGS of recent LA-MRSA ST398 isolates from Southern Italy was performed to gain insights into
169 the transmission dynamics within- and between-country. Our findings strongly suggest the existence
170 of two concurrent modes for LA-MRSA ST398 introduction and spread in Southern Italy. The first
171 could be attributed to multiple introductions of LA-MRSA ST398 strains by trading of piglets with
172 other EU countries, including France and Denmark. The second is related to the expansion of
173 independent ST398 clones, especially in farms trading animals with other Italian farms.

174 With 13 million exported animals per year, Denmark is the leader in pig exports to other EU countries
175 (19, 20), and our analysis revealed that Denmark has been the main provider of pigs to Italy since
176 2015. Denmark has experienced a dramatic increase in the prevalence of LA-MRSA ST398 in pig
177 farms, and in 2018 >80% (83-89%) of the conventional pig farms were found positive for MRSA
178 (18). This increase has been linked to the clonal expansion of three dominant lineages (L1, L2 and
179 L3; ref. 5), which have spread beyond farm level, and reported in the Danish food production chain
180 and healthcare facilities (8, 21, 22). Since Denmark is the primary country of origin of pigs imported
181 to Italy, genomic comparison of our isolates to Danish isolates revealed that isolates from Italian
182 farms who reported pig imports from Denmark, clustered within the dominant Danish lineages L1
183 and L3. This supports transmission of Danish LA-MRSA ST398 along with traded pigs to Italy.
184 Interestingly, also farms purchasing animals from France appeared to be colonized by strains
185 belonging to the Danish lineages L2 and L3. It could be hypothesized that Danish lineages L2 and L3
186 have previously spread to France and from there they were imported to Italy. However, only a few
187 French strains were available in the ST398 reference dataset (16), and they were scattered across the
188 phylogeny. Thus, a larger and more recent collection of French isolates, as well as other national
189 collections of LA-MRSA ST398 isolates from Europe should be inspected to confirm our hypothesis.
190 Interestingly, Italian and Danish isolates clustering within the predominant Danish lineages (L1, L2
191 and L3) also shared a high similarity in antibiotic resistance gene carriage, which confer resistance to
192 commonly used antimicrobials in the Danish pig farming system (18). The different frequency of

193 *ant(9)-Ia* (spectinomycin) and *aph(6)-Ic* (streptomycin) gene carriage between Italian and Danish
194 isolates could reflect the different use of aminoglycosides in the two countries. Moreover, recent
195 isolates from Southern Italy showed higher frequency of the zinc/cadmium resistance gene *czrC*
196 (85%) than previously reported (56%, ref. 23). This is consistent with the predominant SCC*mec* type
197 V found amongst the Italian isolates and the extensive use of zinc oxide to prevent post-weaning
198 diarrhoea in pigs (5, 24), that is still common practice in Italy.

199 This study has some limitations. First, despite the evident genetic relatedness between Danish and
200 Italian MRSA isolates, the skewness of the Danish dataset may have caused a bias towards the
201 suggested geographical origin of MRSA in Italian pigs. Second, unique isolates from only 10 farms
202 in a large region with high density of pig farming were analysed, and the number of isolates per farm
203 was not uniform (2-17 isolates). Third, ST398 has been shown to spread through the environment
204 (e.g. water outflow, manure or dust effusion), contaminated fomites or other hubs of dissemination
205 which we cannot exclude. For example, farm ID 19RC not only imported colonized pigs from
206 Denmark, but also employed a colonized farmer (ID 19RC002U) that regularly visited pig farms in
207 Denmark. In this case, the possibility of travel-associated human colonization and/or human-to-pig
208 transmission cannot be ruled out. However, no such links were documented in the other farms. Thus,
209 our observations strongly suggest that local and transboundary transmission of multidrug-resistant
210 ST398 occurred via trading of colonized animals, as already reported in Denmark, Norway and New
211 Zealand (4, 5, 9).

212 In summary, this study sets the ground for future WGS-based epidemiological investigations of LA-
213 MRSA ST398 in Italy. The dissemination of this lineage is known to be facilitated by animal
214 movements, and ST398-positive pigs trading between Italian pig farms and holdings from other EU
215 countries may have contributed to the spread of this lineage. Our findings underscore the need for
216 control measures on and off the farm to reduce the dissemination of this zoonotic pathogen.

217 **MATERIALS AND METHODS**

218 **Selection criteria of LA-MRSA ST398 isolates from Southern Italy.** A total of 67 different isolates
219 from a previous cross-sectional study conducted in the Calabria region during 2018 were selected for
220 *de novo* WGS (6, 13). Isolates were selected according to the following criteria: *i*) belonging to the
221 predominant *spa* types circulating in EU countries, namely t011 (n=45), t034 (n=12) and t899 (n=10)
222 (3, 15, 16, 25, 26); *ii*) originating from farms (n=10) in which LA-MRSA ST398 was isolated from
223 both pigs and farm workers (13), and; *iii*) showing distinct genotypic (i.e. *spa* type, *SCCmec* type)
224 and phenotypic (antimicrobial susceptibility pattern) traits compared with other isolates from the
225 same farm (6, 13) (Table S1 in supplemental material). Therefore, for each farm, isolates displaying
226 identical epidemiological type and antimicrobial susceptibility profile were considered duplicates,
227 and only one representative isolate (either from pig or worker) was selected for WGS (Table S1).

