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Prevalence, genetic diversity, and antimicrobial susceptibility profiles of *Staphylococcus aureus* isolated from bulk tank milk from Greek traditional ovine farms

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ABSTRACT

Three hundred forty two milk bulk samples were tested for the presence of *Staphylococcus aureus* (*S. aureus*) originating from 114 ovine farms in Greece that followed 'transhumant breeding system'. The contamination of farms was low and was recorded at the level of 25.4%, 23.7% and 20.2% during the three sampling periods showing no significant difference. Eighty six out of 106 isolates (81%) were pansusceptible to the 16 antimicrobials tested, strongly suggesting that antibiotics are not used in a routine basis in this breeding system. Resistance was mainly to penicillin and found only at the rate of 6.6%. PFGE revealed 89 distinct pulsotypes, among 106 different isolates, clustering the majority of them into 6 main groups (80% similarity). PFGE showed that predominant clusters were circulating in this region during the study period and also that antimicrobial resistance didn't affect the predominance of these clusters. Basic biochemical tests can be used only as screening methods to identify *S. aureus* in raw ewe's milk, additional tests are needed such as STAPH-ID or PCR for confirmation. The findings of this work underscore the value of traditional ovine farming systems in food safety and antimicrobial resistance evolution. These traditional farming systems do not have high rates of antimicrobial agent consumption and subsequently low rates of antimicrobial resistance.

1. Introduction

Ovine industry in Greece is an important economic activity as well as in other Mediterranean countries like Spain and Italy. Traditionally, the main use of sheep milk is for cheese making conducted either in industrial scale or in small family dairies, producing protected designation of origin (PDO) cheeses like 'feta' and 'kasseri' adding significantly to the country's gross product. Transhumance was developed by the livestock farmers in order to cope with the grazing seasonality of Mediterranean (Galanopoulos et al., 2011). Nowadays, transhumant sheep and goat populations in Greece move toward mountainous rangelands at spring (especially May) and return to lowlands in the autumn (October–November) (Zervas, 1998). The

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population of transhumant sheep and goats in Greece stands for 7.5% of the total sheep and goat population (~1000,000 animals) using the natural vegetation of mountainous and semi-mountainous rangelands for 5–6 months (Laga et al., 2012).

Raw milk in general is colonized by a variety of microorganisms and hence they represent important sources of foodborne pathogens. The presence of pathogens in milk could either be caused by contamination from various sources, mainly the environment of the farm, or due to the secretion from the mammary gland to the milk in the case of sick animals.

Staphylococcus aureus is one of the most prevalent and economically significant pathogens, causing clinical and subclinical mastitis in small ruminants (Zangerl and Asperger, 2003; Scherrer et al., 2004; Katsuda et al., 2005). The pathogen infects udders and spread the contamination in raw milk and raw milk products (Zottola and Smith, 1993; Jørgensen et al., 2005). Therefore, the bulk milk contamination indicates the presence of *S. aureus* in the farm as a severe agent of clinical or subclinical mastitis. Moreover it is evidence for milk losses and an important contributor that affects the quality and quantity of milk and milk products (Fthenakis and Jones, 1990). In contrast to strains isolated from bovine bulk-tank milk (Stephan et al., 2001), little is known about the occurrence and distribution of various types of *S. aureus* in sheep and goat milk. However, in humans it causes toxic-mediated diseases such as toxic shock syndrome and staphylococcal food poisoning (Le Loir et al., 2003).

1.1. Milk samples

Over the lactation period (December 2011 to June 2012), 342 samples were collected from 114 ovine farms which implemented the transhumant breeding system. The samples were taken three times on each farm, in three different periods coded as period A (December–January 2012), period B (March–April 2013) and period C (May–June 2013). Farms were located in central Greece (region of Thessaly) with herd size varying from 300 to 600 sheep of local breeds. Sampling was carried out from refrigerated tanks before the milk was transported to milk processing plants for cheese (feta) production. Fifty milliliters per sample was aseptically collected and returned to the laboratory at 4 °C in a temperature controlled cool box.

