Extensive countrywide field investigation of subclinical mastitis in sheep in Greece

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ABSTRACT

The objectives of this work were (1) to investigate prevalence of subclinical mastitis, (2) to identify etiological agents involved, and (3) to study factors potentially predisposing ewes to subclinical mastitis. Milk samples were collected from 2,198 ewes in 111 farms with a total population of 35,925 ewes, in all 13 administrative regions of Greece, for bacteriological and cytological examination. Prevalence of subclinical mastitis was 0.260. Main etiological agents were staphylococci (*Staphylococcus aureus* and coagulase-negative species), which accounted for 0.699 of all isolates recovered; prevalence of staphylococcal mastitis was 0.191. In a multivariable mixed-effects analysis, the primary factor found to be associated with increased prevalence of subclinical mastitis was the management system practiced in flocks (flocks under a semi-intensive system had the highest prevalence). Other factors that were included in the multivariable model were the stage of lactation period (ewes in the 2nd month postpartum showed the highest prevalence) and application of postmilking teat dipping. In contrast, measures taken at the end of a lactation period (e.g., intramammary administration of antimicrobial agents) were not found to have an effect on prevalence of subclinical mastitis. The results confirmed the significance of subclinical mastitis as a frequent problem of ewes, with staphylococci as the primary etiological agent. The findings confirm the multifactorial nature of subclinical mastitis and indicate that its control should rely on many approaches.

Key words: dairy sheep, mastitis, prevalence, risk factor

INTRODUCTION

In sheep, mastitis is a multifactorial problem, with many bacteria identified as causal agents and many factors accounting for potential predisposition (Gelasakis et al., 2015; Fthenakis et al., 2017). Mastitis adversely affects production and causes financial problems, especially in dairy farms; it has also been recognized as the most important cause of welfare concerns in ewes (European Food Safety Authority, 2014).

In a recent literature review (Gelasakis et al., 2015), it was reported that field investigations on mastitis in ewes were limited in terms of number of animals sampled and number and geographical extent of farms. In the 15 papers reviewed therein, which described investigations in 10 countries, the median number of animals per study sampled was 380, number of farms was 11, and number of milk samples examined was 703 (Gelasakis et al., 2015).

This paper presents results of an extensive, countrywide study on subclinical mastitis in ewes across Greece. The investigation included 111 farms located in all 13 administrative regions of Greece; the total ewe population in these flocks was approximately 35,000 animals. In Greece, sheep production is the predominant form of agriculture, with over 95% of ewes farmed for dairy production. The objectives of this work were (1) to investigate prevalence of subclinical mastitis, (2) to identify etiological agents involved, and (3) to study factors potentially predisposing ewes to subclinical mastitis.

MATERIALS AND METHODS

Sheep Farms

In total, 111 sheep farms in the 13 administrative regions of Greece were included into the study and visited

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for collection of samples and information. Veterinarians active in small ruminant health management around Greece were contacted by telephone and asked if they wished to collaborate in the investigation. In total, 25 veterinarians were contacted; of these, 23 (0.92) agreed to collaborate. Farms were selected by the collaborating veterinarians on a convenience basis (willingness of farmers to accept a visit by University personnel for sample collection). The principal investigators (NGCV and GCF), accompanied by an assisting investigator (KSI, DAG, DCO, or APP), visited all farms for sample collection. The location of farms around the country is shown in Figure 1.

At start of each visit, breed of animals in the farm was recorded and an interview was carried out with the farmer to obtain various information regarding udder health management (Appendix Table A1). Further, the veterinarian was asked whether ancillary tests for diagnosis of mastitis were performed in samples from animals in the farm (possible answers: yes/no).

Animal Sampling

In each farm, 20 clinically healthy ewes (secundiparous or older) were selected for sampling. For selection of animals, farmers had been asked to remove primiparous ewes and ewes with known udder abnormalities from the main flock. The remaining animals were walked to the milking area and 20 ewes were selected by using an electronic random number generator (www.randomresult.com) among the first 50 (farms with >100 ewes, $n = 99$) or 30 (farms with ≤ 100 ewes, $n = 12$) animals that walked therein.

A standardized clinical examination of the udder (observation, palpation, comparison between glands) was performed, always by the principal investigator (NGCV; Fthenakis, 1994; Mavrogianni et al., 2005) and the first 2 squirts of secretion were drawn on the gloved hand of an assisting investigator and assessed. All investigators involved in sampling procedures wore disposable, nonsterile latex gloves (Alfa Gloves, Karabinis Medical SA, Peania, Greece). The principal investigator, who examined the animals and collected the milk samples, changed gloves after procedures in each animal were completed and before moving to the next one. If udder abnormalities [e.g., abnormal secretion, mammary nodules (i.e., firm space-occupying structures), papilloma-type lesions] were present, the ewe was excluded from sampling. Animals that were found with abnormalities and excluded were not replaced.

