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Staphylococcus aureus and methicillin-resistant *S. aureus* (MRSA) in bulk tank milk, livestock and dairy-farm personnel in north-central and north-eastern Greece: Prevalence, characterization and genetic relatedness

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ABSTRACT

Recently, there has been an increased tendency towards raw-milk consumption, which may pose a consumer risk, due to the possible presence of human pathogenic microorganisms, such as *Staphylococcus aureus* and even methicillin-resistant *S. aureus* (MRSA). The prevalence of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) was investigated in 40 dairy (cattle, sheep and goat) farms in northern Greece. *S. aureus* and MRSA were detected in 47.8% and 4.1% of the 387 samples (raw milk, farmers and animal samples) tested, respectively. Most (81.3%) of the MRSA isolates harbored the *mec*A gene, whereas the *mec*C or Panton-Valentine Leucocidin (PVL) genes were not detected. Seven *spa* types were identified, with t127 being the most prevalent. *Spa* type t034 (CC398) was isolated for the first time from livestock in Greece. Staphylococcal enterotoxin genes were detected in 93.8% of the MRSA isolates. The MRSA isolates were genetically diverse and were all capable of biofilm production. Our results confirm the lurking threat of MRSA in raw milk and dairy farms and suggest the need for surveillance programs starting at the farm level.

1. Introduction

Recently, there has been a growing interest in the consumption of locally produced, minimally processed food and, as a result, raw-milk consumption displays an increasing trend (EFSA, 2015). Moreover, many farmers and their family members often consume raw milk and raw-milk dairy products. In many countries, including Greece, the legislation permits (upon meeting certain requirements) the consumption of raw milk and the manufacture of raw-milk dairy products [Commission Regulation (EC) No.853/2004]. However, raw-milk consumption may pose a risk to the consumer, due to the possible presence of human pathogenic microorganisms, including *Staphylococcus aureus* (Claeys et al., 2013). In addition, the presence of *S. aureus* in raw milk represents a potential source for the introduction of the pathogen into the dairy-food chain (D'Amico and Donnelly, 2011; Johler et al., 2018; McMillan et al., 2016; Song et al., 2015).

S. aureus is associated with a plethora of human and animal diseases (Lowy, 1998; Peton and Le Loir, 2014). In humans, from a food-safety perspective, *S. aureus* is among the major causes of food intoxication, upon ingestion of foods containing pre-formed staphylococcal enterotoxins (SEs) (Le Loir et al., 2003). In dairy animals *S. aureus* constitutes one of the most common causative agents of mastitis, with infected animals frequently shedding *S. aureus* into their milk (Li et al., 2017). Hence, potential sources of raw-milk contamination with *S. aureus* are the lactating ruminants, as well as the farm personnel (Poutrel et al., 2015; Vautor et al., 2005).

Methicillin-resistant *S. aureus* (MRSA) pose serious public-health concerns due to their ability to colonize and infect humans and animals

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(Petinaki and Spiliopoulou, 2012). Following their initial appearance as hospital-associated pathogens (HA-MRSA), new incidents of MRSA infections have emerged in different settings, specifically in the human community (Community-Associated MRSA; CA-MRSA) and in livestock (Livestock-Associated MRSA; LA-MRSA) (Pantosti, 2012). The existence of MRSA-infected or colonized animals can lead to the spread of the pathogens not only to farm personnel, but also to raw milk (Lee, 2003), which represents a potential source of MRSA entrance into the food chain (Oniciuc et al., 2017). MRSA surveillance data in Greek hospitals indicate that the prevalence of MRSA is one of the highest (38.8%) among European countries (ECDC, 2017).

S. aureus produces a wide range of virulence factors such as enterotoxins and leukocidines and is frequently capable of forming biofilms. The five principal SEs are SEA, SEB, SEC, SED and SEE, which are highly stable molecules (resistant to heat and proteolytic enzymes as well as low pH), thus retaining activity in the digestive tract following ingestion (Fisher et al., 2018). These five SEs have been reported to cause 95% of cases of Staphylococcal Food Poisoning (SFP) (Tang et al., 2011).