1.2. Microbiological determinations

Enumeration of CPS (Coagulase Positive Staphylococci) was performed by spreading 0.1 ml of the milk sample on Baird–Parker agar base supplemented with egg yolk telluride emulsion (Merck, Darmstadt, Germany), followed by incubation at 37 °C for 48 h. If present, typical black colonies with or without an opaque halo were picked from plates and stored for further characterization. All the isolates were stored at −80 °C in Trypticase Soy Broth (Merck, Darmstadt, Germany) containing 10% of glycerol. Prior to the testing, the isolates were twice serially cultured on Columbia blood agar medium (Merck, Darmstadt, Germany), containing 5% of sheep blood, for 24 h at 37 °C under aerobic conditions. All suspected colonies were put through a series of preliminary basic tests for *S. aureus* identification; Gram staining, catalase activity, mannitol fermentation, growth in 7.5% saline, hemolytic activity on blood base agar (Merck, Darmstadt, Germany) with 5% defibrinated sheep blood, clumping factor and tube coagulase test (BBL™ Coagulase Plasma, Becton-Dickinson, USA). Further confirmation was carried out by applying the automate Microgen® Staph ID micro well identification system which contains 13 biochemical substrates in12 micro well format (nitrate is incorporated into the glucuronidase well) allowing 13 tests to be provided in the 12 micro wells. In some cases where the identification level was low (<90%) additional tests recommended from Microgen® Staph ID micro well identification system, prepared in house, were applied according to the manufacturer's recommendations.

Extraction of genomic DNA from bacterial cultures were conducted according to the protocol of DNA purification from Gram Positive Bacteria by the Pure Link Genomic DNA kit (Invitrogen, Carlsbad CA). *S. aureus* strains were identified by PCR amplification of *coa* and *nuc* genes. A 500–650 bp fragment of *coa* gene and 416 bp fragment of nuc gene were amplified using primer pairs previously described (12.13) The methicillin resistant *S. aureus* strains were identified by the amplification of a 304 bp fragment of *mecA* gene with the primer pair designed by Killgore et al. (2000).

The PCR reactions were performed in 20 μl final volume. Each reaction consisted of 1× PCR buffer (BioRon, Ludwigshafen, Germany), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 400 nM of each primer, 1 U of SuperHot polymerase (BioRon, Ludwigshafen, Germany) and 200 ng genomic DNA from the bacterial strains. The PCR steps include an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 20 s and extension at 72 °C for 40 s and with a final extension at 72 °C for 7 min. The PCR amplified products were electrophorized in 1.5% agarose gels and after staining with ethidium bromide were visualized under UV illumination by the TEX-20 M (Life Technologies, GibcoBRL System)

1.3. Antimicrobial susceptibility testing

Susceptibility to a panel of 16 antimicrobials was determined by agar dilution method in Mueller-Hinton agar (Merck, Darmstadt, Germany). The final plate concentrations ($\mu g\,ml^{-1}$) used were: penicillin (P) 0.25; sulfomethoxazole (S) 300; tetracycline (T) 8; erythromycin (E) 8; vancomycin (V) 4; chlopamphenicol (C) 32; cephalothin (Kt) 32; ciprofloxacin (Cp) 2; trimethoprim/sulfomethoxazole (Sxt) 4-76; gentamycin (G) 4; trimethoprim (Tm) 2; amoxicillin/clavulanic acid (AMX) 0.25–0.125; cefotaxime (Ct) 64;clindamycin (Cl) 4; rifampicin (R) 4;fosfomycin 32 mg. Minimum inhibitory concentrations of oxacillin (anti-staphylococcal β-lactames) were carried out by agar dilution test with the following dilutions 0.25, 0.5, 1, 2 and 4 $mg\,l^{-1}$, in order to identify suspected MRSA isolates. *S. aureus* ATTC 25923; *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

Table 1
Phenotypic and PCR characterization of suspected colonies.

	Suspected on Baird parker	Preliminary tests	Microgen ID	PCR—genes
A	59	47	39	39
B	54	44	44	40
C	40	31	27	27
Total	153	122	110	106
Sensitivity	N/A	100%	100%	N/A
Specificity	N/A	66%	91%	N/A