228 **DNA extraction and WGS.** Genomic DNA was extracted by the QIAamp DNA Mini Kit (QIAGEN)
229 according to the manufacturer's instructions, except for the addition of 50 µg/ml lysostaphin (Sigma
230 Aldrich) during the lysis step. DNA libraries were prepared using the Nextera XT DNA Library
231 Preparation Kit (Illumina, San Diego, CA, USA), and WGS was performed using a MiSeq (Illumina)
232 platform with paired-end operating mode (2×250bp).

233 **Prediction of antimicrobial resistance genes.** The ResFinder v.3.2 web-based pipeline at the Centre
234 for Genomic Epidemiology (<http://www.genomicepidemiology.org>) was used to search for the
235 presence of known antibiotic resistance genes using default settings (identity threshold $\geq 90\%$;
236 minimum length $\geq 60\%$; ref. 27). Genomes were screened for the *czrC* gene, encoding resistance to
237 cadmium and zinc, by aligning sequence reads against the reference sequence (GenBank accession
238 no. KF593809).

239 **SNP calling and phylogenetic analysis.** For comparative purposes, 89 *S. aureus* ST398 from a
240 worldwide collection (16), plus 45 Italian t899 and t899-related ST398 isolates (15), and 283 Danish
241 ST398 isolates (5) were included in the phylogenetic analysis. Metadata for all isolates is provided in
242 Dataset S1 in supplemental material.

243 Identification of SNPs was performed with NASP version v.1.0.0 (28) using the GATK Unified
244 Genotyper with filtering set to remove SNPs with less than 10-fold sequencing depth and 90%
245 unambiguous variant calls after duplicated regions of the LA-MRSA ST398 reference chromosome
246 S0385 (GenBank accession no. AM990992; ref. 29) were excluded using NUCmer. SNPs caused by
247 recombination events were identified and removed using Gubbins v.2.3.4 (30) prior to phylogenetic
248 reconstruction using IQ-TREE version v.1.5.5 (31) with the best model found by the implemented
249 ModelFinder and bootstrap analysis using 100 replicates. The tree was rooted according to the
250 outgroup used in ref. 16.

251 **Data availability.** WGS data of 10 isolates from farm ID 01CZ and 32RC have previously been
252 submitted to the NCBI Sequence Read Archive (SRA; available at <https://www.ncbi.nlm.nih.gov/sra>)
253 under BioProject PRJNA546229. WGS data for the remaining 57 isolates have been submitted to the
254 NCBI SRA under BioProject PRJNA607440.

255 **Statistical analysis.** Data analyses were performed using GraphPad Prism v.6.1. Categorical data
256 were compared with two-sided Fisher's exact test. Significance was defined as $P \leq 0.05$.

257 **SUPPLEMENTAL MATERIAL**

258 Supplemental material for this article may be found at [XXX](#).

259 Dataset S1, XLSX file.

260 Figure S1, S2 and S3, and Table S1, PDF file.

261

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303 [N.pdf/a9ddd7db-c40c-48c9-8ed5-a8a90f4faa3f](https://ec.europa.eu/eurostat/documents/3217494/9455154/KS-FK-18-001-EN-N.pdf/a9ddd7db-c40c-48c9-8ed5-a8a90f4faa3f) (accessed: 17.02.2020).
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381 **FIGURE LEGENDS**

382 **FIG 1** Rooted maximum-likelihood phylogeny of 67 recent LA-MRSA ST398 isolates from Southern
383 Italy, 89 *S. aureus* ST398 isolates from the international reference collection (16) and 45 Italian LA-
384 MRSA ST398 t899-related isolates (15). The tree was rooted according to the outgroup used in ref.
385 16. Bootstrap values above 90% are illustrated by filled circles at the end of branches. Coloured filled
386 circles at the tips correspond to farm IDs yielding isolates. The scale bar represents the number of
387 nucleotide substitutions per variable site. A to G represent groups of the recent Italian isolates and
388 their closest neighbour from the international reference collection.

389 **FIG 2** Import data of pigs to Italy by country of origin and year. White dots denote the total import
390 value of pigs per year, expressed in millions of US dollars (\$), with solid black line showing the
391 yearly trend. Data were retrieved from the Observatory of Economic Complexity (17).

392 **FIG 3** Rooted maximum-likelihood phylogeny of 67 recent LA-MRSA ST398 isolates from Southern
393 Italy, 283 LA-MRSA ST398 Danish isolates (5), 89 *S. aureus* ST398 isolates from the international
394 reference collection (16) and 45 Italian LA-MRSA ST398 t899-related isolates (15) (see Fig. S2 in
395 supplemental material for further annotations including bootstrap values). The tree was rooted
396 according to the outgroup used in ref. 16. Coloured filled circles at the tips correspond to farm IDs
397 yielding isolates. The scale bar represents the number of nucleotide substitutions per variable site. IT,
398 Italian cluster; L1, Danish lineage 1; L2, Danish lineage 2; L3, Danish lineage 3; t899, Italian t899
399 lineage.