Phenotypic and genotypic study on antibiotic resistance and pathogenic factors of *Staphylococcus aureus* isolates from small ruminant mastitis milk in South of Italy (Sicily)

Alessio Parco,[1] Giusi Macaluso,[1] Maria Foti,[2] Maria Vitale,[1] Vittorio Fisichella,[2] Marco Tolone,[3] Guido Ruggero Loria[1]

[1]Institute for Experimental Veterinary Medicine of Sicily, "A. Mirri" Palermo; [2]University of Messina; [3]University of Palermo, Italy

Abstract

Staphyloccoccus aureus is the major cause of mastitis in small ruminants in the Mediterranean farms causing severe losses to dairy industry. Antibiotic treatment has been the most common approach to control these infections. Aim of this study was to investigate antimicrobial resistance (AMR), virulence factors and biofilm-related genes of 84 Sicilian strains of *S. aureus* isolated from sheep and goats milk during two different periods δT_1 (2006-2009) and δT_2 (2013-2015). Kirby Bauer method and Polymerase Chain Reaction (PCR) were utilized to monitor AMR and related genes (*mecA, tetK, tetM, ermA, ermC*). Moreover, toxin genes (*tsst-1, sea-see, seg-sej*, and *sep*) and biofilm genes (*bap, ica, sasC*) were studied. Twenty-six isolates (30.9%) showed multidrug resistance. The two groups showed similar results with exception for higher values of resistance for tilmicosin and lower for sulfamethoxazole and vancomycin of the second group. *MecA* gene was detected in one isolate. Tetracycline resistance was higher than 20%, with an increase in δT_2 group. Toxin genes were found in 5 isolates (5.9%), belonging of δT_2 group, while 57 of isolates (67.8%) showed biofilm related genes. The high presence of multi-resistant isolates suggests the need of more responsible use of antibiotic therapy for the control of these infections.

Introduction

Sheep and goat farming still represents a valuable opportunity for labour and development of the Mediterranean rural areas of Sicily, Italy. Data from Italian Livestock Register belonging to the Veterinary data base of Ministry of Health, show, on the year 2019, that small ruminant sector in Sicily is growing (8.84%) with a total number of 800,000 heads distributed in 11,803 farms (Anagrafe Nazionale Zootecnica, 2019). To date the main problems for sheep and goat farming are still represented by parasitosis and mastitis. Clinical and more often, subclinical intramammary infections (IMI) in sheep and goats are mainly caused by coagulase-negative staphylococci (CNS) and *Staphylococcus aureus.* IMI caused by *S. aureus* warrant special attention because this aggressive, environmental bacterium is responsible for both acute-hyperacute clinical mastitis (gangrenous mastitis) and subclinical syndromes. *S. aureus* is a Gram-positive coccal bacterium belonging to the Staphylococcaceae family. Normally it is considered, opportunistic pathogen but in the case of mastitis it often becomes highly contagious causing a rapid spread through the flock, characterized by high morbidity. The virulence of *S. aureus* depends on the production of several different factors, such as hemolysins, leukocidins, proteases, and toxins contributing to its pathogenicity. (Some *S. aureus* strains produce toxins, such as superantigen staphylococcal toxic shock syndrome toxin (*tsst-1*), staphylococcal enterotoxins (SEs), or enterotoxin-like proteins (SEl) (Balaban and Rasooly, 2000). Another important virulence factor is related to the ability of some staphylococci to produce biofilms, which affect antibiotic concentrations, allowing for bacterial multiplications within the biofilm population and increasing the chances of its survival within the host (Melchior *et al*., 2006). Several mechanisms such as interaction of antimicrobials with biofilm matrix components, reduced growth rates, the presence of metabolic different subpopulations are responsible for the major resistance of biofilm to antimicrobial (Hall *et al*., 2017). Their formation is a multifactorial event, controlled by quorum sensing and, several proteins, such as the accessory gene regulator protein (agr), the biofilm-associated protein (bap), the intercellular adhesion protein (ica), and the *S. aureus* surface protein (SasC) (Vitale *et al*., 2015). The *ica* operon is responsible for the synthesis of one of them, the intercellular adhesion protein that is an important component of the staphylococcal biofilm (Fitzpatrick *et al*., 2005). In the control of ovine staphylococcal mastitis, antimicrobial therapy continues to play a significant role in limiting the infection spread and moreover to prevent animal death. Antibiotic must be utilized for treatment of the first clinical cases in order to stop the excretion of the pathogen within the whole flock and to avoid contamination of the environment and/or milking machine. Nevertheless, they are too expensive for general treatment of the farm/herd whereas vaccine including autologous vaccine could be the most sustainable way to control the infection on long term. Antibiotic resistance of *S. aureus* isolates from cases of ovine mastitis has been previously described (Azara *et al*., 2017). Since the discovery of penicillin and related antibiotic therapy, *S. aureus* was one of the species which after only 5 years acquired the ability to grow in the presence of this antibiotic due to β-lactamase enzyme, coded at its plasmid level (Pitkala *et al*., 2007). Methicillin resistance was first reported by Jevons (1961) who detected only three methicillin-resistant *S. aureus* strains (MRSA) on a screening of 5,440 isolates. In the last years there was increasing attention due to the emergence of livestock-associated methicillin-resistant