Previous investigations on the prevalence and epidemiology of *S. aureus* and MRSA in the Greek dairy chain (Papadopoulos et al., 2018) and specifically in dairy industries located into the two regions considered in this study (Papadopoulos et al., 2019) reported high isolation frequencies and genetic diversity of these pathogens. These results suggested that further detailed investigations, focusing on the corresponding collaborating dairy farms, might provide more insights on the transmission routes and infection sources. Therefore, the main objectives of this study were: (i) to estimate the prevalence of *S. aureus* and MRSA in raw milk, farmers and animals from dairy cattle, sheep and goat farms in northern Greece, (ii) to assess the genetic variability of the MRSA isolates via pulsed-field gel electrophoresis (PFGE) and *spa* typing and (iii) to characterize the MRSA isolates in terms of selected virulence factors (biofilm formation, Panton-Valentine Leucocidin (PVL) genes and enterotoxin-encoding genes).

2. Materials and methods

2.1. Sample collection

From November 2016 until June 2017, 387 samples were collected from 40 dairy farms located in the regions of Macedonia and Thrace (northern and north-eastern Greece): 11 cattle, 19 sheep, 8 goat and 2 mixed sheep-goat farms. The criterion for selecting the study farms was their collaboration with previously studied dairy industries (Papadopoulos et al., 2019). Samples were taken from bulk tank milk (BTM), from all farm personnel involved and from 10% of each farm's lactating animals (with a maximum of 10 animals per farm). Thus, the collected samples comprised of: 42 BTM samples (12 bovine, 21 ovine, 9 caprine; one cattle farm had two bulk tanks and one mixed sheep-goat farm had only sheep milk at the time of the sampling); 68 farmers' nasal swabs (27 cattle farmers, 22 sheep farmers, 13 goat farmers and 6 sheep-goat farmers) and 277 animal nasal swabs (89 from cows, 104 from sheep and 84 from goats).

Upon clarification of the study's purposes, all human participants gave oral informed consent to participate and none of them reported receiving any recent (within the preceding three months) antimicrobial treatment. The farm owners consented to animal sampling and declared no recent (one month) antimicrobial administration to their animals.

The BTM of each farm was thoroughly stirred prior to sampling and 50 ml were aseptically placed into sterile tubes. Human and animal nasal samples were taken from both nares by means of sterile cotton-tipped swabs. The swabs were immediately placed into tubes containing 10 ml Tryptone Soy Broth (TSB; LAB M Limited, Lancashire, United Kingdom) with 6.5% w/v NaCl (Merck KGaA, Darmstadt, Germany) and 0.3% yeast extract (LAB M). All collected samples were transported to the laboratory in cool boxes (*ca.* 2 °C) within 6 h after collection and processed immediately.

2.2. Isolation, identification and molecular confirmation of S. aureus

Isolation and enumeration (milk samples only) of staphylococci was carried out according to ISO 6888-1 (ISO, 1999) on Baird Parker agar supplemented with egg yolk tellurite (BPA, LAB M) after incubation for 24–48 h at 35 °C as previously described (Sergelidis et al., 2012). Ten-ml portions of milk were aseptically removed from each sample, placed into stomacher bags containing 90 ml TSB with 6.5% w/v NaCl, homogenized for 2 min (Lab Blender 400, Seward & Co. Ltd. London) and then 10-fold serial dilutions were prepared using the same broth. One ml from each dilution was pour-plated into BPA. For the detection of less than 10 CFU (per ml of sample), 1 ml of milk was plated onto BPA. The swabs were incubated in TSB with 6.5% w/v NaCl for 18 h at 35 °C for enrichment; then a loopful was streaked onto BPA and the plates were incubated at 35 °C for 24–48 h.

From each sample, up to four well-isolated, presumptive *S. aureus* colonies (having typical black appearance and surrounded by a clear zone) were selected and sub-cultured onto Tryptone Soya Yeast Extract agar (LAB M) for purification and further characterization. Preliminary identification was based upon biochemical assays (Gram-staining, catalase reaction, mannitol fermentation, coagulase test), morphological and cultural characteristics. Each presumptive *S. aureus* strain was stored at −80 °C in TSB containing 20% glycerol for further molecular characterization.

