Molecular characterization of Methicillin-resistant *Staphylococcus aureus* isolated from the pig production chain in Northern Italy

Ancuta Cezara Simon,[1] Valentina Baldo,[2] Nadia Losio,[2] Virginia Filipello,[2] Angelo Colagiorgi,[1] Federico Scali,[2] Emanuela Zanardi,[1] Sergio Ghidini,[1] Adriana Ianieri,[1] Giovanni Loris Alborali[2]

[1]Department of Food and Drug, University of Parma; [2]Lombardy and Emilia Romagna Experimental Zootechnic Institute (IZSLER), Brescia Section, Italy

Abstract

This study aimed to evaluate the molecular characteristics of methicillin resistant *Staphylococcus aureus* (MRSA) isolated in the swine chain in Northern Italy. A sample of 50 fattening units located in Lombardy was selected. Five cutaneous samples at slaughtering and three environmental samples at farm were collected from each unit giving a total of 250 and 150 samples, respectively. A total of 25 MRSA isolates were isolated from 400 samples, in 17 different fattening units. At farm, 12 out of 250 samples were positive for MRSA (4,8 %), and 13 out of 150 samples at slaughter were identified as MRSA (8,7 %), giving an overall incidence among samples of 6,25 % (n = 25). Molecular characterization was carried out using multi-locus sequence typing (MLST) and *spa*-typing. Outcomes showed that most of the isolates belonged to ST398, carrying *spa*-types t899, t011, t18498, t1939, t1200, and t304. Nonetheless, three isolates were identified as ST97 (t1730 and t4795), and one as ST30, showing *spa*-type t318. Furthermore, a novel ST was identified, namely 5422, showing *spa*-type t1730. Heterogeneity in genotypes within the same farm was also found in different fattening units, with concern for the possibility of the exchange of genetic determinants among different lineages. Genetic diversity among MRSA isolates in pig fattening units has been observed, highlighting the possibility that some isolates could be able to infect different hosts, including human.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most commonly identified multidrug resistant pathogen in many parts of the world (Taylor, 2013). The capability of *Staphylococcus aureus* strains to easily adapt to the selective pressure of antimicrobials made it became a threat to public health. In fact, due to the extensive use of methicillin in clinical settings, *S. aureus* evolved and acquired the resistance to this antimicrobial. Resistance to methicillin in *S. aureus* is mediated by the *mec*A gene and its homologue, *mec*C, which are chromosomally located on the mobile genetic element staphylococcal cassette chromosome (SSCmec). The SSCmec codes for a penicillin binding protein (PBP) 2a, with a low affinity for beta-lactams. (Abdelbary, Basset, Blanc, & Feil, 2017).

Initially, MRSA emerged in healthcare settings (Hospital-acquired, HA-MRSA), but more recently it became also able to colonize humans outside hospitals (community-acquired, CA-MRSA) and animals (Livestock-associated, LA-MRSA) (Zarazaga *et al.*, 2017). In the case of food-producing animals, a specific clone (CC398) has been found in several countries, including Austria, Belgium, Canada, Denmark, France, Germany, The Netherlands and Italy (Oniciuc *et al.*, 2017; Sieber *et al.*, 2018).

Pig herds are an important reservoir for MRSA CC398, that has been frequently found also in cattle and poultry (Lassok and Tenhagen, 2013). In pig farms, it has been reported that dust particles and air systems are possible factors for MRSA transmission (Doulgeraki *et al.*, 2016). Although CC398 has been found to colonize animals, only few isolated cases of clinical infections in animals have been reported (Aires-de-Sousa, 2017). MRSA can also occur in slaughterhouses by entering in or on animals, where it can become part of the resident microbiota (Van den Broek *et al.*, 2009). Furthermore, LA-MRSA clones have been found in raw meat products intended for human consumption (Petternel *et al.*, 2014; Zehra *et al.*, 2019).

The aim of this study was to investigate the molecular characteristics of MRSA isolated in pig fattening units in Northern Italy, through multi-locus sequence typing (MLST) and staphylococcal protein A typing (*spa*-typing).

Materials and Methods

Sample collection and phenotypical identification

A total of 50 fattening units located in Lombardy (Northern Italy) was selected, and overall 400 samples were collected. In particular, dust samples were collected from 3 different sites on farm, *i.e.* barn, aeration device and feeder (n = 150 samples), whereas cutaneous swabs were collected on the neck area from 5 animals in each farm at slaughterhouse right after stunning on the neck area (n = 250 samples) following ISO 18593:2018 norm. All samples were stored at 4°C and processed within 6h at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Area of Diagnosis in Brescia.

