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Occurrence and antibiotic resistance of enterotoxigenic *Staphylococcus aureus* in raw ovine and caprine milk in Greece

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Abstract Ovine ($n=140$) and caprine ($n=35$) raw bulk tank milk samples from farms in central Greece were examined for the occurrence of enterotoxigenic *Staphylococcus aureus*. The *S. aureus* isolates were screened for staphylococcal enterotoxin (SE) production, the presence of enterotoxin genes, antibiotic resistance (AR), and methicillin resistance. *S. aureus* was isolated from 24.3% and 31.4% of ovine and caprine milk samples, respectively. Among the *S. aureus* isolates of ovine milk ($n=34$) and caprine ($n=11$) milk, the enterotoxigenic (SEA-SED) isolates were 21 (61.8%) and 7 (63.6%) for the ovine and caprine milk, respectively. Most toxigenic isolates harbored more than one toxin gene and a total of 11 distinct toxinotypes were detected. The most frequent toxin-gene combinations were "*sec, tst*" (8 isolates), "*seb, seg, sei, tst*" (4), "*seb, seg, sei*" (3), and "*seb*" (3). Six isolates displayed multiple AR towards up to five antimicrobials. Among ovine milk isolates, the highest resistance frequency was observed towards erythromycin (11.8% of the isolates) and tetracycline (8.8%). Among caprine milk isolates, the most frequent resistance was observed towards erythromycin (18.2%). One methicillin-resistant *S. aureus* (MRSA) isolate was detected in an ovine milk sample and belonged to *spa* type t4038. This *spa* type

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has been isolated for the first time in Greece and, to our knowledge, has not been previously reported among MRSA isolates from raw milk or dairy products worldwide.

1 Introduction

Staphylococcus aureus is a Gram-positive, opportunistic pathogen for both animals and humans resulting in a plethora of clinical manifestations, whereas from a food safety perspective, *S. aureus* is a frequent cause of bacterial food intoxication in humans. Staphylococcal food poisoning (SFP) is caused by the ingestion of food containing one or more preformed staphylococcal enterotoxins (SEs) produced by enterotoxigenic strains of staphylococci and, in particular, *S. aureus*. Many serologically distinct SEs or SE-like toxins have been identified, which include the classical SEs (SEA through SEE), newer SEs (SEG through SEJ), and more recent ones (SEK through SEX). All enterotoxins share superantigenic activity, whereas some (SEA-SEE, SER, SES, SET) have been proven to be emetic. Toxic shock syndrome toxin-1 (TSSA-1), initially designated as SEF, is considered the major cause of toxic shock syndrome, and lacks emetic activity. Another public health concern is the presence of antibiotic-resistant (AR) and, in particular, methicillin-resistant *S. aureus* (MRSA) strains in food-producing animals and foods of animal origin (EFSA 2009).

S. *aureus* can be isolated from various environmental sites such as dust, water, air, and feces. A large percentage of the human population is a permanent or intermittent carrier of *S. aureus. S. aureus* is also present on the skin and mucosae of food-producing animals, and it is a frequent etiological agent of intramammary infections in dairy animals, including small ruminants. Consequently, *S. aureus* can gain access to the raw milk supply either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or via contamination from the environment during improper handling and processing of raw milk. Raw milk is a good substrate for *S. aureus* growth and, under permissive conditions, enterotoxin production as well (Pexara et al. 2012). Although pasteurization inactivates *S. aureus* cells, the SEs are resistant to heat and generally retain their biological activity even after pasteurization. As result, dairy products, including raw milk, have frequently been implicated in SFP (De Buyser et al. 2001).

S. *aureus* appears to be isolated at high frequencies from raw small ruminants' milk, and studies conducted in various countries have shown that *S. aureus* isolates from caprine milk (CM) and ovine milk (OM) frequently produce SEs or harbor genes encoding for their synthesis (Jørgensen et al. 2005; Morandi et al. 2007). In addition, AR and MRSA *S. aureus* strains have been isolated from both bovine and small ruminants' milk in various countries, as summarized in the review of Pexara et al. (2013). To our knowledge, no such data are available from Greece.