A DNA purification protocol for Gram-positive bacteria (Pure Link Genomic DNA kit, Invitrogen, Carlsbad, CA) was used for genomic DNA extraction. The PCR conditions previously described by Zdragas et al. (2015) were used for the detection of the *coa* (Hookey et al., 1998) and *nuc* (Sudagidan and Aydin, 2009) genes, in order to confirm the identification of all presumptive *S. aureus* isolates. A sample (raw milk or swab) was defined as positive if it contained at least one *S. aureus* isolate. Furthermore, for a better representation, only one *S. aureus* isolate per every phenotypically positive sample was selected and subjected to further characterization.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the agar-dilution method in Mueller-Hinton agar (MHA, Merck) according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2009). Briefly, plates were prepared (in the laboratory) by incorporating the appropriate amount of antimicrobial agent into MHA. For each bacterial isolate the inoculum was prepared by adjusting the turbidity to 0.5 McFarland and was applied rapidly to the agar surfaces using a multi-channel pipet (Eppendorf, Merck) capable of transferring multiple inocula to each plate. The results were evaluated after incubation at 35 °C for 24 h. Susceptibility towards the following 14 antimicrobials was evaluated: penicillin, P; oxacillin, Ox; amoxicillin/clavulanic acid, Amc; tetracycline, T; erythromycin, E; vancomycin, V; chloramphenicol, C; ciprofloxacin, Cp; trimethoprim/sulfamethoxazole, Sxt; trimethoprim, Tm; gentamicin, G; amikacin, Ak; kanamycin, K; rifampicin, R. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) minimum inhibitory concentration breakpoints (EUCAST, 2018). Multidrug-resistance (MDR) was defined as previously proposed (Magiorakos et al., 2012). *S. aureus* ATTC 25923, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

2.4. Molecular confirmation and genetic characterization of MRSA

2.4.1. Detection of the mecA and mecC ($mecA_{LGA251}$) genes

The methicillin-resistance phenotype was molecularly verified, using previously described PCR conditions (Zdragas et al., 2015) targeting the *mec*A (Murakami et al., 1991) and *mec*C (Stegger et al., 2012)

genes. In cases where neither the *mec*A nor the *mec*C genes were detected, the isolates were further tested onto MHA plates with 6 μg/ml of oxacillin and 4% NaCl, according to the CLSI guidelines. The PCR amplicons were separated in 1.5% agarose gels stained with ethidium bromide and visualized under UV illumination (TEX-20 M, Life Technologies, Gibco BRL System).

2.4.2. Detection of virulence-associated genes (PVL and SEs) and assessment of biofilm-formation ability

The PCR protocol described by Stegger et al. (2012) was used for the detection of the PVL gene. Five specific primer sets, previously described by Jarraud et al. (2002), were used for the detection of genes encoding for the five classic SEs (*sea*, *seb*, *sec*, *sed*, *see*). Amplifications of SE-coding genes were performed as single PCR assays.

Biofilm production ability by MRSA strains was determined using a semi-quantitative, microtiter-plate, adherence assay according to the protocol described by Wang et al. (2010), which measures the optical density (OD) of adherent biofilms stained with 0.3% (w/v) crystal violet at 570 nm. The cut-off (ODc) was defined as the mean OD value of the negative control (plain broth medium) + 3 × standard deviation (SD) of ODs of negative control (Stepanović et al., 2000). Depending on the resulting OD readings, MRSA strains were classified as either no biofilm producers ($OD < ODc$), weak- ($ODc < OD \leq 2 \times ODc$), moderate- ($2 \times ODc < OD \leq 4 \times ODC$) or strong biofilm producers ($4 \times ODc < OD$).

2.4.3. Spa and Pulsed Field Gel Electrophoresis (PFGE) typing

Spa typing was conducted according to Aires-de-Sousa et al. (2006). Typing was performed through the publicly available Ridom Spa Server (www.spaserver.ridom.de) (Harmsen et al., 2003) and the RidomStaphType software (Rıdom GmbH, Würzburg, Germany) was used for *spa* sequence analysis. PFGE analysis was conducted following the PulseNet protocol (McDougal et al., 2003) using the size standard, electrophoretic conditions, dendrogram construction and comparison criteria previously described (Papadopoulos et al., 2018), with the exception that a newer version of the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) was used (ver.7.5) to analyze restriction patterns. Clusters were selected using a cutoff at the 80% level of genetic similarity.

3. Results

3.1. Prevalence of S. aureus and MRSA

3.1.1. Occurrence of S. aureus

S. aureus was isolated from 185 out of the 387 (47.8%) samples analysed: from 17 (40.5%) of the BTM samples, from 35 (51.5%) of the farmers and from 133 (48.0%) of the animals. At the farm level, *S. aureus* was detected in 34 out of the 40 (85.0%) farms. The isolation frequencies per sample type are shown in Table 1.

