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

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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 slaughter 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 of 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 mecC, which 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 et al., 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).

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 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 mecA gene and its homologue, mecC, 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).

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, mecA and mecC...
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/t138, 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 is depicted 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               |
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 (Alkharshash 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).](image-url)
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 the 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 t1899 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 spread the control 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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