The objectives of this study were to estimate the prevalence of *S. aureus* in raw, bulk tank OM and CM produced in farms of central Greece and to examine the isolated strains in terms of (a) enteroxigenicity, (b) antimicrobial susceptibility, (c) the presence of encoding SE genes, and (d) the presence of MRSA encoding genes.

2 Material and methods

2.1 Sample collection

Samples from 136 OM and 35 CM farm bulk tanks (200–250 L milk) and 4 OM collection center (250–300 L milk) in the region of Thessaly, central Greece, were collected between February and May 2014. Milk was sampled from farms using hand milking. Sampling of milk was performed according to the International Dairy Federation guidelines (ISO 2008). Milk samples (100 mL each) were placed in sterile glass containers and transported to the laboratory at *ca*. 4 °C within 6–12 h after sampling.

2.2 Isolation of *S. aureus*

Isolation of coagulase-positive staphylococci (CPS) was performed according to ISO 6888-1 (ISO 1999) on Baird-Parker agar supplemented with egg yolk tellurite emulsion (Oxoid, UK). Confirmation of the isolates to the species level was based upon Gram staining, catalase reaction, hemolysis test on Columbia agar with defibrinated sheep blood (Oxoid, UK), and biochemical characterization (API ID 32 Staph, bioMérieux, France). One *S. aureus* isolate from each positive milk sample was selected for further characterization. All isolates were stored at −70 °C in Brain Heart Infusion broth (BHIB, Merck, Germany) with 25% glycerol.

2.3 Detection of enterotoxigenic *S. aureus* strains

The *S. aureus* isolates were tested for the production of classical staphylococcal enterotoxins using a commercial enzyme immunoassay kit Ridascreen, SET-total (R-Biopharm, Germany). Before testing, isolates were grown aerobically in BHIB for 24 h at 37 °C. Fully grown cultures were filtered through 0.2-μm membranes (Whatman FP30/0.2 CA-S, UK), and the filtrates were analyzed according to the manufacturer's instructions without quantification. The kit has a detection limit *ca*. 0.25 ng.mL^{-1}. Isolates found positive for SE production were tested using a different commercial enzyme immunoassay kit Ridascreen, SET-A,B,C,D,E (R-Biopharm, Germany) in order to determine the type of enterotoxin produced (A B, C, D, and E). The detection limit for this kit is in the range of 0.2–0.7 ng.mL^{-1}.

2.4 Detection of staphylococcal enterotoxin genes (*se*) and of the toxic shock syndrome toxin-1 gene (*tst*)

The *S. aureus* strains found to be enterotoxigenic based on the Ridascreen SET-total kit results were further examined for the presence of the *se* and *tst* genes by PCR using previously published primer sequences for *sea* (Tsen and Chen 1992), *seb-see* plus *tst* (Johnson et al. 1991), *seg-sei* (Jarraud et al. 1999), and *sej* genes (Monday and Bohach 1999). DNA extraction was conducted using a QIAamp DA minikit (Qiagen, Germany) according to the manufacturer's instructions. DNA amplification was performed in a Perkin-Elemer GeneAmp 2400 thermocycler (Applied Biosystems, UK). The reaction and amplification conditions used for the PCR assays were those described by Akineden et al. (2008).

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Positive controls used were *S. aureus* ATCC 19095 FRI 137 (*sec*, *seh*, *seg*, *sei*), ATCC 700699 (*sea*, *sec*, *seg*, *sei*, *sel*), ATCC 23235 (*sed*, *seg*, *sei*, *sej*), ATCC 14458 (*seb*), and ATCC 27664 (*see*).

2.5 Determination of antibiotic resistance

The antibiotic susceptibility of the *S. aureus* isolates to 13 antibiotics/antibiotic combinations was determined by the disk diffusion method according to the Clinical Laboratory Standards Institute guidelines (CLSI 2013) on Mueller-Hinton agar (Merck, Germany) plates. The antibiotics and the respective quantities (in μg) per impregnated disk (BBL, Becton Dickinson, USA) were as follows: tetracycline (TE, 30), amikacin (AK, 30), gentamicin (GM, 10), cephalothin (CF, 30), tobramycin (NN, 30), fusidic acid (FA, 10), amoxicillin+clavulanic acid (AMC, 20+10), ciprofloxacin (CIP, 5), sulfamethoxazole/trimethoprim (SXT, 23.75+1.25), ampicillin (AM, 10), cefoxitin (FOX, 30), clindamycin (DA, 2), and erythromycin (E, 15).