1.4. Pulsed-field gel electrophoresis

PFGE was performed using *SmaI* (Takara, Tokyo, Japan) as the restriction endonuclease as described previously (Sergelidis et al., 2014). Briefly, bacteria were grown overnight in 5-ml brain–heart infusion broth at 37 °C, harvested by centrifugation and washed by TE buffer (100 mmol l^{-1} Tris-base, 100 mmol l^{-1} EDTA, pH8). 100 μl of cell suspension in TE containing 1 μg of lysostaphin (Sigma Aldrich, Poole, UK) were mixed with an equal volume of 1.8% Seakem Gold agarose (FMC Bio-Products, Rockland, MD) in TE. The cell–agarose suspension was added into a block mould and allowed to solidify at 4 °C for 15 min, two plugs made for each strain. Agarose plugs were placed in 3 ml EC buffer (6 mmol l^{-1} Tris–HCl, pH 8; 1 mmol l^{-1} NaCl; 100 mmol l^{-1} EDTA, pH 8; 0.5% Brij58 (Sigma Aldrich, Poole, UK); 0.2% sodium desoxycolate (Difco Laboratories, Detroit, MI); 0.5% sodium *N*-lauroylsarkosine) and lysed overnight at 37 °C. Subsequently, the plugs were washed four times in TE buffer (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA pH8), each one for 15 min with gentle agitation at 54 °C and finally stored in fresh TE buffer at 4 °C until further analysis. Digestion of DNA was performed with 30 U of the restriction enzyme *SmaI* (TaKaRa, Kyoto, Japan) for 4 h at 25 °C. Restriction fragments of DNA were separated by PFGE using 1% Seakem Gold agarose gels (FMC Bio-Products) in 0.5× Tris–borate–EDTA buffer with CHEF-DRIII (Bio-Rad, Hercules, CA). *Salmonella* serotype Branderup strain H9812 digested with 40 units *XbaI* (TaKaRa, Kyoto, Japan) was used as size standard. Electrophoresis conditions were 14 °C for 21 h, with pulse time ranging from 5 to 40 s at an angle of 120°, and the voltage was 6 V cm^{-1}. Gels were stained with ethidium bromide and photographed. A database containing all the *SmaI* PFGE patterns was created by using Bionumerics software (ver.6.6 Applied Maths, Sint-Martens-Latem, Belgium), where band patterns over the multiple gels were normalized and compared. Clustering was performed using the Dice's similarity coefficient and the unweighted pair group method with arithmetic means (UPGMA), with 1.7% of tolerance and optimization. The diversity of PFGE type distribution was calculated using the Simpson's diversity index (D), which ranges from 0 (no diversity) to 1 (extreme diversity) as a measure for PFGE type diversity (Hunter and Gaston, 1988). Statistical comparison between different time periods and prevalence rates of *S. aureus* strains was analyzed by chi-square test (Microsoft Excel, 2007).

2. Results

One hundred and fifty three suspected colonies, according to the criteria described by ISO 6888-1: 1999, focusing on typical black colonial appearance with egg yolk positive or negative reaction, were collected and stored in Baird Parker medium in deep freezers (or −80 °C). The phenotypic analysis of these isolates using basic biochemical tests revealed 122 presumptive *S. aureus* out of 153 strains from Baird Parker, but only 106 of them were confirmed to be *S. aureus* by PCR (87%), while Microgen® Staph ID resulted in 110 positive *S. aureus* (96%) (Table 1, Fig. 1). Sensitivity for both tests was 100% but specificity was 66% for basic biochemical tests and 91% for Microgen® Staph

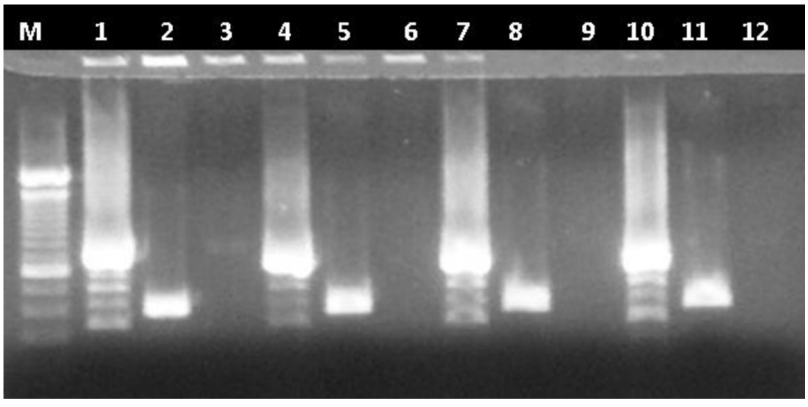


Fig. 1. PCR reactions performed on bacterial genomic DNA for the amplification of *nuc*, *coa* and *mecA* genes. Lanes 1, 4, 7 and 10: amplified PCR products of *nuc* gene. Lanes 2, 5, 8 and 11: amplified PCR products of *coa* gene. Lanes 3, 6, 9 and 12: amplified PCR products of *mecA* gene. Lane M: 100 bp DNA ladder. mecA gene was not detected.

Table 2
Contamination rates with *S. aureus* for sheep farms.

Sampling period ($N = 114$)	Staph. positive—PCR	Staph. negative—PCR	Total
A	29 (25.4%)	85 (74.6%)	114
B	27 (23.7%)	87 (76.3%)	114
C	23 (20.2%)	91 (79.8%)	114

Table 3
Antimicrobial resistance rates of *S. aureus* strains.

Antimicrobial agent(s)	*N*	%
Pansusceptible	86	81.1
Penicillin	7	6.6
Oxacillin (0.25 mg)	7	6.6
Erythromycin	3	2.8
Fosfomycin	2	1.9
Trimethoprim/sulfomethoxazole	1	0.9
Tetracycline	1	0.9

ID. The bulk tank milk of 114 farms were contaminated with *S. aureus* at the level of 25.4% during the A sampling period, 23.7% during the B and 20.2% during the C sampling period respectively. Farms that were free of *S. aureus* were 74.6% during A period, 76.2% during B period while at the C period was 79.8% (Table 2). Significant difference was not found in the isolation rates among different sampling periods ($P < 0.05$).