3.1.2. Antimicrobial susceptibility

Out of 185 *S. aureus* isolates (one for each positive sample) tested, 183 (98.9%) were found to be resistant to at least one antimicrobial and 72 (38.9%) were MDR (Table 2). Higher resistance frequencies were observed against P (97.8%), Amc (84.3%), T (43.8%) and G (40.0%). Sixteen (8.6%) of the *S. aureus* isolates were identified as presumptive MRSA based on their resistance to Ox. These strains were isolated from 16 different samples (16/387, 4.1%) of BTM (1/42), animals (6/277) and farmers (9/68). All these strains carried the *coa* and *nuc* genes; the *mec*A gene was detected in 13 out of the 16 samples (81.3%), whereas the *mec*C gene was not detected. The three *mec*A-negative MRSA isolates tested positive onto MHA with 6 μg/ml of Ox and 4% NaCl. At the farm level, MRSA were detected in 14 out of the 40 farms (35%).

Table 1
Isolation frequency of *S. aureus* and MRSA from bulk tank milk (BTM), animals and farm personnel.

Sample type	No of samples	No *S. aureus* (%)	No MRSA (%)
Bovine BTM	12	3 (25.0)	1 (8.3)
Ovine BTM	21	11 (52.4)	0
Caprine BTM	9	3 (33.3)	0
Cows	89	22 (24.7)	1 (1.1)
Sheep	104	53 (51.0)	3 (2.9)
Goats	84	58 (69.0)	2 (2.4)
Cattle farmers	27	17 (63.0)	4 (14.8)
Sheep farmers	22	12 (54.5)	3 (13.6)
Goat farmers	13	5 (38.5)	2 (15.4)
Sheep-goat farmers	6	1 (16.7)	0
Total[a]	387	185 (47.8)	16 (4.1)

[a] Positive samples for *S. aureus* and MRSA originated from 34 (85%) and 14 (35%) out of 40 participating farms, respectively.

3.1.3. Cattle farms

In cattle farms, *S. aureus* and MRSA were recovered from 25.0% (mean count of 3.7 log CFU/ml) (Table 3) and 8.3% (1/12) of BTM samples, respectively and the corresponding isolation frequencies from the animals' nares were 24.7% and 1.1%, respectively. Additionally, *S. aureus* and MRSA were recovered from 63.0% to 14.8% of the tested cattle farmers, respectively. At the farm level, *S. aureus* and MRSA were detected in 10 (90.9%) and 6 (54.5%) out of the 11 cattle farms, respectively.

3.1.4. Small ruminants' farms

In sheep farms *S. aureus* and MRSA were recovered from 52.4% (mean count of 4.3 log CFU/ml) (Table 3) and 0% of BTM samples, from 51.0% to 2.9% of sheep and from 54.5% to 13.6% of farmers, respectively. The corresponding isolation frequencies in goat farms were 33.3% (mean count of 4.0 log CFU/ml) (Table 3) and 0% of BTM samples, 69.0% and 2.4% of goats and 38.5% and 15.4% of farmers, whereas the isolation frequencies from farmers in the two-mixed sheep-goat farms were 16.7% and 0%, respectively. At the farm level, *S. aureus* and MRSA were detected in 15 (78.9%) and 4 (21.1%) out of the 19 sheep farms, in 7 (87.5%) and 3 (37.5%) out of the 8 goat farms and in 2 and 1 out of the 2 mixed sheep-goat farms, respectively.

3.2. Carriage of the PVL and SE genes and biofilm formation ability

All 16 MRSA isolates in the present study were PVL-negative and their screening for SE genes showed that 93.8% carried at least one SE gene, with the isolates displaying a total of six different toxin-gene profiles, including one isolate that tested negative for all tested SE genes (Fig. 1). The majority of the MRSA isolates (56.3%) carried only one SE gene, 5 (31.3%) harbored two SE genes and 1 (6.3%) carried three SE genes. The most frequently detected SE gene was sec (present in 15 isolates), alone or in combination with other SE genes, followed by *sed*, *seb* and *sea*, which were only detected in combination with sec (Fig. 1). The *see* gene was not detected.

All MRSA isolates in our study were found capable of biofilm-production, with 5 (31.3%) characterized as moderate- and the remaining 11 (68.7%) as weak-biofilm producers (Fig. 1).