Correspondence: Giusi Macaluso, Institute for Experimental Veterinary Medicine of Sicily, via Gino Marinuzzi 3, 90129 Palermo, Italy.
Tel.: +393497641520.
E-mail: giusi.macaluso@izssicilia.it

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strains (LA-MRSA) (Guardabassi *et al*., 2013). High-level resistance to methicillin or other β-lactam antimicrobials in *S. aureus* strains is conferred by the *mecA* gene (Peacock and Paterson, 2015). Staphylococcal resistance to other antimicrobials such as cephalosporins, tetracyclines, erythromycin, lincomycin and kanamycin have also been recently reported (Jones *et al*., 2006; Guo *et al*., 2020; Karaman *et al*., 2020). High costs of modern antibiotics do not always guarantee the expected results, but they are still the only chance to stop clinical disease and moreover to avoid a massive pathogen spread in the flock. All bactericidal antibiotics could be very useful to sterilize the clinical cases at the beginning of the infection and associating proper biosecurity practices in the farm to stop the contagion. On the other hand, the use of antibiotic classified as "bacteriostatic" such as tetracyclines and macrolides, could be helpful in recovering clinical signs for a while, but after few weeks may concern the risk of a reoccurrence of the outbreak in the farm. The prolonged use of these bacteriostatic products may also drive to drug-resistance phenomena. Recently, the risk of antibiotic failure has increasingly emphasized the importance of targeted, specific treatments by identifying the most effective product to stop/resolve the outbreak. The aim of this study is to analyse the antibiotic resistance profile of *S. aureus* isolates collected from ovine milk samples in Sicily from 2006 to 2015 and to provide a contribution by updating data on mastitis antibiotic treatments in small ruminants. In addition, the isolates were studied for the presence of toxin genes and biofilm-related genes.