2.6 Detection of *mec*A gene and *spa* typing

DNA extraction from staphylococcal cells was performed according to Casey et al. (2006), and the detection of the *mec*A gene was carried out by PCR according to Murakami et al. (1991). A MRSA isolate from a previous study, *spa* type t127, was used as a positive control.

The amplification and sequencing of the *spa* gene was performed using a method based on DNA sequence typing of the *S. aureus* protein A gene (*spa* typing) which is currently considered a reliable single method for identifying the MRSA lineage (Aires-de-Sousa et al. 2006). Typing was performed through the publicly available Ridom Spa Server (www.spaserver.ridom.de) (Harmsen et al. 2003).

2.7 Statistical analysis

The 95% confidence intervals (CI) for the estimated proportions of *S. aureus*-positive OM and CM samples were calculated using the exact probability method of Minitab (version 14, Minitab Inc., State College, PA, USA). The estimated proportions and their respective CIs were multiplied by 100 to yield percentage values.

3 Results

3.1 Occurrence of enterotoxigenic *S. aureus* in ovine and caprine milk

S. aureus was isolated from 34 out of the 140 (24.3%) (95% CI=17.4–32.2%) samples of OM and from 11 out of the 35 (31.4%) (95% CI=16.9–49.3%) samples of CM. One isolate per positive sample was further characterized, and the number of enterotoxigenic *S. aureus* isolates along with the SEs produced is listed in Table 1.

Thirteen out of the 34 (38.2%) OM isolates were negative for enterotoxin production whereas the remaining 21 isolates (61.8%) were able to produce one of the "classical" SEs. SEB and SEC were the enterotoxin types most frequently detected, produced by

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Table 1 Production of enterotoxins (SEA-SEE) in *S. aureus* isolates from raw ovine (OM) and caprine milk (CM)

Sample origin	No of isolates	Enterotoxin types and number (%) of isolates producing the respective toxin						
		SEA	SEB	SEC	SED	SEE	SEA, SEC	Total enterotoxigenic (%)
OM	34	2 (9.5)	10 (47.6)	8 (38.1)	1 (4.8)	–	–	21 (61.8)
CM	11	1 (14.3)	–	5 (71.4)	–	–	1 (14.3)	7 (63.6)

10 and 8 *S. aureus* isolates, respectively, whereas none of the isolates were able to produce SEE.

For CM, seven out of the 11 (63.6%) tested isolates were found to be enterotoxigenic (Table 1) and SEC was the enterotoxin type most frequently detected. None of the CM *S. aureus* isolates could produce SEB, SED, or SEE.

3.2 Detection of *se* and *tst* genes

The toxin-gene profiles of enterotoxigenic *S. aureus* isolates are shown in Table 2. In CM isolates, the detection of *se* genes via PCR (*sea*, *sec*, and *sea+sec*) paralleled the isolates' enterotoxin production phenotype that was determined by the application of immunoassays. In contrast, in six of the OM isolates, no correspondence was observed between the presence of enterotoxin genes and their enterotoxigenic phenotype. Thus, only the *seg* gene was detected (via PCR) in one SEA-positive isolate (isolate O1) while only the *seb*, *seg*, and *sei* genes were detected in two SEC-positive (O19 and O20) and one SED-positive isolate (O21). It is important to note that one SEC-positive isolate (O18) yielded negative PCR results for all *se* genes tested, while the *sec* gene was detected in two SEC-negative isolates (O3 and O4).

Among all tested isolates, the genes most frequently detected were *tst* (53.6%), *seg* (50%), and *seb*, *sei*, and *sec* (46.4%). None of the isolates harbored *see*. In OM isolates, the genes most frequently detected (61.9%) were *seb* and *seg*. In CM isolates, *sec* was the gene most commonly detected (71.4%) whereas *seb* was not detected in any of the tested isolates.