3.3. Spa types and PFGE clusters

The 16 MRSA isolates were assigned to seven different *spa* types, whereas two isolates were non-typeable and may belong to the 1–2% of the *S. aureus* isolates that are non-typeable via *spa* typing (Votintseva et al., 2014). The most common *spa* types were t127 (n = 6), t034 (n = 2) and t3586 (n = 2), while t012, t192, t253 and t13336 were represented only once (Fig. 1). To our knowledge, this is the first time that the *spa* types t012, t192, t253 and t13336 were detected in Greece.

Table 2
Numbers and percentages of *S. aureus* isolates resistant to different antimicrobials according to their isolation source.

Origin of *S. aureus* isolates	No of *S. aureus* isolates	No of *S. aureus* isolates resistant to different antimicrobials[a] (%)													
		P	Ox	Sxt	G	E	Ak	K	T	Tm	Amc	Cp	R	V	C
Bovine BTM	3	3 (100)	1 (33.3)	0	1 (33.3)	2 (66.6)	0	1 (33.3)	3 (100)	1 (33.3)	3 (100)	0	0	0	0
Ovine BTM	11	11 (100)	0	0	2 (18.2)	1 (9.1)	0	0	0	0	11 (100)	0	0	0	0
Caprine BTM	3	3 (100)	0	0	1 (33.3)	0	1 (33.3)	1 (33.3)	0	0	2 (66.6)	0	0	0	0
Cows	22	20 (90.1)	1 (4.5)	2 (9.1)	4 (18.2)	4 (18.2)	0	2 (9.1)	18 (81.8)	2 (9.1)	17 (77.3)	0	0	10 (45.5)	0
Sheep	53	52 (98.1)	3 (5.7)	0	22 (41.5)	3 (5.7)	6 (11.3)	13 (24.5)	12 (22.6)	4 (7.5)	45 (84.9)	0	2 (3.8)	1 (1.9)	0
Goat	58	57 (98.3)	2 (3.4)	1 (1.7)	29 (50.0)	9 (15.5)	0	2 (3.4)	29 (50.0)	13 (22.4)	43 (74.1)	0	1 (1.7)	1 (1.7)	0
Farm personnel	35	35 (100)	9 (25.7)	1 (2.9)	15 (42.9)	9 (25.7)	0	13 (37.1)	19 (54.3)	4 (11.4)	35 (100)	0	0	1 (2.9)	2 (5.7)
Total	185	181 (97.8)	16 (8.6)	4 (2.2)	74 (40.0)	28 (15.1)	7 (3.8)	32 (17.3)	81 (43.8)	24 (13.0)	156 (84.3)	0	3 (1.6)	13 (7.0)	2 (1.1)

[a] Antimicrobial concentration breakpoints (μg/ml): P, penicillin 0.125; Ox, oxacillin 2; Sxt, trimethoprim/sulfamethoxazole 4/76; G, gentamicin 1; E, erythromycin 2; Ak, amikacin 16; K, kanamycin 8; T, tetracycline 2; Tm, trimethoprim 4; Amc, amoxicillin/clavulanic acid 0.5/0.25; Cp, ciprofloxacin 1; R, rifampicin 0.5; V, vancomycin 2; C, chloramphenicol 8.

Table 3
Staphylococcus aureus populations in contaminated bulk tank milk (BTM) samples from ruminant farms located in the regions of Macedonia and Thrace.

BTM type	BTM sample ID	Farm ID[a]	*S. aureus* counts (log cfu/ml)
Bovine	611	T036	3.6
Bovine	838	M014	3.6
Bovine	860	M012	4.0
Mean bovine			3.7
Ovine	492	T017	3.0
Ovine	506	T040	3.6
Ovine	520	T039	4.7
Ovine	608	T038	4.7
Ovine	609	T037	4.4
Ovine	612	T035	4.3
Ovine	614	T034	4.7
Ovine	667	T022	4.3
Ovine	899	M011	4.1
Ovine	962	M010	4.6
Ovine	985	M009	4.7
Mean ovine			4.3
Caprine	641	T024	4.3
Caprine	666	T022	3.7
Caprine	1058	M002	4.0
Mean caprine			4.0
Overall mean			4.1

[a] M, farm located in the region of Macedonia; T, farm located in the region of Thrace.

