PAPER

PREVALENCE AND CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATES FROM RETAIL MEAT IN SOUTH ITALY

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ABSTRACT

This study aimed to estimate the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) from 500 retail meat products in South Italy from June 2016 to June 2018, including 150 raw bovine, 120 pork, 150 chicken and 80 horse meat.

After bacteriological analysis, 12 (2.4%) samples tested positive for MRSA. Isolates were characterized by antimicrobial susceptibility, *spa* typing and MLST. MRSA were also investigated by PCR for the presence of enterotoxins, *lukF-PV-lukS-PV* and *icaA-icaD* genes. Antimicrobial susceptibility testing showed that MRSA isolates were multidrug resistant. One strain harboured PVL-encoding genes (8.3%). Seven MRSA isolates of 12 (58.3%) carried *seh* enterotoxin encoding gene. The *icaA* and *icaD* genes were both present in 10 isolates (83.3%).

MRSA isolates in retail meat may serve as a potential source of exposure to MRSA for humans and monitoring of food-producing animals and hygiene standards should be strictly and carefully considered throughout the entire meat chain to ensure food safety.

Keywords: biofilm, food safety, Methicillin-resistant *Staphylococcus aureus*, Panton Valentine Leukocidin, retail meat

1. INTRODUCTION

Staphylococcus aureus is considered as one of the major foodborne pathogens and is responsible for a wide spectrum of infections worldwide (Wu *et al*., 2018). Methicillin-resistant *S. aureus* (MRSA) poses a public health issue because of its multiple antimicrobial resistance and data on the occurrence of MRSA in food-producing animals and food is underestimated as the report is currently voluntary (EFSA and ECDC, 2019).
Traditionally, MRSA strains are distinguished into two distinct epidemiological groups, hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA) (Tang *et al*., 2017). CA-MRSA frequently harbour staphylococcal cassette chromosome *mec* (SCC*mec*) types IV or V and the genes *lukS-PV-lukF-PV* encoding the subunits of the Panton-Valentine leucocidin, a cytotoxin that causes leukocyte lysis or apoptosis via pore formation (BOYLE-VAVRA and DAUM, 2007). HA-MRSA typically possess larger-size SCC*mec* types I, II or III and often exhibit resistance to multiple classes of antimicrobial agents strains (Shore *et al*., 2014). A third group of MRSA, known as livestock-associated MRSA (LA-MRSA), has recently been identified and it infects livestock and companion animals, as well as some other farm animal species and wild animals. LA-MRSA have mainly SCC*mec* types IVa or V, although, non-typeable cassettes and SCC*mec* type XI have also been found (BUTAYE *et al*., 2016).
Methicillin resistance is primarily attributed to the altered penicillin binding protein (PBP2a), encoded in the *mecA* gene, which has a reduced affinity for β-lactam antibiotics. Recently, a homolog of the *mecA*, *mecC* ($mecA_{LGA251}$) was identified in MRSA strains from humans and livestock that were phenotypically resistant to methicillin, but tested negative for the *mecA* gene. The *mecC* gene shares about 70% nucleotide homology with *mecA* and is located in SCC*mec* XI (Velasco *et al*., 2015).
The *nuc* gene is considered a marker for the detection of *S. aureus* and encodes for a thermostable nuclease (COSTA *et al*., 2005).
S. aureus has the ability to form biofilms on various materials and surfaces. Biofilms in the food industry can cause serious hygienic problems as the bacteria could adhere to the food contact surfaces and contaminate foodstuffs (RODE *et al*., 2007).
The mechanism of biofilm formation is promoted by *ica* locus containing four genes, namely *icaA*, *icaB*, *icaC*, *icaD*. The product of *ica* locus is the polysaccharide intracellular adhesin (PIA), that mediates intercellular aggregation of bacterial cells. PIA was found to be the main exopolysaccharide component of the staphylococcal biofilm (ARCIOLA *et al*., 2015). The *icaA* gene encodes for a transmembrane enzyme, N-acetylglucosaminyltransferase that contribute to the synthesis of the poly-N-acetylglucosamine polymer and requires *icaD* for full functioning (CIFTCI *et al*., 2009).
In recent years, MRSA isolation from fresh retail meat has been reported in U.S.A., Saudi Arabia, Korea, Denmark, Finland, Germany, Spain and Switzerland (EFSA and ECDC, 2019; GE *et al*., 2017; KIM *et al*., 2015; TANG *et al*., 2017), suggesting that these products may pose a potential risk for MRSA transmission to humans (BUYUKCANGAZ *et al*., 2013).
To the best of our knowledge, there is little data available on the prevalence of MRSA contamination in fresh meats sold at retail prices in Italy.
Genotyping of *S. aureus* isolated from retail meat is an important tool in epidemiological studies of infection and contributes to better understanding of the pathogen's dissemination. Several molecular methods have been developed for typing *S. aureus* isolates, such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing

(MLST) and *spa* typing (STROMMENGER *et al*., 2006; ENRIGHT *et al*., 2000; BANNERMAN *et al*., 1995).
The aims of this study were to evaluate the prevalence of MRSA in fresh meat samples sold at retail prices in southern Italy and investigate the molecular characteristics of MRSA isolates as regards some virulence-associated genes, and antimicrobial resistance profiling for epidemiological studies and risk assessment purposes in the "One Health" perspective.

2. MATERIALS AND METHODS

2.1. Isolation and identification of MRSA

A total of 500 fresh meat samples, over a two-year period (June 2016-June 2018), were collected at retail markets by local health officials, and transported to the laboratories of the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (IZS PB) and analysed for the detection of *S. aureus*. These samples comprised 150 raw bovine, 120 pork, 150 chicken, and 80 horse meat samples.
Isolation and identification of *S. aureus* were performed according to EN ISO 6888 1-2 1999. Presumptive *S. aureus* colonies (black colonies with a zone of clearing of the medium) were identified by conventional biochemical methods and plated onto blood agar. After 18-24 h of incubation at 37°C, *S. aureus* isolates were subcultured on CHROMagar™ MRSA (CHROMagar, Paris, France). DNA was extracted from an isolated bacterial colony using the InstaGene Matrix™ (Bio-Rad, Segrate (MI), Italy), following the manufacturer's instructions. All *S. aureus* isolates were screened by multiplex PCR for 16S rRNA (MONDAY and BOHACH, 1999), *nuc* (COSTA *et al*., 2005) and *mecA*/*mecC* (GARCÍA-ÁLVAREZ *et al*., 2011) genes in order to confirm *S. aureus* species and to detect methicillin resistance. Confirmed MRSA isolates (one strain per sample) were further characterized and tested for antimicrobial susceptibility.

2.2. In vitro antimicrobial susceptibility

Antimicrobial susceptibility of MRSA isolates was determined by disc diffusion method according to the guidelines of Clinical Laboratory Standards Institute (2013). A total of eleven antibiotics were included: penicillin (10 units), oxacillin (1 μg), cefoxitin (30 μg), cephalothin (30 μg), gentamicin (10 μg), kanamycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (25 μg), chloramphenicol (30 μg), enrofloxacin (5 μg). The MIC of teicoplanin of the MRSA isolates was determined by Etest® (bioMérieux Italia Spa, Bagno a Ripoli (FI) Italy), following CLSI interpretative breakpoints (2017). *S. aureus* ATCC 25923 was included for quality control.

2.3. Genotyping

The polymorphic X region of the protein A gene (*spa* typing) was amplified according to a published protocol (STROMMENGER *et al*., 2006). Amplification of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqi*L) by Multilocus sequence typing (MLST) was performed as described by ENRIGHT (2000). The DNA sequences were submitted to the *Staphylococcus* MLST database (http://saureus.mlst.net/) to obtain the allelic profiles of MRSA strains.