Five of the enterotoxigenic isolates (17.9%) possessed only a single enterotoxin gene (*sea*, *seb* or *seg*). Eight isolates (28.6%) carried the *sec-tst* gene combination, and seven (25%) were positive for three genes (as shown in Table 2). Genotypes encoding four (*seb*, *seg*, *sei*, and *tst* or *seb*, *seg*, *seh*, and *sei*) and five (*seb*, *sec*, *seg*, *sei*, and *tst*) toxins were detected in five (17.9%) and two (7.1%) strains, respectively. The gene combinations most frequently detected (alone or in addition to other toxin genes) in toxin-gene-positive isolates were the *seg-sei* (detected in 12 OM isolates and one CM isolate) and the *tst-sec* (detected in six OM and five CM isolates).

3.3 Antibiotic susceptibility of *S. aureus* isolates

None of the 45 tested *S. aureus* isolates showed resistance to CF, FA, AMC, or SXT (Table 3). For the remaining of the antimicrobials tested, the resistance

Table 2 Toxin-gene profiles of enterotoxigenic *S. aureus* isolates from raw ovine and caprine milk

Isolate	Gene	Enterotoxin production phenotype[a]
O1	*seg*	A
O2	*sea, seg, sei*	A
O3	*seb, sec, seg, sei, tst*	B
O4	*seb, sec, seg, sei, tst*	B
O5	*seb, seg, sei, tst*	B
O6	*seb, seg, sei, tst*	B
O7	*seb, seg, sei, tst*	B
O8	*seb, seg, sei, tst*	B
O9	*seb*	B
O10	*seb*	B
O11	*seb*	B
O12	*seb, seg, seh, sei*	B
O13	*sec, tst*	C
O14	*sec, tst*	C
O15	*sec, tst*	C
O16	*sec, tst*	C
O17	*sec, seg, sei*	C
O18	–	C
O19	*seb, seg, sei*	C
O20	*seb, seg, sei*	C
O21	*seb, seg, sei*	D
C1	*sea*	A
C2	*sea, sec, tst*	A, C
C3	*sec, tst*	C
C4	*sec, tst*	C
C5	*sec, tst*	C
C6	*sec, tst*	C
C7	*sec, seg, sei*	C

O ovine milk isolate, *C* caprine milk isolate

[a] Enterotoxin-production phenotype as determined by the application of immunoassays

profiles of AR isolates were quite variable both in terms of the antimicrobial number and the type. For instance, whereas three isolates were found to be resistant to only a single antimicrobial (TE, E, or CIP), six isolates displayed multiple AR (to up to five antimicrobials, i.e., AK, NN, FOX, DA, and E in isolate O18). Overall, the highest resistance frequencies were observed for E (11.8%) and TE (8.8%) in OM isolates and for E (18.2%) in CM isolates. Interestingly, O18, the only isolate which displayed resistance to FOX, was found to carry the *mec*A gene (MRSA). The s*pa* typing of this isolate revealed type t4038. The MRSA isolate was able to synthesize SEC, but no *sec* gene was detected.

Table 3 Antibiotic resistance profiles of *S. aureus* isolated from raw ovine (OM) and caprine milk (CM)

Isolate origin	Resistance profile	No. of resistant isolates (isolate ID)
OM (*n*=34)	AK, NN, FOX, DA, E	1 (O18)*
	NN, DA, CIP, E	1 (O3)
	AK, CIP, E	1 (O12)
	TE, E	1 (O9)
	AM, TE	2 (O19, O16)
	TE	1 (O21)
CM (*n*=11)	GM, E	1 (C4)
	E	1 (C5)
	CIP	1 (C2)

TE tetracycline, *AK* amikacin, *GM* gentamicin, *CF* caphalothin, *NN* tobramycin, *FA* fusidic acid, *AMC* amoxicillin/clavulanic acid, *CIP* ciprofloxacin, *SXT* sulphamethoxazole/trimethoprim, *AM* ampicillin, *FOX* cefoxitin, *DA* clindamycin, *E* erythromycin

**mec*A-positive (MRSA) strain

4 Discussion

4.1 Prevalence of *S. aureus* in ovine and caprine milk

S. aureus was isolated from 24.3% of the OM samples and from 31.4% of the CM samples. Similar to our results, *S. aureus* was detected in 24% of the raw OM samples collected from Epirus, Greece (Fotou et al. 2011), whereas all milk samples collected from 21 sheep farms from the regions of Xanthi and Evros were positive for *S. aureus* (Alexopoulos et al. 2011).