Materials and methods

Sampling, isolation, and characterization

Eighty-four isolates of *S.aureus* coming from individual and/or bulk milks belonging to sheep and goat farms of different area of Sicily were tested for antimicrobial susceptibility against a panel of the most utilized antimicrobials. Eighty-one isolates of Staphylococci were obtained from milk samples of sheep or goat related to clinical mastitis and isolated during the diagnostic activity carried out at the Istituto Zooprofilattico Sperimentale della Sicilia (IZS). Three samples of *S.aureus* were isolated from bulk milk during auto-control programs. Milk samples were collected after cleaning and disinfection of the udders and discharge of the first milk; samples were taken at the morning by manual milking and collected in sterile vials. Milk samples were screened for the presence of mastitic pathogens including *Staphylococcus* spp. as well as the other pathogens which cause mastitis in small ruminants comprising, *Corynebacterium* spp. *Streptococcus* spp. and mycoplasmas. After isolation, field strains were identified through standard procedures and stored at –80°C for further studies. Samples (10 ml) were diluted with 90 ml of buffered peptone water (Oxoid, Basingstoke, UK) and homogenized. They were seeded into Baird-Parker RPF agar (Oxoid) and incubated aerobically at 35°C for 24 and 48 h. The isolates of *S. aureus* were identified using conventional biochemical methods including Gram staining, catalase test (Biomerieux), oxidase (oxidase strips Oxoid), coagulase and Test VP (Voges – Proskauer – Biomerieux). Before testing all isolates were subcultured in 10 ml of brain–heart infusion (BHI) broth (Oxoid) for 24 h at 37°C. All strains of *S. aureus* selected for this survey were isolated from 2006 to 2009 (δT_1) and from 2013 to 2015 (δT_2).

Antimicrobial susceptibility

Susceptibility tests were performed by disk diffusion method (Bauer *et al*., 1966) on Mueller-Hinton Agar (Liofilchem®, Teramo, IT). Susceptibility to 19 different molecules belonging to 9 classes of antibiotics was evaluated by placing on agar plate antibiotic discs of: aminoglycosides [gentamicin (10 µg), spectinomycin (10 µg), streptomycin (10 µg), tobramycin (10 µg)]; carbapenems [imipenem (10 µg)]; cephalosporins [cefepime (30 µg)]; fluoroquinolones [enrofloxacin (5 µg)]; glycopeptides [vancomycin (30 µg)]; lincosamides [lincomycin (15 µg)]; macrolides [erythromycin (15 µg); tilmicosin (15 µg)]; tylosin (30 µg)]; penicillins [ampicillin (10 µg), oxaxillin (1 µg)]; phenicols [florfenicol (30 µg)]; rifamycins [rifampicin (30 µg)]; sulfonamides [sulfamethoxazole (25 µg)]; tetracyclines [oxytetracycline (30 µg), tetracycline (30 µg)] (Liofilchem®, Teramo, IT). For investigation on antibacterial activity, the diameter of inhibition zone was measured after incubation at 37°C for 24 h. Isolates were considered either susceptible (S), intermediate (I) or resistant (R) according to Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standards Institute-CLSI, 2017). Additionally, isolates were considered multidrug resistant (MDR) when a resistance to three or more antimicrobial classes was detected (Magiorakos *et al*., 2012).

The data obtained from the antimicrobial susceptibility tests were grouped into two periods intervals δT_1 (2006-2009 - 21 isolates) and δT_2 (2013-2015 - 63 isolates) and compared to show the behavior of the different isolates in different periods with a time interval of 5 years. Statistical analysis was carried out using T-Test to check the significance of the differences ($P<0.05$).

Detection of antibiotics resistance and toxin genes

Further genetic analysis was carried out in order to evaluate possible acquired genetic resistance. In particular, erythromycin (*ermA* and *ermC*) and tetracycline (*tetK* and *tetM*) resistance genes were investigated by multiplex PCR (Ardic *et al*., 2005). The DNA was extracted by the Instagene Matrix (Biorad) according to the manufacturer's instructions. PCRs were performed following the multiplex PCR method described by Strommenger *et al*. (2003). An internal control primer pair specific to 16S rDNA (16S rDNA F 5′- CAG CTC GTG TCG TGA GAT GT-3, 16S rDNA R 5′- AAT CAT TTG TCC CAC CTT CG-3′) was added to the multiplex PCR protocol to determine amplification effectiveness and likely PCR inhibition. The PCR mixture was prepared using PuReTaq Ready-To-Go, GE Healthcare, in 25 ul total volume containing 0.4M of each primer and 21 µl of water. To this mixture 2 µl of extracted bacterial DNA were added. The PCR protocol consisted of 3 min of preliminary denaturation at 95°C, 30 cycles of 30 s denaturation at 95°C, 30 s of primer binding at 54°C and 30 s of polymerization at 72°C followed by a final 4 min polymerization at 72°C. Amplification products were subjected to 1.5% agarose gel electrophoresis in a fixed 100V electrical field; the resulting bands for *ermC* , *ermA*, *tetK*, *tetM* were examined under an ultraviolet-transilluminator (UV Transilluminator 2000; Bio-Rad, Milan, Italy) and photographed with a Kodak digital camera. The results were grouped into two-time intervals δT1(2006-2009) - δT2(2013-2015) and compared to show the behavior of the isolates in different periods.