Based on the antimicrobials tested 86 out of 106 isolates (81%) were pansusceptible and only 7 of them showed resistance to penicillin (6.6%). Seven of the strains showed potential resistance to oxacillin (0.25 $\mu g\,ml^{-1}$—low dose) but *mecA* gene has not been detected by PCR, so these cannot be characterized as MRSA (Fig. 1). Sporadically a small number of strains showed resistance to other antimicrobials (Table 3). Resistance to penicillin in *S. aureus* strains was around 15% in B period but only 2.6% and 0% in A and C respectively showing significant difference ($P < 0.05$). The 106 *S. aureus* isolates could be divided into 31 PFGE groups in 6 main clusters (80% cut of value) and 89 different pulsotypes. Each pulsotype differed by at least one band from the other pulsotypes. The six main clusters consisted of 65 out of the 106 isolates. Cluster A consisted of 5 isolates, B and E with 12 isolates each, C 9 isolates, D 8 isolates and F 19 isolates. (Fig. 2) Simpson index of diversity was calculated with a value of 0.927. Resistance to penicillin was found in only 7 of the 106 investigated *S. aureus* strains and all of them represented by different single pulsotypes and 3 of them belonged to 3 of the main clusters, basically indicating that penicillin resistance was not due to a single clone. (Fig. 2)

3. Discussion

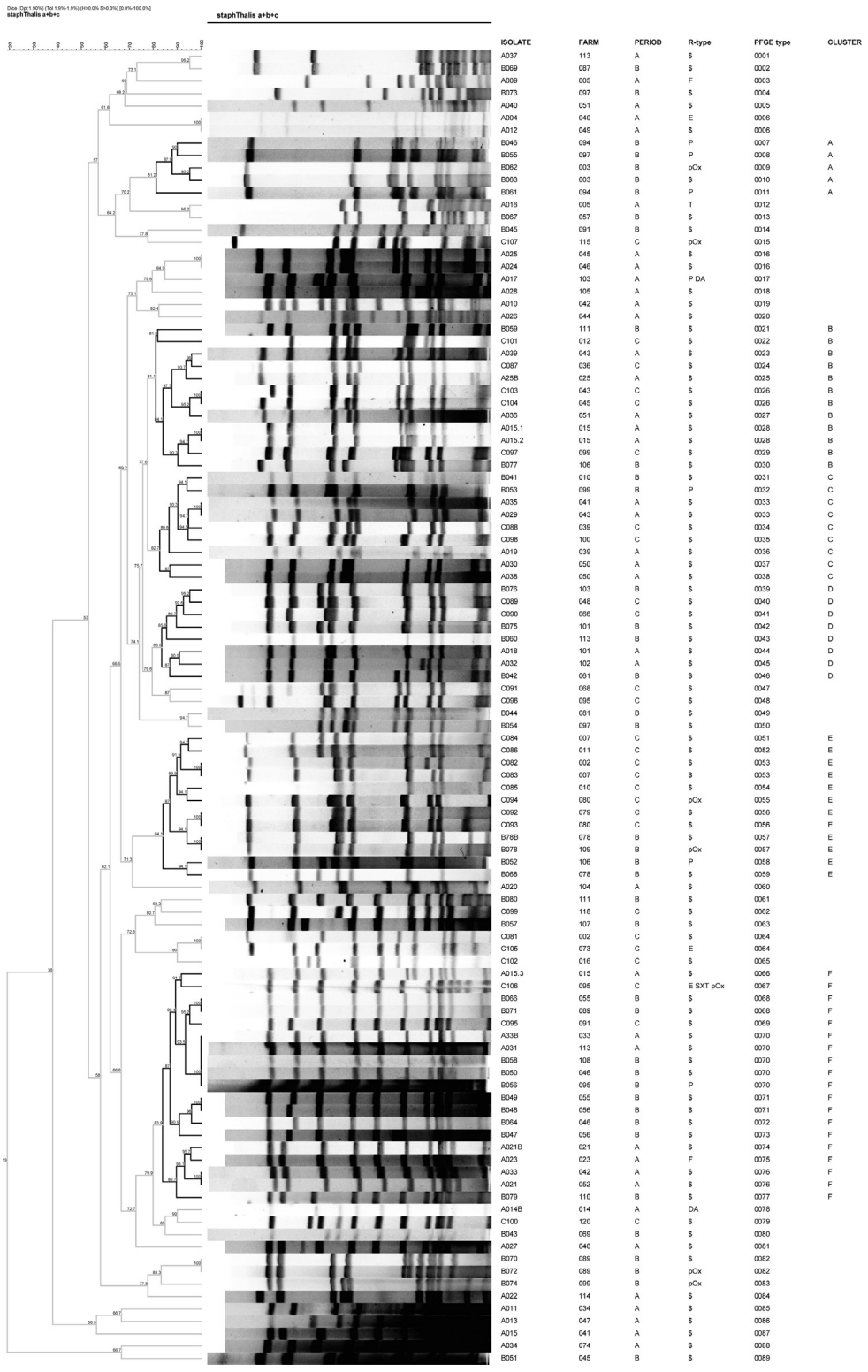
Staphylococci are the main etiological agents of small ruminants intramammary infections (IMI), the more frequent isolates being *S. aureus* in clinical cases and coagulase negative species in subclinical IMI (Bergonier et al., 2003). The pathogen is the main causative agent of clinical mastitis (20–94%) in ewe's meat (Bergonier and Berthelot, 2003) and also recovered from 65.3% of 547 clinically affected mammary glands in Norway (Mork et al., 2007).