Eleven different pulsotypes (PT; designated as 01–11) were identified in 14 of the 16 MRSA isolates; these were assigned into eight clusters, designated as I -VIII (Fig. 1), whereas the remaining two, *spa* type t034 MRSA isolates proved to be non-typeable by *Sma*I-PFGE. Cluster IV was the most represented, comprising six isolates, followed by cluster VIII (two isolates), while the remaining clusters were only represented once (Fig. 1). An association between PFGE clusters and *spa* types was also observed. Cluster IV consisted of six isolates, which were all ascribed to *spa* type t127. Cluster VIII consisted of two *spa* type t3586 isolates and all the other clusters consisted of isolates of different *spa* types.

4. Discussion

S. aureus was detected in 47.8% of the tested samples and among these *S. aureus*, isolates from 4.1% of the samples were identified as MRSA. All the MRSA isolates were capable of biofilm formation, 81.3% (13/16) harbored the *mec*A gene and 93.8% (15/16) carried enterotoxin genes. The *mec*C or Panton-Valentine Leucocidin (PVL) genes were not detected. The predominant *spa* type was t127, and t034 (CC398) was isolated for the first time from livestock in the country. PFGE analysis confirmed a considerable level of genetic diversity among the MRSA isolates (11 pulsotypes and eight clusters).

The BTM results from cattle farms showed that the *S. aureus* prevalence estimates were lower, but those of MRSA were within the range of previously reported estimates (ranging from 41% to 62% for *S. aureus* and from 0.8% to 3.8% for MRSA) in the US (Haran et al., 2012) and

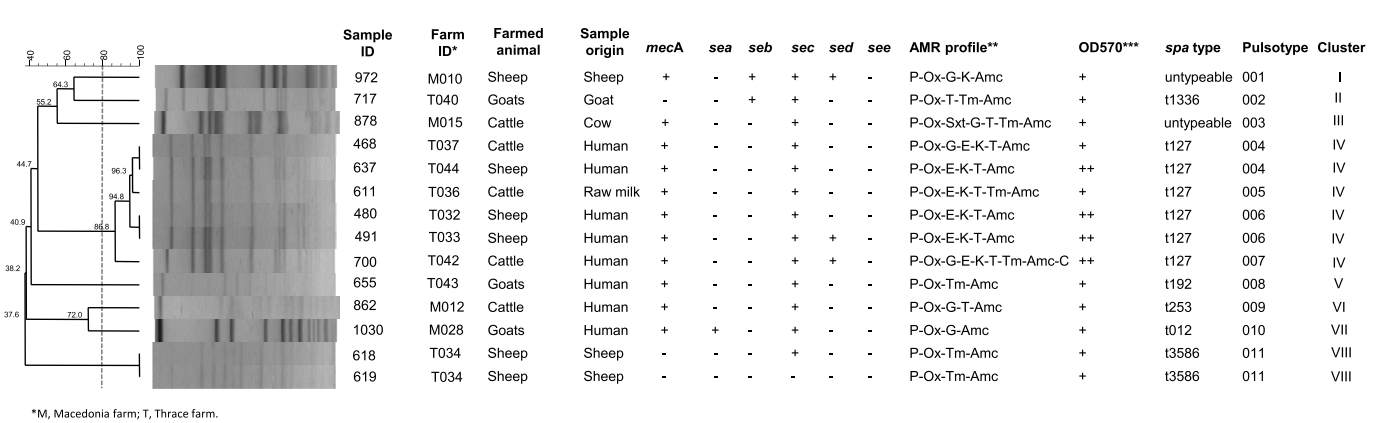

Fig. 1. Dendrogram of *Sma*I Pulsed Field Gel Electrophoresis (PFGE) pulsotypes (PT) and characteristics of the 14 MRSA isolates examined in this study (two *spa* type t034, CC398, MRSA isolates were non-typeable). The vertical dashed line indicates the cutoff (80% level of similarity). All 16 MRSA strains tested positive for the presence of the *nuc* and *coa* genes and negative for the presence of the *mecC* and PVL genes.

Italy (Cortimiglia et al., 2016; Parisi et al., 2016; Traversa et al., 2015). In Germany, the prevalence of MRSA in BTM from dairy herds was estimated to be 4.1% (in 2009) and 4.7% (in 2010) (Kreausukon et al., 2012; Tenhagen et al., 2014) and a recent study in conventional and organic dairy herds reported isolation frequencies of 9.7% and 1.7%, respectively (Tenhagen et al., 2018). Contrary to our results, a zero prevalence of *S. aureus* in cows' nares was reported in Norway (Jorgensen et al., 2005). In the Netherlands, 28% of the veal calves and 33% of the farmers tested carried MRSA in their nares (Graveland et al., 2010). In Italy, MRSA were isolated from 44% of the BTM samples, from 61% of the cows and from 36% of cattle farmers (Antoci et al., 2013). In contrast, 0% and 4.7% MRSA isolation frequencies have been reported from cattle farmers in Germany (Dahms et al., 2014) and South Korea (Lim et al., 2013), respectively.