2.4. SCC*mec* typing and detection of enterotoxins, *lukF-PV-lukS-PV* and *icaA-icaD* genes

SCC*mec* elements were typed by multiplex PCR as previously described (GARCÍA-ÁLVAREZ *et al*., 2011; KONDO *et al*., 2007). The SCC*mec* type IV was subtyped according to ZHANG (2012).
MRSA isolates were screened by three multiplex PCR for the detection of 12 genes encoding staphylococcal enterotoxins (for *sea* to *see*, for *seg* to *sej*, for *sem* to *seo*), using twelve specific primer sets as previously described (BOEREMA *et al*., 2006; JARRAUD *et al*., 2002; LØVSETH *et al*., 2004; MONDAY and BOHACH, 1999; ROSEC and GIGAUD, 2002). All MRSA strains were subjected to a PCR assay to test the presence of *lukF-PV-lukS-PV* and *icaA-icaD* genes encoding respectively, Panton-Valentine Leukocidin (PVL) and polysaccharide intercellular adhesin (PIA), as described elsewhere (HESJE *et al*., 2011; ZMANTAR *et al*., 2008). Furthermore, the ability of the MRSA strains to form biofilm was tested using a semi-quantitative adherence assay by microtiter plate (MTP) according to ZMANTAR (2010). The assay recorded the optical density (OD) at 570nm of adherent biofilm after incubation for 24 h at 37°C. Biofilm formation was classified as highly positive ($OD_{570} \geq 1$), low grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$).

3. RESULTS AND DISCUSSION

Methicillin-resistant *Staphylococcus aureus* is a public health concern and food contaminated by MRSA may serve as a potential vehicle for transmission to humans (EFSA, 2009). This study reports on the prevalence of MRSA isolated from fresh meat and sold at retail prices as well as the characterization of some virulence-associated genes and antimicrobial resistance profiling. In this survey, among 500 retail fresh meat samples subjected to bacteriological analysis, 72 (14.4%; 72/500) tested positive for *S. aureus* and 12 (2.4%; 12/500) for MRSA. Total *S. aureus* counts performed using standard microbiological procedures were below 10^3 colony forming units per gram (CFU/g) in all tested samples. All MRSA isolates carried the *mecA* gene and none carried the *mecC* gene. Several surveys have estimated the prevalence of MRSA in retail meat worldwide. In a study from U.S.A., GE *et al*. (2017) found that 27.9% of the retail meats examined was contaminated by *S. aureus* and 1.9% tested positive for MRSA. Another study from U.K. recovered MRSA from 7.3% of retail meat samples (FOX *et al*., 2016). Previous studies conducted in Italy reported a contamination rate of meat product samples of 10% and 0.5%, but no MRSA strain was found among the isolates (NORMANNO *et al*., 2007a; NORMANNO *et al*., 2007b; TRAVERSA *et al*., 2015). Probably, these differences in the prevalence of MRSA in retail meats could be due to the different geographical area, sampling and collection period.
In this report, most MRSA isolates (5/12; 41.7%) were t127/ST1/SCC*mec* type IVa, *seh* positive and PVL-negative. The 16.7% (2/12) of the isolates was t174/ST1/SCC*mec* type IVa, SEs and negative PVL genes. Other recovered MRSA strains were: t386/ST1/SCC*mec* type IVa (1/12; 8.3%) and t599/ST1/SCC*mec* type IVa (1/12; 8.3%), both *seh* positive and PVL-negative, t044/ST80/SCC*mec*type IVc (1/12; 8.3%), SEs negative and PVL positive, ST97 t1236/ST97 (1/12; 8.3%) and t899/ST398 SCC*mec* type V, both SEs and PVL-negative (1/12; 8.3%) (Table 1). ST1 is a clone frequently implicated in human infections and spa-type 127 is the prevalent clone involved in cases of invasive MRSA infections in Europe (MONACO *et al*., 2013). MRSA t127/ST1 was also found in cows, sheep, goats and pigs in