The data regarding the prevalence of *S. aureus* in CM and OM from other countries are also quite variable (Mørk et al. 2003; Jørgensen et al. 2005; de Garnica et al. 2013; Spanu et al. 2013; Cortimiglia et al. 2015). Although differences in the sensitivity and specificity of the protocols used for bacterial isolation may have contributed to these variant results, the different husbandry practices applied in different countries may at least partly account for the aforementioned differences in prevalence estimates. Difficulties in managing the sanitary quality of CM and OM arise from a number of factors, including the low level of milk yield per animal, the milking process (frequently hand milking), the difficulties involved in machine milking, and the conditions under which herds or flocks are raised (Alexopoulos et al. 2011; Fotou et al. 2011).

4.2 Detection of enterotoxigenic *S. aureus*

A high abundance of SE-positive strains was observed among the *S. aureus* isolates in our study. These results are in accordance with literature data, particularly for CM, suggesting that goats are an important reservoir of enterotoxigenic staphylococci (Valle et al. 1990). Hence, SE production (SEA-SED) was demonstrated in 57.3–64% of the *S. aureus* isolates from Norwegian bulk CM (Jørgensen et al. 2005; Mørk et al. 2003). Forty five and 35% of the *S. aureus* isolates from bulk CM were characterized by

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enterotoxin production in Italy (Spanu et al. 2013) and Northern Ireland (Harvey and Gilmour 1988), respectively. However, other investigators reported a lower percentage of enterotoxigenic isolates (Foschino et al. 2002).

Among the types of SE produced by enterotoxigenic CM isolates, a high prevalence of SEC (85.7%) was observed, which is consistent with previous studies (Foschino et al. 2002; Harvey and Gilmour 1988; Morandi et al. 2007; Scherrer et al. 2004; Valle et al. 1990). High proportions (85.2–94%) of SEC production in SE-positive *S. aureus* isolates from bulk CM have also been reported in the studies of Jørgensen et al. (2005) and Spanu et al. (2013).

In our study, among the total enterotoxigenic OM isolates, a high percentage of SEC producers was observed (38.1%); however, the enterotoxin type detected at the highest frequency was SEB (47.6%). It should be noted, however, that since only one isolate per *S. aureus*-positive bulk tank milk sample was further characterized in our study, it is quite likely that more than one *S. aureus* genotype could have been present in the respective *S. aureus*-positive bulk tank milk samples. According to literature data, SEC appears to be the predominant toxin type detected in *S. aureus* isolated from OM and particularly from mastitic OM (Orden et al. 1992). It has been proposed that considerable geographical variation exists in the distribution of enterotoxigenic *S. aureus* (Jørgensen et al. 2005). The characterization of a greater number of OM isolates from different regions in Europe is necessary to determine the extent of the heterogeneity of *S. aureus* in terms of SE production.

In our study, with the exception of one CM isolate (C2) that was found capable of producing two enterotoxins (SEA and SEC) (Table 2), all SE-producing isolates produced only a single enterotoxin. Nonetheless, the ability of *S. aureus* strains to produce more than one SE has been reported and the combination of SEA- and SEC-producing strains was shown to be the most common among CM isolates producing two enterotoxins (Jørgensen et al. 2005; Valle et al. 1990). The low production frequency of other enterotoxins (especially SED) and the lack of SEE synthesis for both OM and CM isolates are in agreement with previous studies (Harvey and Gilmour 1988; Normanno et al. 2007a; Valle et al. 1990).

Published studies have attributed outbreaks of SFP after consumption of small ruminants' raw milk and raw milk products to the production of SEA, SEB, and SEC (De Buyser et al. 2001; Schønberg and Wåltorp 2001). Hence, in the present study, the *S. aureus* isolates found capable of producing any of these SE can pose public health risks. In particular, the high percentage of SEC-positive *S. aureus* isolates among other enterotoxigenic types (from both OM and CM) is of great importance because SEC has been recognized as an important cause of SFP associated with the consumption of dairy products (Manfreda et al. 2005).