The *S. aureus* isolates were also analyzed for the presence of different toxin genes and the presence of methicillin resistance *mecA* gene. Genes for pyrogenic toxins: staphylococcal enterotoxins (SE) from A to E and toxic shock syndrome toxin 1 (*tsst-1*) were detected by Multiplex PCR A and Exfoliative toxins *eta* and *etb and mec*A genes by Multiplex PCR B as described by Mehrotra *et al*. 2000. For detection of *seg*, *seh*, *sei*, *sej*, and *sep*, a multiplex PCR assay described by De Buyser (2009) was followed. Detection of

femA was used as an internal positive control and *S. aureus* ATCC 25923 as quality control. The following reference strains for SE genes kindly provided by the Italian reference laboratory for Coagualse positive and negative Staphylococci were used: FRIS6 (*sea*, *seb*); FRI137 *(sec*, *seg*, *seh*, *sei)*; HMPL280 *(sed*, *seg*, *sei*, *sej*, *sep*, *ser)*; and FRI326 *(see)*. The amplicons were detected using a 2.5% agarose gel containing ethidium bromide and visualized under ultraviolet light as described by Vitale *et al*. (2018).

Detection of genes involved in biofilm formation

Ica operon was detected with primers including a region from *ica R* to *ica A* (Position from 1889 to 2486 *S. aureus* ica operon sequence GenBank: AF086783). (Cramton *et al*.1999). PCRs for *bap* and *SasC* genes were performed as described by Cucarella *et al*. (2004) and Schroeder *et al*. (2009) respectively.

Statistical analyses

Statistical analysis was carried out using T-Test to check the significance of the differences (P<0.05) between δT_1(2006-2009) - δT_2(2013-2015).

Duplicate tests were performed on each sample by IZS of Sicily and Department of Veterinary Science of University of Messina. One-way paired T-test was used to compare multidrug resistance patterns from bacterial isolates in groups δT_1 and δT_2 using R (R Core Team, 2019).

Results

Staphylococcus aureus antimicrobial susceptibility

Isolates displayed high frequencies of antibiotic resistance (Table 1). The resistance patterns varied from 1 to 10 of the 19 antibiotics tested. The largest number of resistances were recorded against spectinomycin (100% of the isolates) and streptomycin (86,9% of the isolates) both belonging to the aminoglycosides family. The isolates showed high susceptibility to florfenicol, imipenem, rifampicin, and tobramycin. Among the 84 isolates, 31 (36.9%) were resistant to sulfamethoxazole, 18 (21.4%) to tetracycline and 19 (22.6%) to oxytetracycline and to erythromycin. Remaining isolates showed resistance lower of 20,0% to the other molecules. Multidrug resistance occurred in 26 isolates (30.9%). The most abundant MDR pattern observed was combined resistance to aminoglycosides, macrolides and tetracyclines in 19 isolates (22.6%). T-test revealed significant increase of the resistance to tilmicosin and decrease to sulfamethoxazole and vancomycin between the two periods intervals (P<0.05). Although they were not significant, the results showed as the resistances to oxytetracycline and tetracycline tend to increase between δT_1 and δT_2.