Our data from small ruminants' farms show higher *S. aureus* and MRSA prevalence estimates compared to those previously reported in ovine and caprine BTM in Greece (Zdragas et al., 2015; Pexara et al., 2016). The reported isolation frequencies of *S. aureus* and MRSA in small ruminants' milk in other countries are quite variable, ranging from 43.1% to 76.9% and from 0% to 2%, respectively (Cortimiglia et al., 2015; Giacinti et al., 2017; Spanu et al., 2013). The *S. aureus* and MRSA isolation frequencies from the ruminants' nasal cavities in the present study were comparable to those reported from Denmark (Eriksson et al., 2013) and Tunisia (Gharsa et al., 2012, 2015). Regarding MRSA carriage by farmers, MRSA were isolated from 2.9% of goat farmers in the Czech Republic (Stastkova et al., 2009) and from all (three) goat farmers, but not from a sheep farmer in Italy (Caruso et al., 2016). In our study, the overall (cattle, sheep and goat farms) *S. aureus* and MRSA isolation frequencies from farmers were 51.5% and 13.2%, respectively.

The aforementioned differences in the reported *S. aureus* and MRSA prevalence estimates among different studies may be attributed to the sensitivity of the detection methods used i.e., selective isolation of MRSA vs. targeting *S. aureus* and testing of isolated *S. aureus* for MRSA phenotypic traits (Furuya et al., 2007).

All 16 MRSA isolates in our study were found to be PVL-negative, which is consistent with previous findings regarding milk-associated MRSA isolates (Antoci et al., 2013; Traversa et al., 2015).

Milk and dairy products are considered as the main source of enterotoxigenic MRSA isolates (Oniciuc et al., 2016). In dairy microbiology, sec has been the most frequently detected SE-gene among milk-associated isolates (Carfora et al., 2015; McMillan et al., 2016; Mehli et al., 2017). In agreement with our results, studies in Italy reported that almost all MRSA strains isolated from dairy cattle (Feltrin et al., 2016), bovine (Normanno et al., 2007) and ovine (Carfora et al., 2015; Macori et al., 2017) milk and dairy products were enterotoxigenic and previous investigations have documented the ability of MRSA to produce enterotoxins and cause staphylococcal foodborne outbreaks (Jones et al., 2002; Kerouanton et al., 2007). In contrast to our results, other researchers have reported low carriage of SE-genes among LA-MRSA isolates (Basanisi et al., 2017; Kreausukon et al., 2012).

Similar to our results, previous studies have reported the ability of MRSA isolates from milk and dairy products to form biofilms (Antoci et al., 2013; Parisi et al., 2016). Biofilm production not only enables bacteria to tolerate sanitation processes but also promotes horizontal spread of antibiotic-resistance determinants (Savage et al., 2013).

MRSA *spa* types are normally associated with specific MLST types (Hasman et al., 2010). The most prevalent *spa* type in our study was t127 (6/16), all of which were isolated from farms in the region of Thrace. Of note, *spa* type t127, which belongs to MLST ST1, was also isolated in the same time period from a food handler of a local (Thrace) dairy industry which receives raw milk from the investigated farms (Papadopoulos et al., 2019), indicating possible MRSA transmission through the dairy production chain, although further characterization via Whole Genome Sequencing may be required. It should be also noted that *spa* type t127 was also the dominant type among the MRSA recently isolated from dairy plants and farms in the region of Epirus (Papadopoulos et al., 2018), suggesting that it may represent the prevalent *spa* type in the dairy production chain in Greece. Interestingly, *spa* type t127 has been previously isolated from a patient in the internal medicine ward of a hospital in northern Greece (Kachrimanidou et al., 2014). *Spa* type t127 represents a prevalent lineage of LA-MRSA in Italy (Franco et al., 2011) and has been isolated from sheep milk (Caruso et al., 2016), goat milk (Cortimiglia et al., 2015), from both humans and animals in dairy sheep farms (Macori et al., 2017) and, recently, from raw bovine milk in Norway (Mehli et al., 2017).