4.3 Detection of *se* and *tst* genes

For all SE-positive OM isolates, the PCR assay results confirmed the presence of the corresponding *se* gene. However, this was not the case for five out of the 21 SE-positive CM isolates. Differences between the results obtained via PCR and those obtained via the application of immunoassays have been also reported previously (Cremonesi et al. 2007; Jørgensen et al. 2005; Morandi et al. 2007; Poli et al. 2007). In our study, the observation that the corresponding genes were not detected by PCR in

one SEA-positive and in three SEC-positive isolates by enzyme immunoassay (all from OM) is in agreement with previous studies. Morandi et al. (2007) proposed that the lack of detection of the corresponding genes in three SEA- and SEC-positive OM isolates could indicate the existence of sequence variations in *se* genes or the production of an unknown toxin that cross reacts during the immunological detection of SEA and SEC. Isolates testing positive for SEA by reversed passive latex agglutination but negative for *sea* by PCR have also been observed in animals other than sheep (Jørgensen et al. 2005).

On the contrary, the *sec* gene was detected in two SEC-negative OM isolates in our study. Similarly, Poli et al. (2007) and Cremonesi et al. (2007) did not detect SEC by SET-RPLA in the *sec*-positive isolates from cheeses made with raw bovine and CM, respectively. This finding may be explained by the lower sensitivity of the immunoassay methods used or by the fact that the detection of *se* genes does not necessarily indicate toxin production and biological activity (Morandi et al. 2007).

In agreement with previous studies, we found that enterotoxigenic CM isolates mainly harbored the *sec* gene (Akineden et al. 2008; Lyra et al. 2013; Scherrer et al. 2004). None of our CM isolates carried the *seb* gene. Similarly, several studies reported that none of the strains isolated from bovine and CM and dairy products harbored the *seb* gene (Akineden et al. 2001; Lyra et al. 2013). In other studies, however, the *seb* gene was detected in *S. aureus* isolates from milk and dairy products at various frequencies (Jørgensen et al. 2005; Zouharova and Rysanek 2008). The fact that *seb* was the most commonly detected SE genotype in isolates from OM in our study is in agreement with the findings of previous studies (Arcuri et al. 2010; Peles et al. 2007).

None of the strains in our study harbored the *see* gene, in agreement with other investigations on *S. aureus* isolates from milk and dairy products (Arcuri et al. 2010; Carfora et al. 2015; Cremonesi et al. 2007; Jørgensen et al. 2005; Poli et al. 2007; Zouharova and Rysanek 2008).

In the present work, half of the enterotoxigenic isolates carried two or more (up to four) *se* genes (Table 2). The presence of two or more *se* genes in *S. aureus* strains from milk and dairy products was also reported in other studies (Carfora et al. 2015; Cremonesi et al. 2007; Morandi et al. 2007; Poli et al. 2007).

The frequent combination of *seg* and *sei* genes in toxigenic *S. aureus* strains in our study is consistent with previous reports (Lyra et al. 2013; Rosec and Gigaud 2002; Scherrer et al. 2004). Some authors reported a predominant and systematic co-detection of *seg* and *sei* genes (Rosec and Gigaud 2002) whereas others have reported their presence individually or in different combinations with other *se* genes (Arcuri et al. 2010; Cremonesi et al. 2007; Zouharova and Rysanek 2008). The association between *seg* and *sei* has been attributed to their location within the same gene cluster (*egc*) in the genomic island mSA*b* (also known as SaPIn3/m3) (Malachowa and DeLeo 2010). In our study, one isolate harboring only the *seg* gene was found. The occurrence of strains harboring only *seg* or *sei* may be explained by mispriming due to a point mutation in one of these genes or the existence of variants in the *egc* cluster, combinations of toxin-gene-containing mobile elements such as plasmids and genomic islands in the same strain, or even a new type of genetic mobile element (Arcuri et al. 2010).

The co-detection of *sec* and *tst* genes observed in our study is consistent with other studies of *S. aureus* from mammary secretions of dairy animals and from bulk milk (Akineden et al. 2008; Jørgensen et al. 2005; Orden et al. 1992; Scherrer et al. 2004).