Italy and other European countries (AGERSØ *et al*., 2012; ALBA *et al*., 2015; PAPADOPOULOS *et al*., 2018). MRSA t127/ST1 clone is often detected in Italian pig industry and the presence of a pig reservoir from this lineage has been hypothesized (ALBA *et al*., 2015; FRANCO *et al*., 2011). Moreover, *seh* gene is considered to be constitutive of ST1, independently from the host origin (MONECKE *et al*., 2011). MRSA with genotype t044/ST80/SCC*mec*type IVc PVL-positive belongs to CC80 and lacks enterotoxin genes. First recognized in Denmark in 1993, now it is widely spread throughout Europe, North Africa, sub-Saharan Africa and the Middle East (MONECKE *et al*., 2011). It is mainly associated with skin infections in the community, but rarely causes invasive infections (DAVID *et al*., 2010). The presence of this clone in retail horse meat underlies the spread of this MRSA-ST80 clone. Indeed, there are studies from various countries about nosocomial infections in horses due to MRSA causing a variety of infections (CUNY *et al*., 2017; ISLAM *et al*., 2017; STEINMAN *et al*., 2015). In this survey, only one isolate (1/12; 8.3%) harboured PVL-encoding genes. The finding of MRSA ST80/t044 PVL-positive suggests human handlers as potential source of contamination of meat, with PVL being a marker of CA-MRSA. Another genotype recovered in this study from bovine retail meat was MRSA t1236/ST97. In this isolate, SCC*mec* was not detected. Other studies reported this genotype to be associated with sheep, goats and cows as methicillin-susceptible *S. aureus* (FELTRIN *et al*., 2016; PORRERO *et al*., 2012). ST97 (CC97) is generally responsible for bovine mastitis. Less commonly, it was found in small ruminants, pigs, and humans. This clonal complex is the second most prevalent MRSA lineage in pig finishing holdings in Italy and one of the *S. aureus* lineages associated with cattle, particularly with bovine mastitis (FELTRIN *et al*., 2016). The finding of MRSA strains in raw fresh meat should be considered carefully since meat may expose humans to this microorganism. It would be desirable monitoring health status of animals and implement control measures for breeding and slaughtering to avoid contaminations of their meat by *S. aureus*. MRSA t899/ST398 SCC*mec* type V PVL-negative is considered an important livestock-associated (LA)-MRSA present in pigs, poultry, calves, companion animals, horses and other farm animal species in many countries. This clone was found in retail chicken meat in England (FOX *et al*., 2016), in Germany (KRAUSHAAR *et al*., 2017) and China (WANG *et al*., 2014). LA-MRSA may also pose an occupational risk for those people in close contact with livestock and their derived carcasses, especially pig farmers, cattle farmers, poultry farmers, slaughterhouse workers and veterinarians (HADJIRIN *et al*., 2015).

These persons are more likely to be colonized with MRSA and spread the microorganism in the community. Hence, retail meat may be a route for transmission of CA-MRSA (e.g. MRSA ST1-t127) and LA-MRSA to humans.

Several foods are implicated in Staphylococcal food poisoning (SFP) such as raw meat, sausages, raw milk and raw milk cheese, in which contamination could be due to animal carriage or to infections of animal origin. Colonized food handlers, rather than animals, are likely sources of contamination after heat treatment of the food (BASANISI *et al*., 2017). The emetic activity of enterotoxins has been demonstrated only for SEA, SEB, SEC, SED, and SEE (JOHLER *et al*., 2015). The 58.3% (7/12) of the MRSA isolated in this study harboured *seh* gene, but in literature, there is little data available on the prevalence of MRSA in SFP (Table 1) (JØRGENSEN *et al*. 2005; and OSTYN *et al*. 2012). Nevertheless, the risk of human infection cannot be ignored. Consequently, more attention should be paid during food handling and storage in order to reduce the potential role of food in the dissemination of successful MRSA lineages.

Biofilm matrix is considered to be a significant virulence factor because when growing in this mode of life, microorganisms become more tolerant to antimicrobial agents and extremely difficult to eradicate. In this survey, the 83.3% (10/12) of MRSA isolates carried both the *icaA* and *icaD* genes; the16.7% (2/12) of the isolates harboured only *icaD* gene (Table 1). As regard the ability to form biofilm, the MRSA strains were biofilm producers with MTP method, although, production level varied. Among these, 83.3% of isolates (10/12) were low grade biofilm positive and 16.7% (2/12) were strongly biofilm producers (Table 1). These strains could be of concern for the meat industry since bacteria in biofilms can be resistant to the normal disinfection and prophylaxis methods. Special attention should be paid to hygiene procedures in farms and food facility.