Limited information is available about the contamination of ewe's raw bulk tank milk with *S. aureus*. Our results showed that the mean value of positive bulk tank milk samples from 114 flocks were 22.8%, higher than 14.85% referred by Linage et al. (2012) and related to 26.46% in Spain (de Garnica et al., 2013), but lower than 31.7–33.3% in Switzerland (Muehlherr et al., 2003). In contrast to our results a survey in Northern Greece showed that all the tested bulk tank samples from 26 flocks were contaminated with *S. aureus* (Alexopoulos et al., 2011). Another survey in Greece showed that *S. aureus* occurred in 24% of individual udder from healthy samples from 25 farms traditionally breaded and located in the mountain territory of Epirus (Fotou et al., 2011). However, in other countries higher contamination rates were recorded with 60% in Malaysia (Chye et al., 2004), 76.9% in Italy (Spanu et al., 2013) and sometimes reaching 100% of the examined flocks in Turkey (Gündoğan et al., 2006).

One hundred and twenty two colonies (79.7%) of the 153 colonies selected from Baird parker agar plates were considered to be *S. aureus* after the basic biochemical tests while 110 (71.8%) considered to be *S. aureus* using the Microgen® Staph ID kit. In total 106 of them (69.3%) were finally confirmed to be *S. aureus* by PCR amplification. The sensitivity for both methods were calculated to be 100% in this study while specificity was 66% for basic biochemical tests and 94% for Microgen® Staph ID kit. It was concluded that basic biochemical tests, including coagulase test and mannitol salt agar growth, can be used only as screening methods to identify *S. aureus* in raw ewe's milk. In order to avoid false negative results additional tests are needed such as Microgen® Staph ID or PCR. The findings of this survey are in line with the data recorded by Muehlherr et al. (2003) who found that 65.9% of *S. aureus* isolates from raw sheep milk were egg yolk negative. However, egg yolk reaction and phenotypical detection of clumping factor and/or protein A are not sufficient tests to identify *S. aureus* strains from any origin (Scherrer et al., 2004). The efficacy of Microgen® Staph ID kit has been well documented with 100% sensitivity and specificity in clinical human's specimen (Anyanwu and John, 2013) but it seems to have some limitation in strains isolated from sheep milk as specificity was around 94%.

It has been stated that PFGE of *S. aureus* is the most discriminant genotyping tool and a good method to resolve clonal relationships (Hennekinne et al., 2003; He et al., 2014). Generally, little is known about the occurrence and distribution of various types of *S. aureus* in sheep. Although a wide diversity of isolates was observed, six main clusters represented the majority of the isolates and were distributed throughout the regions included in this study.

The results of this survey indicates a strong genetic relationship of *S. aureus* isolates of raw bulk tank ewe's