Briefly, samples were enriched in Brain Hearth Infusion (BHI, Oxoid, Italy) containing 7.5 % NaCl and incubated for 18-24 h at 37°C. Next, 0,1 mL of the resulting broth was plated onto CHROMagar MRSA (bioMérieux, France) and Mannitol Salt Agar (MSA, Oxoid, Italy) as specified in ISO 6888-1:1999 norm. Suspected pink colonies grown on CHROMagar and yellow colonies surrounded by bright yellow zones on MSA were subjected to Gram staining, coagulase and urease tests. Phenotypical identification was performed by miniaturized biochemical system Api Staph (bioMérieux, France).

Molecular characterization

Detection of nuc, mecA and mecC (species confirmation and methicillin resistance)

The detection of *nuc*, *mec*A and *mec*C

Correspondence: Ancuta Cezara Simon, Department of Food and Drug, University of Parma. Via del Taglio, 10 43126 Parma, Italy.
Tel: +39.0521.302754.
E-mail: ancutacezara.simon@studenti.unipr.it

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(*mec*A homologue) was carried out with a multiplex using specific primers as reported by Pichon *et al.* (2012). Briefly, the PCR reaction mix (final volume 20 µL) contained 1X HotStarTaq Master Mix (Qiagen INC, Hilden, Germany), 0.5 µM of each primer, and 1 µL DNA. The thermic profile was 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 1 min. The final elongation step was performed at 72°C for 10 min. The amplified PCR products were distinguished by electrophoresis in a 2.5 % agarose gel (Agarose Multi-Purpose, Roche) 120 V for 40 minutes stained with Eurosafe Nucleic Acid Stain (Euroclone, 1X). 100 bp DNA ladder (Invitrogen, 0.5 µg/µL) was included.

For this work, twenty-five isolates identified as positive for *nuc*, *mec*A and *mec*C genes, were processed for further analysis.

Multilocus Sequence Typing (MLST) and staphylococcal protein A (spa)-typing

MLST analysis was carried out as described by Enright *et al.* (2000). The Sequence Types (STs) were determined with the database available on the *Staphylococcus aureus* MLST website (https://pubmlst.org/saureus) sited at the University of Oxford. For *spa* typing, the *spa* gene was amplified by PCR as described by Shopsin and colleagues (Shopsin *et al.*, 1999) and *spa* types were determined with the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). All DNA sequences were obtained with a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Novel MLST and *spa* sequences were submitted to the respective database for the designation of the new profile.

Phylogenetic analysis of the isolates

The *spa*-typing data from MRSA isolates were analyzed by the BURP (Based Upon Repeat Pattern) algorithm, using the StaphType software v. 2.2.1 (Ridom GmbH, Germany). Neighbor-Joining tree of the isolates was constructed using MEGA 6 (Molecular Evolutionary Genetics Analysis; Tamura *et al.*, 2013).

Results

Overall, 400 samples were collected, 150 from farm environment and 250 from animals at slaughterhouses. For the purposes of this work, a fattening unit was considered positive if MRSA were isolated at any stage from farm and/or from slaughterhouse, without considering the contamination source. A total of 17 out of 50 pig fattening units were positive for MRSA and overall 25 samples were positive for MRSA. At farm, 12 out of 250 samples were positive for MRSA (4,8 %), and 13 out of 150 samples at slaughter were identified as MRSA (8,7 %), giving an overall incidence among samples of 6,25 % (n=25).

The results of the MLST characterization allowed the identification of 5 different Sequence Types (STs). ST398 was the most frequent ST recovered among samples (n=19), followed by ST97 (n=3), ST4894 (n=1) and ST30 (n=1) (Table 1). In addition, a new ST, namely ST5422, was detected in one isolate (Figure 1).

The isolates were also characterized by *spa*-typing, which allowed to identify 9 different profiles (Figure 2). Within the ST398, the most frequent *spa*-type detected was t899 (n=6), followed by t011 (n=5), t18498 (n=4) and t1939 (n=2). *Spa*-types t318 and t034 were detected only in one sample each. Regarding ST97, two *spa*-types were identified: t1730 (n=1) and t4795 (n=2). Other subtypes identified among isolates were ST30/t318, ST4898/t18494, and ST5422/t1730.