The orifice, edge, and lower half of the body of the teat were disinfected by single-use sterile gauzes, onto which povidone iodine 7.5% (Betadine surgical scrub, Mundipharma Medical Company, Basel, Switzerland)

had been poured, followed by wiping off by means of a new sterile gauze; different gauzes were used for each teat. Then, 10 to 15 mL of secretion was collected into a sterile container; separate samples were collected from each mammary gland into separate containers. Milk samples were then drawn directly onto a paddle for performing the California Mastitis Test (CMT).

Transportation of samples to the laboratory in Karditsa was always handled by the principal investigators. Samples were stored in portable refrigerators with ice packs and transported by car; for samples collected in islands, airplane (farms in Crete, Lesvos, or Rhodes) or boat (farms in Cephalonia) transportation, with accompanying luggage (but always ice-packed), was also involved.

Microbiological Examination

Laboratory procedures started within 24 h after collection. Milk samples (10 μ L) were cultured using Columbia blood agar plates incubated aerobically at 37°C for 48 h. If nothing had grown, media were re-incubated for another 24 h. Bacterial identifications were performed by using standard methods (Barrow and Feltham, 1993; Euzeby, 1997).

In total, 115 CNS isolates [91 from cases of subclinical mastitis (0.200 of such isolates) and 24 from cases of mammary carriage (0.145 of such isolates)] recovered in pure culture during the study were selected at random and identified to species level by using the Vitek 2 automated system (BioMerieux, Marcy-l'Étoile, France; definitions of subclinical mastitis/mammary carriage are detailed in the "Data Management and Analysis" section below). For selection of isolates for speciation among all those recovered, an electronic random number generator was employed.

Cytological Examination

After sample collection, at ewe-side, all samples were tested by use of the CMT. The test was performed as previously described for ewe milk (Fthenakis, 1995); it was always carried out and scored by the same person (i.e., the principal investigator, NGCV). Five degrees of reaction (negative, trace, 1, 2, and 3) were described (Schalm et al., 1971). Milk smears were also produced and dried.

Subsequently, the microscopic cell counting method (Mccm; IDF reference method; International Dairy Federation, 1984; Contreras et al., 2007; Raynal-Ljutovac et al., 2007) was performed in 894 samples (0.203 of all samples). The milk smears were stained by the Giemsa method for estimation of leukocyte subpopulations; proportion of leukocyte types therein was

Figure 1. Location of 111 sheep farms around Greece that were included in an investigation on subclinical mastitis. Color version available online.

calculated by observing at least 10 fields of each milk film under magnification 10 $\times$.

Data Management and Analysis

Subclinical mastitis was considered in ewes in which a bacteriologically positive milk sample [(1) >10 colonies of the same organism and (2) no more than 2 different types of colonies] with concurrently increased CMT score (≥ 1) plus neutrophil and lymphocyte proportion ($\geq 65\%$ of all leukocytes) was detected (Fragkou et al., 2014). Mammary carriage was considered in ewes in which a bacteriologically positive milk sample with no increased CMT score ($\leq$ trace) or neutrophil and lymphocyte proportion ($< 65\%$ of all leukocytes) was detected. Isolation of ≤ 10 colonies of an organism or isolation of over 2 different types of colonies from a sample was considered to indicate contamination.

Staphylococcal subclinical mastitis and staphylococcal mammary carriage were considered among the above ewes, respectively, in which staphylococci were identified in milk samples. All of the above definitions referred to ewes (hence, animals with both glands affected were counted as 1 case).

Quantitative information on the cellular content of ewe milk was obtained by using 2 sets of data: the CMT results and the results of the Mccm. Although it is generally established that CMT results are reliable proxy measurements for SCC (Fthenakis, 1995; Gonzalez-Rodríguez and Carmenes, 1996), we further confirmed that in the present study. Following assignment of numerical values to CMT scores (value 0 to score negative, value 1 to score trace, value 2 to score 1, value 3 to score 2, and value 4 to score 3) and $\log_{10}$ transformations, the correlation between CMT scores and Mccm SCC was $r = 0.913$ (95% CI: 0.902–0.923; $P < 0.001$) and the corrected R^2 was 83.4%.