In our study, a high occurrence of "new" SE genes (especially *seg* and *sei*) was observed in isolates found positive for the production of classical SEs. Since, to date, no commercial tests for the "new" types of SEs exist, *S. aureus* isolates containing the corresponding genes should be considered as potential SE producers. A review of the literature seems to indicate an increase in the number of potentially enterotoxigenic *S. aureus* isolates from milk and dairy products carrying "new" SE genes in addition to the classical ones (Arcuri et al. 2010; Rosec and Gigaud 2002; Zouharova and Rysanek 2008). It should be noted, however, that in our study the prevalence of *se* genes may have been underestimated because only the isolates testing positive for SE production via immunoassays were tested with PCR, as well as due to the fact that the isolates were not tested for the presence of the recently described *se* genes (*sek-ser* and *seu*). Although Commission Regulation (EC) No 1441/2007 (EC 2007) specifies the term "staphylococcal enterotoxins" as those compounds which are detectable by commercial immunoassays (toxins SEA-SEE), further investigations are needed to evaluate the ability of *S. aureus* isolates to produce "new" SEs in milk and evaluate their role in food safety.

4.4 Antibiotic susceptibility of *S. aureus* isolates

The results of the present study revealed that the highest resistance frequency was observed towards E (11.8%) and TE (8.8%) among OM isolates and towards E (18.2%) among CM *S. aureus* isolates. When testing the antibiotic susceptibility of *S. aureus* isolates from OM and CM, Vyletělova et al. (2011) reported that 20% and 30% of the OM and CM isolates, respectively, was found resistance to E. Rola et al. (2015) reported that 6.3% of CPS isolated from raw CM was resistant to TE and FOX. TE and E are commonly used antibiotics in animals for the treatment of infections. The long-term usage of these antibiotics is considered to be the greatest risk for the selection of resistant strains.

The present study showed that three *S. aureus* isolates (6.7%) were resistant to three or more antimicrobial agents. Rola et al. (2015) reported that only 2.9% of CPS isolated from raw CM was resistant to three or more antimicrobial agents.

In our study, the MRSA isolate was resistant to AK, NN, FOX, DA, and E. Cortimiglia et al. (2015) reported that three out of four MRSA isolates from bulk CM were resistant to TE, besides being resistant to penicillin and FOX. In addition, two of them were resistant to trimethoprim and one to DA. The AR profile of their third MRSA isolate was different, displaying additional resistance to kanamycin, streptomycin, E, and DA.

The observation in our study that the MRSA isolate was able to produce SEs (SEC) is in accordance with previous studies. Normanno et al. (2007b) reported that all MRSA strains isolated from foods of animal origin in Italy were able to produce SEs. The most frequently detected enterotoxin was SED followed by SEA and SEC. MRSA frequently contain genes encoding for SE production (Fey et al. 2003). These findings provide further evidence that MRSA may also be involved in food poisoning outbreaks, as reported by Jones et al. (2002) and Kluytmans et al. (1995).

In our study, the detected MRSA *spa* type t4038 has been isolated for the first time in Greece and is not among the reported *spa* types from raw milk or dairy isolates (www.spaserver.ridom.de). According to Ridom Spa Server, the global frequency of

occurrence of t4038 is low and has been reported in the Netherlands. More frequently, other MRSA *spa* types seem to be associated with raw milk, dairy products, and farm personnel such as t127 (Carfora et al. 2015; Cortimiglia et al. 2015), t899 (CC398) (Cortimiglia et al. 2015), t011 (CC398), t567, and t108 (Bardiau et al. 2013) and *spa* types t011, t034, and t2576 (Fessler et al. 2010). Despite the fact that the *spa* type detected in our study is not among those associated with increased virulence in humans, typing of MRSA isolates can provide useful data on the epidemiology and monitoring of MRSA in foods.

In conclusion, the presence of enterotoxigenic *S. aureus* and MRSA in CM and OM and their potential introduction into the dairy chain could pose public health risks. The implementation of Good Hygiene Practices and Pre-Harvest Food Safety Principles at the farm level, the prudent use of antibiotics in veterinary medicine, and the avoidance of raw milk consumption are of paramount importance in order to minimize public health risks.

Compliance with ethical standards This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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