Two MRSA strains, one isolated from a goat (farm no 22) and the other from a cattle farmer (farm no 24) belonged to *spa* type t034, a type strongly associated with the livestock-associated clonal complex (CC) 398 (Stegger et al., 2013). Both isolates were non-typeable with *Sma*I-PFGE (in three separate electrophoretic runs), in agreement with findings from previous studies which demonstrated that, due to DNA methylation, isolates belonging to CC398, ST398, including *spa* type t034, are generally non-typeable by standard *Sma*I-PFGE (Wulf and Voss, 2008). To the best of our knowledge, this is the first time that CC398 is isolated from animals in Greece, although it is considered as the most prevalent livestock-associated CC in Europe (Cuny et al., 2013). CC398 MRSA strains have been isolated in Greece from human patients with or without prior contact with animals (Drougka et al., 2012; Sarrou et al., 2015). *Spa* type t3586 has also been previously detected in Greece, in MRSA isolated from two sheep from different farms in the region of Epirus (Papadopoulos et al., 2018).

PFGE analysis revealed genetic diversity among the MRSA isolates. Overall, no specific association was noted between PTs and farms, with the detection of common PTs among farms being observed in some cases (Fig. 1). All MRSA isolates originated from different farms, except for two sheep MRSA strains that were isolated within the same farm, shared the same PT and belonged to cluster VIII. However, an association was noted between sample type and PFGE cluster: clusters I and VIII consisted of sheep isolates, clusters II and III consisted of goat and cow isolates, respectively, and clusters IV-VII consisted exclusively of human isolates, except for cluster IV which additionally contained a bovine-milk isolate.

PFGE cluster IV consisted of five human MRSA isolates and one bovine BTM isolate, which were all ascribed to *spa* type t127. All corresponding contaminated samples originated from different cattle or sheep farms in the region of Thrace, indicating the widespread distribution of this *spa* type in this geographical area. This significant clonal similarity among human and bovine BTM isolates indicates possible cross-contamination between farmers and milk and transfer into the food chain. Similar to our findings, a study in Italy reported that most of the *spa* type t127 MRSA isolates associated with cattle showed very high genetic relatedness (90–100% PFGE similarity) to human isolates (Alba et al., 2015). Interestingly, association between PFGE clusters, samples and *spa* types of LA-MRSA isolates has been previously suggested (McMillan et al., 2016). In contrast, and in agreement with previous findings (Xie et al., 2011), no association between SE gene-profile and PFGE cluster was noted (Fig. 1). However, this lack of association could be a result of the relatively low power (effect size) in our study, given that only 16 strains were analysed.

Although both *spa* and PFGE typing were able to distinguish all the MRSA isolates of the present study and confirm their high genetic diversity, PFGE further differentiated MRSA isolates of the same *spa* type. The genetic diversity of MRSA isolates, which was observed not only among animal species, but also among different farms, suggests the existence of multiple sources of contamination (Locatelli et al., 2016).

5. Conclusion

At the farm level, *S. aureus* and MRSA were detected in 34 (85%) and 14 (35%) out of the 40 participating farms, respectively. MRSA was isolated only from bovine BTM, but high MRSA isolation frequencies

were noted from animals and farm personnel in farms of all three species of lactating ruminants, pointing to the likelihood that besides raw bovine milk, milk from the other two species of dairy animals may constitute a possible source for the dissemination of MRSA into the dairy production chain and, subsequently, to the community. Furthermore, the isolation of MRSA strains with biofilm-formation ability is of concern and stresses the need for the continuous monitoring and evaluation of the applied cleaning and sanitizing procedures, both at the farm- and at the dairy-plant-facility level. The detection of SE genes in almost all MRSA isolates reveals the lurking threat (upon favorable conditions) of SFP, suggesting the need for implementing surveillance programs and prevention strategies even at the farm level.

The dominant *spa* type, at least in the region of Thrace, was t127. In association with our previous findings in other geographical areas, this finding suggests that t127 is the predominant *spa* type in the dairy production chain in Greece. Furthermore, MRSA *spa* type t034, CC398, which constitutes a major LA-MRSA lineage in Europe, was detected for the first time in animals in Greece. Finally, *spa* types t012, t192, t253 and t13336 were isolated for the first time in Greece.

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Declarations of interest

None.

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