Genotypic detection of erythromycin and tetracycline resistance genes

Tetracycline resistance genes (TRg) were detected in 19 samples (22.6%) (Table 2); *tetK* (360 bp) and *tetM* (158 bp) were simultaneously highlighted in almost all positive samples (17 out of 19), while no sample present only *tetM* gene. There is a significant difference (P<0.05) of presence of TRg between δT_1 (4.8%) and δT_2 (28,6%). Regarding the Erythromycin Resistance genes (ERg), detection rates of *ermC* (299 bp) were 15.5%, only an isolate showed *ermA* (190 bp) resistance gene. The difference between δT_1 and δT_2 was only significant for *ermC* gene. The contemporanous presence of *tet*(*K*) and *tet*(*M*) were detected in 17 isolates (20.5%), with significant increase between the two periods intervals (Table 2). As shown in Table 3, the contemporaneous presence of resistance genes for tetracycline and erythromycin was detected in seven samples (8.3%), belonging to group δT_2. The *mecA* was found only in one sample belonging to δT_1 (Table 3).

Detection of biofilm formation and toxin genes

Enterotoxin genes were detected only in four isolates (4.8%) of δT2 group while Toxic shock syndrome toxin (*tsst-1*) gene was present in five samples (5.9%), all belonging to δT2 group. No exfoliative toxin gene was detected. Fifty-seven isolates (67.9%) result positive for *ica* operon, thirty isolates (35.7%) presented *sasC* gene. In the group δT1 eight isolates (38.0%) are simultaneously positive for both genes while the bap gene was not present in any

Table 1. *S. aureus* Isolates resistance to 19 antibiotics.

Classes	Antimicrobials	δT_1			δT_2			$\delta T_1 + \delta T_2$		
		I + R	Total Strains	%	I + R	Total Strains	%	I + R	Total Strains	%
Aminoglycosides	Gentamicin	1	21	4.8	1	63	1.6	2	84	2.4
	Spectinomycin	21	21	100	63	63	100	84	84	100
	Streptomycin	19	21	90.5	54	63	85.7	73	84	87
	Tobramycin	-	21	-	-	63	-	-	84	-
Carbapenems	Imipenem	-	21	-	-	63	-	-	84	-
Cephalosporins	Cefepime	2	21	9.5	-	63	-	2	84	2.4
Fluoroquinolones	Enrofloxacin	-	21	-	2	63	3.2	2	84	2.4
Glycopeptides	Vancomycin	6	21	28.6*	3	63	4.8*	9	84	10.7
Lincosamides	Lincomycin	2	21	9.5	11	63	17.5	13	84	15.4
Macrolides	Erythromycin	5	21	23.8	14	63	22.2	19	84	22.6
	Tilmicosin	1	21	4.8*	13	63	20.6*	13	84	16.7
	Tylosin	3	21	14.3	12	63	19.0	15	84	17.8
Penicillins	Ampicillin	3	21	14.3	4	63	6.3	7	84	8.3
	Oxaxillin	1	21	4.8	-	63	-	1	84	1.2
Phenicols	Florfenicol	-	21	-	-	63	-	-	84	-
Rifamycins	Rifampicin	-	21	-	-	63	-	-	84	-
Sulfonamides	Sulfamethoxazole	15	21	71.4*	16	63	25.4*	31	84	36.9
Tetracyclines	Oxytetracycline	3	21	14.3	16	63	25.4	19	84	22.6
	Tetracycline	2	21	9.5	16	63	25.4	18	84	21.4

Strains resulted intermediates and resistant (I) + (R) grouped in two intervals δT_1 (2006-2009) and δT_2 (2013-2015). *P-value <0.05 – T-test was performed for comparisons of the resistance among δT_1 and δT_2.

isolate. No significant differences (P>0.05) were observed between the two groups (Table 4).

Discussion

Staphylococcus aureus is a main responsible for mastitis in ruminant herds with big economic loss in dairy farms due to lower or absent milk production. In this study several *S.aureus* isolates from clinical mastitis cases showed resistance to different antibiotics and a prevalence of multidrug resistant strains (30.9%) much higher than those reported in previous studies (Azara *et al*., 2017; Zdragas *et al*., 2015). Moreover, according to other reports (Rajala-Schultz *et al*., 2004; Ceniti *et al*., 2017), a high resistance and multiresistance for Tetracycline, Macrolides and Sulfamethoxazole was observed, although these molecules are not specifically prescribed for the treatment of Gram-positive, bacterial mastitis. The significant increase of tilmicosin resistance, between δT_1 (4.8%) and δT_2 (20.6%), suggests an increasing use of this antibiotic in the health management of small ruminant farms in Sicily.