Table 1. Antimicrobial resistance profiles, genotypic characteristics and virulence-associated genes of the 12 MRSA isolates analyzed in this study.

N°	Source of meat	Resistance to*:	SCC*mec*	*spa* type	MLST	*SEs*	*lukF-PV/lukS-PV*	*icaA*	*icaD*	#OD_{570}
1	Horse	P, OX, FOX, KF, K, E, TE	Iva	t174	ST1	-	-	-	+	++
2	Horse	P, OX, FOX, KF, K, E, TE	Iva	t174	ST1	-	-	-	+	++
3	Horse	P, OX, FOX, KF, CN, K, E, ENR	IVc	t044	ST80	-	+	+	+	++++
4	Bovine	P, OX, FOX, KF, E, TE	ND	t1236	ST97	-	-	+	+	++++
5	Bovine	P, OX, FOX, K, TE, KF, STX	Iva	t127	ST1	*seh*	-	+	+	++
6	Bovine	P, OX, FOX, K, TE, KF, STX	Iva	t386	ST1	*seh*	-	+	+	++
7	Pork	P, OX, FOX, CN, K, E, KF, TE	Iva	t127	ST1	*seh*	-	+	+	++
8	Pork	P, OX, FOX, CN, K, E, KF, TE	Iva	t127	ST1	*seh*	-	+	+	++
9	Pork	P, OX, FOX, CN, K, E, KF, TE	Iva	t127	ST1	*seh*	-	+	+	++
10	Pork	P, OX, FOX, CN, K, E, KF, TE	Iva	t127	ST1	*seh*	-	+	+	++
11	Pork	P, OX, FOX, K, E, TE, KF	Iva	t599	ST1	*seh*	-	+	+	++
12	Chicken	P, OX, FOX, TE, KF, STX	V	t899	ST398	-	-	+	+	++

*Antibiotic abbreviations: P, penicillin; OX, oxacillin; FOX, cefoxitin; KF cephalothin; CN, gentamicin; K, kanamycin; E, erythromycin; TE, tetracicline; ENR, enrofloxacin; STX, trimethoprim-sulfamethoxazole. #Strongly biofilm positive (++++), low grade biofilm positive (++).

Global consumption of antimicrobials has increased worldwide causing the development of resistance to several antimicrobial agents in bacteria and this might result to serious problems. In this study, MRSA isolates were resistant to penicillin, oxacillin, cefoxitin, cephalothin followed by tetracycline (11/12; 91.7%), kanamycin (10/12; 83.3%), erythromycin (8/12; 66.7%), gentamicin (5/12; 41.7%), trimethoprim-sulfamethoxazole (3/12; 25%), enrofloxacin (1/12; 8.3%). The MIC value of teicoplanin was ≤ 1.5 μg/ml for all MRSA isolates (Table 1). Multidrug resistance in retail meat was also observed in other studies (JACKSON *et al.*, 2013; TANG *et al.*, 2017). Aminoglicosides, macrolides,

penicillins, and tetracyclines are some of the classes of antimicrobial agents extremely important for veterinary medicine considering the wide range of applications and diseases to be treated (WENDLANDT *et al*., 2015). Therefore, controlling the use of antibiotics in farming could limit the risk of transmission of multidrug resistant pathogens among animals and potentially to humans through the food chain.

4. CONCLUSIONS

In this study, raw bovine, pork, chicken and horse meat samples were positive for MRSA, although, the level of prevalence was low and varied between meats of different origin. The data obtained from this survey suggest that the presence of MRSA in fresh retail meats could be the result of human contamination due to colonized food handlers or cross-contamination of carcasses during food processing. Furthermore, the most MRSA clonal complexes found in the present survey are responsible for community infections, suggesting that food may contribute to the spread of MRSA in the environment. Further studies should be designed to collect more exhaustive data on the prevalence and evolution of these pathogens.
In conclusion, monitoring of food-producing animals and strict hygienic standards should be carefully considered throughout the entire meat chain, from primary production to retail in order to prevent or reduce the transmission of multidrug resistant pathogens to consumers from the "One Health" perspective.

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