ISOLATE	FARM	PERIOD	R-type	PFGE type	CLUSTER
A037	113	A	$	0001	
B069	087	B	$	0002	
A009	005	A	F	0003	
B073	097	B	$	0004	
A040	051	A	$	0005	
A004	040	A	E	0006	
A012	049	A	$	0006	
B046	094	B	P	0007	A
B055	097	B	P	0008	A
B062	003	B	pOx	0009	A
B063	003	B	$	0010	A
B061	094	B	P	0011	A
A016	005	A	T	0012	
B067	057	B	$	0013	
B045	091	B	$	0014	
C107	115	C	pOx	0015	
A025	045	A	$	0016	
A024	046	A	$	0016	
A017	103	A	P DA	0017	
A028	105	A	$	0018	
A010	042	A	$	0019	
A026	044	A	$	0020	
B059	111	B	$	0021	B
C101	012	C	$	0022	B
A039	043	A	$	0023	B
C087	036	C	$	0024	B
A25B	025	A	$	0025	B
C103	043	C	$	0026	B
C104	045	C	$	0026	B
A036	051	A	$	0027	B
A015.1	015	A	$	0028	B
A015.2	015	A	$	0028	B
C097	099	C	$	0029	B
B077	106	B	$	0030	B
B041	010	B	$	0031	C
B053	099	B	P	0032	C
A035	041	A	$	0033	C
A029	043	A	$	0033	C
C088	039	C	$	0034	C
C098	100	C	$	0035	C
A019	039	A	$	0036	C
A030	050	A	$	0037	C
A038	050	A	$	0038	C
B076	103	B	$	0039	D
C089	048	C	$	0040	D
C090	066	C	$	0041	D
B075	101	B	$	0042	D
B060	113	B	$	0043	D
A018	101	A	$	0044	D
A032	102	A	$	0045	D
B042	061	B	$	0046	D
C091	068	C	$	0047	
C096	095	C	$	0048	
B044	081	B	$	0049	
B054	097	B	$	0050	
C084	007	C	$	0051	E
C086	011	C	$	0052	E
C082	002	C	$	0053	E
C083	007	C	$	0053	E
C085	010	C	$	0054	E
C094	080	C	pOx	0055	E
C092	079	C	$	0056	E
C093	080	C	$	0056	E
B78B	078	B	$	0057	E
B078	109	B	pOx	0057	E
B052	106	B	P	0058	E
B068	078	B	$	0059	E
A020	104	A	$	0060	
B080	111	B	$	0061	
C099	118	C	$	0062	
B057	107	B	$	0063	
C081	002	C	$	0064	
C105	073	C	E	0064	
C102	016	C	$	0065	
A015.3	015	A	$	0066	F
C106	095	C	E SXT pOx	0067	F
B066	055	B	$	0068	F
B071	089	B	$	0068	F
C095	091	C	$	0069	F
A33B	033	A	$	0070	F
A031	113	A	$	0070	F
B058	108	B	$	0070	F
B050	046	B	$	0070	F
B056	095	B	P	0070	F
B049	055	B	$	0071	F
B048	056	B	$	0071	F
B064	046	B	$	0072	F
B047	056	B	$	0073	F
A021B	021	A	$	0074	F
A023	023	A	F	0075	F
A033	042	A	$	0076	F
A021	052	A	$	0076	F
B079	110	B	$	0077	F
A014B	014	A	DA	0078	
C100	120	C	$	0079	
B043	069	B	$	0080	
A027	040	A	$	0081	
B070	089	B	$	0082	
B072	089	B	pOx	0082	
B074	099	B	pOx	0083	
A022	114	A	$	0084	
A011	034	A	$	0085	
A013	047	A	$	0086	
A015	041	A	$	0087	
A034	074	A	$	0088	
B051	045	B	$	0089	

Fig. 2. Dendrogram of *SmaI* Pulsed Field Electrophoresis (PFGE) types and characteristics of the 106 *S. aureus* isolates examined in this study.

milk at the level of 80% in the majority of the isolates (61.3%). Closely related or identical isolates were detected in all sampling periods in different farms and probably this is an evidence that certain genotypes dominated in ewes. A study of genetic relationships between isolates of *S. aureus* recovered from mammary secretions was performed in sheep dairy farms in France and showed that in most of the investigated farms, a broad distribution of *S. aureus* types with identical or closely related banding patterns, were responsible for most of the *S. aureus* intramammary infections (Vautor et al., 2003). Same results were also shown in Spain and in Italy which found a large heterogeneity in genotypes among *S. aureus* isolated from ovine dairy flocks (Goñi et al., 2004; Spanu et al., 2013). It is interesting that 81% of isolates were susceptible to all the antimicrobials tested. Penicillin resistance at a rate of 6.6% was shown to be similar to other studies (Mørk et al., 2005) but low comparing to the 96.3% of Gündoğan et al. (2006). Surprisingly, *S. aureus* isolates were susceptible to the antibiotics usually used to cure mastitis such as ampicillin, sulbactam–ampicillin, tetracycline, amynoglycosides, trimethoprim and fluoroquinolones. This is a strong evidence of the low pressure of antibiotics in sheep industry in Greece especially in traditional breeding systems like transhumant. The full sensitivity to oxacillin at the concentration of 2 $\mu g\,ml^{-1}$, an antibiotic used for screening methicillin resistant *Staphylococcus aureus* (MRSA), shows the absence of the pathogen in raw ovine milk in Greece. In contrast, MRSA has been detected in bovine mastitic milk in Korea (Moon et al., 2007), Belgium (Vanderhaeghen et al., 2010), Germany (Spohr et al., 2011), Turkey (Türkyilmaz et al., 2010), Hungary (Juhász-Kaszanyitzky et al., 2007) as well as in bulk tank milk in Minnesota (Haran et al., 2012). Our data suggest that ovine milk from traditional farming is not a common source of MRSA and also indicating that penicillin resistance is not spreading through a single clone.