Heterogeneity was observed in the *spa* profiles within fattening units. In fact, molecular characterization showed the presence of different *spa*-types and/or STs within the same farm in 6 out of 21 farms

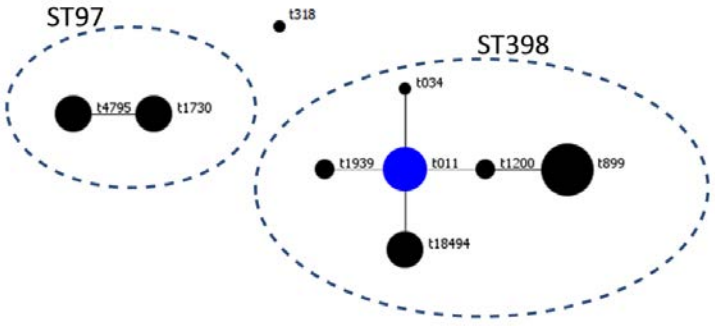

Figure 1. BURP clustering of spa types identified in this study. The spa typing data from *S. aureus* isolates were analyzed by the BURP algorithm (StaphType software v. 2.2.1, Ridom). Each spa type identified is depicted with a circle. The size of the circle is proportional to the frequency of the spa type in the population. Related *spa* types are connected with a black line. A blue circle corresponds to the clonal complex founder.

Table 1. Spa-types and MLST profiles of MRSA isolates.

Spa-type	ST	*n* farms where it was detected	% among positive farms	% farms surveyed
t899	398	5	29	10
t011	398	5	29	10
t18494	398	3	18	6
t1200	398	2	12	4
t1939	398	2	12	4
t4795	97	1	6	2
t1730	97	1	6	2
t1730	5422	1	6	2
t318	30	1	6	2
t18494	4894	1	6	2
t034	398	1	6	2

(Table 2). Results from BURP analysis showed that five farms were positive for MRSA presenting unrelated *spa*-types, whereas relatedness has been found among the strains isolated from the remaining unit (Figure 1, Table 2).

Discussion

Pig herds are an important reservoir for MRSA, with ST398 representing the most frequently reported subtype at European level in swine production chain (EFSA, 2009a; Battisti *et al.*, 2010; Parisi *et al.*, 2019). Although pig colonization with MRSA ST398 is frequent, infection in swine is rare. Nonetheless, it has been observed that such MRSA are able to colonize and cause infection in other species, including human, especially in areas with intense livestock-farming (Pan *et al.*, 2009; Van Cleef *et al.*, 2010). Although people professionally exposed to animals (owners, farmers, veterinarians and abattoir workers) are more likely to be colonized by MRSA ST398, colonization may also occur in family members not in direct contact with pigs (Cuny *et al.*, 2009; Aires-de-Sousa, 2017).

In this work, it has been observed that the 42 % of the sampled fattening units were positive for MRSA. Such prevalence is higher than the MRSA level in European production holdings reported by European Food Safety Authority (26.9 %; EFSA, 2009), but results are in line with previous findings that have reported different levels of MRSA prevalence in Italy, ranging from 33.9 % to 64.7 % (EFSA, 2009a; Parisi *et al.*, 2019).

In order to investigate the genetic diversity of the MRSA isolates, molecular characterization by MLST and *spa*-typing was performed. Similarly to many other studies, the LA-MRSA ST398 was the ST most frequently recovered among pig farms (EFSA, 2009a; Battisti *et al.*, 2010; Parisi *et al.*, 2019). Within ST398, *spa*-typing revealed the presence of 6 different profiles, of which *spa*-type t899 was the most prevalent (57.1 %), which is the fifth most common genotype throughout Europe, following *spa*-types t011, t108, t034 and t127 (EFSA, 2009a). Among the other identified profiles, the *spa*-type t011, that is the most diffused in Europe (EFSA, 2009a), was found in the 23.8 % of MRSA ST398. *Spa*-types t034 and t1939 were found in two and one isolates, respectively, in line with the European levels (EFSA, 2009a). To the best of authors' knowledge, *spa*-type t1200 has never been reported in scientific literature as recovered from pigs. Conversely, isolates presenting such *spa*-type have been collected from infection sites in hospital settings in Saudi Arabia, and thus t1200 is considered as HA lineage, raising concern for public health (Alkharsah *et al.*, 2019). Finally, a new *spa*-type, namely t18494, has been found in three isolates belonging to ST398. This profile was also identified in one other isolate carrying ST4894.

Two *spa*-types belonging to the LA-MRSA lineage ST97 (i.e., t1730 and t4795) were recovered in three samples from two different farms. Both *spa*-types were previously reported by Locatelli *et al.* (2017) in samples collected from dairy cattle herds, swine farms related to dairy herds, and humans in contact with herds, suggesting that these genotypes could be transmitted from animals to humans and vice versa. Furthermore, an isolate belonging to ST30/t318 was reported for the first time in Italian pig farms with this work, although ST30 carrying other *spa*-types (*i.e.*, t012 and t093) were isolated from swabs collected in slaughterhouses located in Southern Italy (Normanno *et al.*, 2015). Isolates belonging to ST30 have been described as CA-MRSA; in particular, ST30/t318 has been reported to be responsible for abscesses, bloodstream or necrotic infections in different parts of the world (*e.g.,* Czech Republic, China) (Jiang *et al.*, 2013; Rájová *et al.*, 2016), and the presence of an isolate collected from farm