The low percentage of resistance to oxacillin and enrofloxacin is consistent with the results obtained by other authors (Zdragas *et al*., 2015; Ceniti *et al*., 2017). Moreover, all isolates showed susceptibility to florfenicol, imipenem, rifampicin and tobramycin suggesting that these antibiotics are not used in a routine basis in Sicilian small ruminant farming. No correlation was found between the isolates resistant to β-lactams and tetracycline classes, according with previous studies conducted on *S. aureus* isolates isolated from raw sheep milk and /or cheese samples (Spanu *et al*., 2014). Despite a variety of available antibiotics, success of treatment of *S. aureus* mastitis particularly during lactation is argued (Pengov and Ceru, 2003). Clearly, there are several factors that influence the outcome of the therapy. Bacterial strains resistant to antimicrobial agents used in mastitis treatment might be one of the important reasons for therapy failure. Thus, information on susceptibility trends for a bacterial species within a given population is important. Antimicrobial susceptibility testing of the causal pathogens should help to identify the most appropriate treatment for therapy of mastitis. The *mecA* gene was detected only in one of the *S. aureus* isolates studied. In this study, the *mecA* gene was detected in one of the *S. aureus* strains only in contrast to a *mecA* positive prevalence of 4.5% detected in *S. aureus* isolated from food sources (Vitale *et al*., 2018), mainly derived from cow-milk of Ragusa province where a higher prevalence of *mecA* has been detected in people working in bovine farms (Antoci *et al*., 2013). This result agrees also with what was found in other studies on small ruminants (Vyletelová *et al*., 2011; Virdis *et al*., 2010; Kotzamanidis *et al*., 2021) and confirms low prevalence of MRSA in sheep and goats' milk (Caruso *et al*., 2016) and dairy products (Basanisi *et al*., 2016). The high presence of *tet*(*K*) and *tet*(*M*) (20.5%) suggests an overuse of broad-spectrum antibiotics such as tetracyclines in sheep and goat diseases. Moreover, only in δT2 group, the 11.1% of the samples present a simultaneous presence of TRg and ERg. Not always there is correspondence between the presence of *tet* and *erm* genes and phenotypic resistance to related antimicrobials as found by Ardic (2005) and Sekiguchi (2003). It's possible to hypothesize that resistance to these antibiotics may depend on different mechanisms as found by other authors (Mathews *et al*. 2010). Maybe different bacterial pathways are

Table 2. Presence of resistance genes *tetK, tetM, ermA, erm C*, with the corresponding phenotypic antibiotic resistance.

	T.R.g[1]	%	*tetK*[2]	%	*tetM*[3]	%	*Tet(K) + Tet(M)*[6]	%
δT_1	1/21	4.7	0/21	-	0/21	-	1/21	4.7
δT_2	18/63	28.6	2/63	3.2	0/63	-	16/63	25.4*
Total	19/84	22.6	2/83	2.4	0/84	-	17/83	20.5
	E.R.g[1]	%	*ermA*[4]	%	*ermC*[5]	%	*Erm(A) + Erm(C)*[6]	%
δT_1	1/21	4.7	1/21	4.7	0/21	-	0/21	-
δT_2	12/63	19	0/63	-	12/63	19*	0/63	-
Total	13/84	15.5	1/84	1.2	12/84	14.3	0/84	-

[1]Presence of at least one resistance gene, [2]Presence of the only gene tetK,[3]Presence of the only gene tetM,[4]Presence of the only gene ermA, [5]Presence of the only gene ermC, [6]Presence of both genes,. T-test was performed for comparisons of the resistance among δT_1 and δT_2. * P-value < 0.05.