The findings of this work underscore the value of traditional ovine farming systems in food safety and antimicrobial resistance evolution. These traditional farming systems do not have high rates of antimicrobial agent consumption and subsequently low rates of antimicrobial resistance. These findings could also be a useful tool in understanding and controlling clinical and sub clinical mastitis in dairy ewes caused by *S. aureus* and to reduce milk losses due to mastitis as well as to avoid the contamination of milk products with this pathogen. Further studies should be performed in order to investigate the reservoirs of *S. aureus* in sheep flocks and apply strategies for contamination control.

Conflict of interest statement

None.

Acknowledgments

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References

Alexopoulos, A., Tzatzimakis, G., Bezirtzoglou, E., Plessas, S., Stavropoulou, E., Sinapis, E., Abas, Z., 2011. Microbiological quality and related factors of sheep milk produced in farms of NE Greece. Anaerobe 17, 276–279.

Anyanwu, N.C.J., John, W.C., 2013. Conventional and rapid methods for identification of *Staphylococcus aureus* from clinical specimens. AJBLS 1, 41–43.

Bergonier, D., Berthelot, X., 2003. New advances in epizootiology and control of ewe mastitis. Livest. Prod. Sci. 79, 1–16.

Bergonier, D., de Cremoux, R., Rupp, R., Lagriffoul, G., Berthelot, X., 2003. Mastitis of dairy small ruminants. Vet. Res. 34, 689–716.

Chye, F.Y., Abdullah, A., Ayob, M.K., 2004. Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol. 21, 535–541.

de Garnica, M.L., Linage, B., Carriedo, J.A., Santos, J.A., Gonzalo, C., 2013. *Staphylococcus aureus* and *Escherichia coli* prevalence in ovine bulk tank milk. Small Ruminant Res. 115, 108–112.

Fotou, K., Tzora, A., Voidarou, C., Alexopoulos, A., Plessas, S., Avgeris, I., Bezirtzoglou, E., Akrida-Demertzi, K., Demertzis, P.G., 2011. Isolation of microbial pathogens of subclinical mastitis from raw sheep's milk of Epirus (Greece) and their role in its hygiene. Anaerobe 17, 315–319.

Fthenakis, G.C., Jones, J.E.T., 1990. The effect of experimentally induced subclinical mastitis on milk yield of ewes and on the growth of lambs. Br. Vet. J. 146, 43–49.

Gündoğan, N., Citak, S., Turan, E., 2006. Slime production, DNase activity and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk, pasteurised milk and ice cream samples. Food Control 17, 389–392.

Galanopoulos, K., Abas, Z., Laga, V., Hatziminaoglou, I., Boyazoglu, J., 2011. The technical efficiency of transhumance sheep and goat farms and the effect of EU subsidies: do small farms benefit more than large farms? Small Ruminant Res. 100, 1–7.

Goñi, P., Vergara, Y., Ruiz, J., Albizu, I., Vila, J., Gómez-Lus, R., 2004. Antibiotic resistance and epidemiological typing of *Staphylococcus aureus* strains from ovine and rabbit mastitis. Int. J. Antimicrob. Agents 23, 268–272.

Haran, K.P., Godden, S.M., Boxrud, D., Jawahir, S., Bender, J.B., Sreevatsan, S., 2012. Prevalence and characterization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. J. Clin. Microbiol. 50, 688–695.

He, Y., Xie, Y., Reed, S., 2014. Pulsed-field gel electrophoresis typing of *Staphylococcus aureus* isolates. In: Ji, Y. (Ed.), Methicillin-Resistant *Staphylococcus aureus* (MRSA) Protocols. Humana Press, pp. 103–111, http://link.springer.com/book/10.1007%2F978-1-62703-664-1

Hennekinne, J.A., Kerouanton, A., Brisabois, A., De Buyser, M.L., 2003. Discrimination of *Staphylococcus aureus* biotypes by pulsed-field gel electrophoresis of DNA macro-restriction fragments. J. Appl. Microbiol. 94, 321–329.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466.

Jørgensen, H.J., Mørk, T., Høgåsen, H.R., Rørvik, L.M., 2005. Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. J. Appl. Microbiol. 99, 158–166.

Juhász-Kaszanyitzky, É., Jánosi, S., Somogyi, P., Dán, Á., Van Der Graaf-van Bloois, L., Van Duijkeren, E., Wagenaar, J.A., 2007. MRSA transmission between cows and humans. Emerg. Infect. Dis. 13, 630–632.

Katsuda, K., Hata, E., Kobayashi, H., Kohmoto, M., Kawashima, K., Tsunemitsu, H., Eguchi, M., 2005. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. Vet. Microbiol. 105, 301–305.