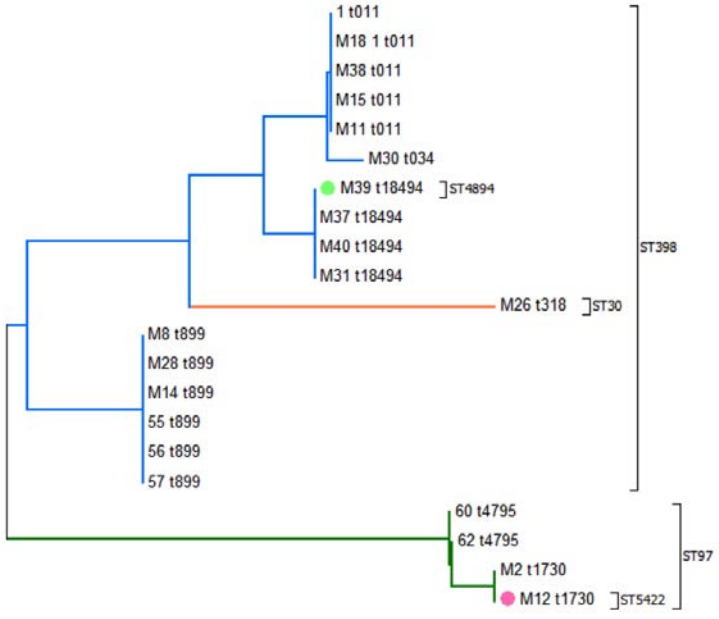

Figure 2. Neighbor-Joining tree of the isolates included in this study. Different colors correspond to the Sequence Types (STs). The analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

Table 2. Molecular pattern distribution among farms with more than one spa-type or ST detected.

Farm code	*n spa*-types	*spa*-types	*spa*-type relatedness	STs
1	2	t1730, t899	unrelated	5422, 398
2	2	t1939, t034	unrelated	398
3	2	t18494, t011	related	398
4	2	t1730, t011	unrelated	97, 398
5	2	t18494, t1200	unrelated	398
6	1	t18494	unrelated	398, 4894

belonging to this lineage is of concern for the health of people coming into contact with animals. Finally, the molecular characterization allowed to identify an isolate belonging to a novel ST, further characterized as ST5422, showing *spa*-type t1730. The presence of different *spa*-types and/or STs within the same pig farm was observed in 6 out of 50 fattening units (Table 2). Unrelated *spa*-types (up to three *spa*-types) either belonging to ST398, ST97, ST4894 or ST5422 have been found within a same farm (Figure 1, Table 2).

It is not clear what the presence of different genotypes in a fattening unit is related to, although in other works it has been found that genetic variability was due to mutations in the strains already present in the farm (*e.g.* deletion of one repeat in *spa*-type t18498 results in *spa* type t034, and the deletion of one repeat in t899 results in t1939) or to the introduction of new strains by other animals or humans visiting the farms (van Duijkeren *et al.*, 2008; Verhegghe *et al.*, 2013). The presence of different MRSA genotypes within farms could favor the exchange of virulence determinants among different lineages (van Duijkeren *et al.*, 2008; Verhegghe *et al.*, 2013), with potential implications for both animal and human health.

The reduction of MRSA animal-human transmission is possible through the use and the implementation of management practices aimed at increasing on-farm biosecurity. Also the adoption and the improvement of measures such as good husbandry practices, Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP) on farms, in slaughterhouses and in food production areas are useful in controlling the spread of bacteria (EFSA, 2009b), leading to a reduction of related health risks and to a higher microbiological quality and safety of swine products. In fact, a serious concern is related to the occurrence of foodborne transmission of MRSA, since MRSA presence in pork meat and meat products has been reported by different authors (Petternel *et al.*, 2014; Tang *et al.*, 2017). The ST398 lineage is the most commonly found among the foodborne-associated MRSA, including some of the genotypes identified in this work, such as the *spa*-types t011 and t034 (EFSA, 2009b; Petternel *et al.*, 2014; Tang *et al.*, 2017).

These findings stress once more that practical measures should be taken within pig farms to reduce the spread of MRSA.

Conclusions

Although ST398 colonization in humans is mostly related to professional exposure, in some cases it can cause severe infections (Pan *et al.*, 2009). Furthermore, other molecular profiles have been found among the isolates, such as the CA-MRSA ST30, that is also responsible for different infection in humans. The results of this work highlight the necessity of monitoring both the community and the animal reservoirs, and of adopting and implementing measures able to control the spread of MRSA among animals, in order to reduce zoonotic transmission of this pathogen by direct or indirect human-animal contact and through the consumption of contaminated foodstuffs.

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