Table 3. Presence of resistance genes for tetracycline (TRg) and erythromycin (ERg) and of *mecA* gene.

Samples	*TRg + Erg*	%	*mecA*	%
δT_1	0/21	-	1/21	4.8
δT_2	7/63	11.1	0/63	-
Total	7/84	8.3	1/84	1.2

Table 4. Presence of different biofilm formation genes in *Staphylococcus aureus* isolates.

Samples	*ica operon*	%	*sas C*	%	*Bap*	%
T1	14/21	66.7	9/21	42.9	0/21	-
T2	43/63	68.3	21/63	33.3	0/63	-
Total	57/84	67.9	30/84	35.7	0/84	-

used to reach resistance. We cannot exclude also that Single-Nucleotide Polymorphisms (SNP) at level of the primer's sequences are responsible for the lack of amplification in some cases. To this end, and to fully characterize the isolates, whole genome sequencing analysis are planned for the future studies.

IMI caused by *S. aureus* may concern severe implications for public health because of the risk of enterotoxins production and toxic shock syndrome toxin (Balaban and Rasooly, 2000). In contrast with previous studies conducted on *S.aureus* strains isolated from bulk-tank milk samples of goats and sheep (Scherrer *et al*., 2004) and on isolates from dairy products and tissue samples in Sicily the prevalence of toxin genes detected in the present study was not particularly high. However, increased in δT_2 group suggesting the possibility that toxigenic *S. aureus* isolates are spreading with the time in animal herds. In a previous report in Greece the presence of the staphylococcal enterotoxin C (SEC) was more related to the mastitis milk (Kotzamanidis *et al*., 2021). Exfoliative toxins genes were not detected, according with previous studies on *S. aureus* isolates from bovine mastitis (Endo *et al*., 2003; Vitale *et al*., 2019). The high involvement of *S. aureus* in IMI may concern severe implications for public health and food safety because of the risk of enterotoxins production and toxic shock syndrome toxin (Balaban and Rasooly, 2000). In subclinical inflammation milk production is almost normal and *S.aureus* can be transmitted into the dairy food chain, leading to contaminated dairy products, particularly when they are made with raw milk like in traditional and artisanal cheese. Staphylococcal enterotoxins (SEs) are a major cause of food poisoning world-wide (Agurdin *et al*., 2010; Mehli *et al*., 2017). Biofilm formation in strains from cases of sheep and goats is a poorly investigated aspect if compared with other virulence factors. According to Tel (2012) the *ica* operon was detected with high percentage (67.9%) and the *bap* gene was not present in any isolates. These data may justify how, in cases of sheep and goat mastitis, the ability of *S. aureus* to resist against therapy by forming biofilm is mainly mediated by the *ica* operon.

Conclusions

This study showed that there is a high prevalence of MDR in *S.aureus* isolates collected in Sicily from mastitic milks of small ruminants, with an increase of resistance to broad spectrum antibiotic, as tetracyclines. This "commercial" induced resistance is proved by the evidence to find β-lattamines susceptible strains of Staphylococcus showing resistance to tetracyclines. It suggests that an evaluation of antimicrobial susceptibility is always recommended before treating the flock. Products like florfenicol, rifampicin, tobramycin or imipenem are not all registered for veterinary use because of the risk to introduce AMR in food of animal origin. In addition, the high cost of herd or flock treatment is not sustainable for the farming economy in Sicily especially for small ruminants. Florfenicol has been recently introduced in Veterinary practice mainly addressed to respiratory syndromes of cattle and/or pets and its cost is justified if related to the loss of one calf or to welfare of companion animals. An increase in toxigenic potential was highlighted by the presence of *sec*, *tsst-1* genes in some isolates of δT_2 group. Biofilm-forming ability could be another important virulence factor of *S. aureus* strains which underlines the importance of cleaning and disinfection of milking parlour, equipment, and the other farm facilities and moreover, the responsibility by farmer to implement biosecurity practices.

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