Killgore, G.E., Holloway, B., Tenover, F.C., 2000. A 5′ nuclease PCR (TaqMan) high-throughput assay for detection of the mecA gene in staphylococci. J. Clin. Microbiol. 38, 2516–2519.

Laga, V., Ragkos, A., Skapetas, V., Mitsopoulos, I., Kiritsi, S., Abas, Z., Mazaraki, K., Bambidis, V., 2012. Current trends in the transhumant sheep and goat sector in Greece. In: Acar, Z., López-Francos, A., Porqueddu, C. (Eds.), New Approaches for Grassland

Research in a Context of Climate and Socio-economic Changes. CIHEAM, Zaragoza, pp. 473–476, ⟨http://www.iamz.ciheam.org/samsun2012/files/OPTIONS%20Mediterranneennes%20A-102.pdf⟩ (accessed 12.2.2015).

Le Loir, Y., Baron, F., Gautier, M., 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. 2, 63–76.

Linage, B., Rodríguez-Calleja, J.M., Otero, A., García-López, M.L., Santos, J.A., 2012. Characterization of coagulase-positive staphylococci isolated from tank and silo ewe milk. J. Dairy Sci. 95, 1639–1644.

Moon, J.S., Lee, A.R., Kang, H.M., Lee, E.S., Kim, M.N., Paik, Y.H., Park, Y.H., Joo, Y.S., Koo, H.C., 2007. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J. Dairy Sci. 90, 1176–1185.

Mørk, T., Tollersrud, T., Kvitle, B., Jørgensen, H.J., Waage, S., 2005. Comparison of *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine, and caprine mastitis. J. Clin. Microbiol. 43, 3979–3984.

Mork, T., Waage, S., Tollersrud, T., Kvitle, B., Sviland, S., 2007. Clinical mastitis in ewes; bacteriology, epidemiology and clinical features. Acta Vet. Scand. 49, 23.

Muehlherr, J.E., Zweifel, C., Corti, S., Blanco, J.E., Stephan, R., 2003. Microbiological quality of raw goat's and ewe's bulk-tank milk in Switzerland. J. Dairy Sci. 86, 3849–3856.

Scherrer, D., Corti, S., Muehlherr, J.E., Zweifel, C., Stephan, R., 2004. Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. Vet. Microbiol. 101, 101–107.

Sergelidis, D., Abrahim, A., Papadopoulos, T., Soultos, N., Martziou, E., Koulourida, V., Govaris, A., Pexara, A., Zdragas, A., Papa, A., 2014. Isolation of methicillin-resistant Staphylococcus spp. from ready-to-eat fish products. Lett. Appl. Microbiol. 59, 500–506.

Spanu, V., Scarano, C., Virdis, S., Melito, S., Spanu, C., De Santis, E.P.L., 2013. Population structure of *Staphylococcus aureus* isolated from bulk tank goat's milk. Foodborne Pathog. Dis. 10, 310–315.

Spohr, M., Rau, J., Friedrich, A., Klittich, G., Fetsch, A., Guerra, B., Hammerl, J.A., Tenhagen, B.A., 2011. Methicillin-resistant *Staphylococcus aureus* (MRSA) in three dairy herds in Southwest Germany. Zoonoses Public Health 58, 252–261.

Stephan, R., Annemüller, C., Hassan, A.A., Lämmler, C., 2001. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. Vet. Microbiol. 78, 373–382.

Türkyilmaz, S., Tekbiyik, S., Oryasin, E., Bozdogan, B., 2010. Molecular epidemiology and antimicrobial resistance mechanisms of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk. Zoonoses Public Health 57, 197–203.

Vanderhaeghen, W., Hermans, K., Haesebrouck, F., Butaye, P., 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. Epidemiol. Infect. 138, 606–625.

Vautor, E., Abadie, G., Guibert, J.M., Huard, C., Pépin, M., 2003. Genotyping of *Staphylococcus aureus* isolated from various sites on farms with dairy sheep using pulsed-field gel electrophoresis. Vet. Microbiol. 96, 69–79.

Zangerl, P., Asperger, H., 2003. Chapter 6 media used in the detection and enumeration of *Staphylococcus aureus*. In: Janet, E.L., Corry, G.D.W.C., Rosamund, M.B. (Eds.), Prog. Ind. Microbiol. Elsevier, Amsterdam, pp. 91–110.

Zervas, G., 1998. Quantifying and optimizing grazing regimes in Greek mountain systems. J. Appl. Ecol. 35, 983–986.

Zottola, E., Smith, L., 1993. Growth and survival of undesirable bacteria in cheese. In: Fox, P.F. (Ed.), Cheese: Chemistry, Physics and Microbiology. Springer, US, pp. 471–492.