Data were entered into Microsoft Excel (Microsoft Corp., Redmond, WA) and analyzed using STATA15 (StataCorp Inc., College Station, TX). Basic descriptive analysis was performed. The outcome of “subclinical mastitis” or “staphylococcal subclinical mastitis” was considered. Exact binomial confidence intervals were obtained. To estimate the prevalence of subclinical mastitis among all the ewes in the study farms, we declared farms to be strata and sampling weights to be $[1/(\text{number of ewes on the farm})]$ and used Jackknife analysis with STATA’s “SVY proportion” command. A preliminary assessment of the importance of predictors was performed by using cross-tabulation with the chi-squared test, and with simple logistic regression without random effects. Subsequently, mixed-effects logistic regression was employed to perform the same comparisons, using the different farms ($n = 111$) as a

random effect. The same procedure was then followed for evaluation of significance of predictors for staphylococcal subclinical mastitis, as well as for mammary carriage in farms into the study. In the above computations, specifically for evaluation of the significance of stage of the lactation period, the following categories were taken into account: 1st month after lambing, 2nd month after lambing, 3rd and 4th month after lambing, and 5th to 8th month after lambing. No other categories were collapsed during the analysis. Further, specifically for evaluation of significance of administration of antimicrobial agents at end of lactation period, the procedure was repeated by taking into account only farms in which ewes were at the 1st month of the lactation period. Moreover, specifically for evaluation of significance of anti-mastitis vaccination, 4 farms in which inappropriate vaccination schedules were performed were excluded from calculations. Finally, a further model was constructed to evaluate whether anti-mastitis vaccination status might have affected the possible effect of other predictors.

A multivariable model was created using mixed-effects logistic regression with farm as the random effect, and initially offering to the model all variables that achieved a significance of $P < 0.2$ in the univariable analysis. Variables were removed from this initial model by backward elimination. The P -value of removal of a variable was assessed by the likelihood ratio test, and for those with a P of > 0.2 the variable with the largest probability was removed. This process was repeated until no variable could be removed with a P -value of > 0.2 . The final multivariable model required the following variables: (1) management system in flock, (2) stage of lactation period in ewes in flock, and (3) application of postmilking teat dipping. Statistical significance was defined at $P < 0.05$.

RESULTS

Prevalence and Etiology of Subclinical Mastitis

The 111 farms had a total of 35,925 ewes. Ewe population per farm varied from 40 to 1,500 (mean: 323 ± 21.5) animals. In total, 2,220 ewes were examined. Among these, in 9 farms, in 22 animals, which the respective farmers believed to be normal, clinically evident mammary abnormalities were detected; prevalence was 0.010 (95% CI: 0.007–0.015). These included 5 animals with abnormal secretion and 17 animals with intramammary nodules or hardness.

Then, 2,198 ewes with clinically normal udders were sampled. Among these, 572 ewes were detected with subclinical mastitis; its prevalence in the population sampled was 0.260 (95% CI: 0.242–0.279). Within

Table 1. Frequency of bacterial isolates recovered from milk samples from ewes with subclinical mastitis or mammary carriage in Greece

Microorganism	No. of isolates (frequency of isolation) ¹	
	Isolates from subclinical mastitis ²	Isolates from mammary carriage ²
CNS	454 (0.597)	166 (0.597)
<i>Staphylococcus aureus</i>	77 (0.101)	11 (0.040)
<i>Streptococcus</i> spp.	36 (0.047)	1 (0.004)
<i>Corynebacterium</i> spp.	27 (0.036)	40 (0.144)
<i>Escherichia coli</i>	26 (0.034)	10 (0.036)
<i>Micrococcus</i> spp.	20 (0.026)	15 (0.054)
<i>Mannheimia haemolytica</i>	19 (0.025)	2 (0.007)
<i>Trueperella pyogenes</i>	19 (0.025)	2 (0.007)
<i>Acinetobacter</i> spp.	16 (0.021)	5 (0.018)
<i>Bacillus</i> spp.	12 (0.016)	17 (0.061)
<i>Enterococcus</i> spp.	9 (0.012)	0 (0.000)
Other ³	45 (0.059)	9 (0.032)
Total	760	278

¹Results presented on an animal basis. A sample from each mammary gland of 2,198 ewes in 111 farms was collected and examined separately; subclinical mastitis was detected in 0.260 of ewes.

²Subclinical mastitis was considered in ewes in which a bacteriologically positive milk sample (>10 colonies of the same organism and no more than 2 different types of colonies) with concurrently increased California Mastitis Test (CMT) score (≥ 1) plus neutrophil and lymphocyte proportion ($\geq 65\%$ of all leukocytes) was detected. Mammary carriage was considered in ewes in which a bacteriologically positive milk sample (>10 colonies in pure culture) with no increased CMT score ($\leq$ trace) or neutrophil and lymphocyte proportion ($< 65\%$ of all leukocytes) was detected.

³*Klebsiella* spp., *Kokuria* spp., *Proteus* spp., *Peptococcus* spp., *Pseudomonas* spp., *Rhodococcus* spp., and *Burkholderia* sp.

farms into the study, its prevalence varied from 0.000 to 0.850 (median value among farms: 0.250). The estimated overall proportion of ewes with subclinical mastitis in the study farms combined was 0.250 (95% CI: 0.157–0.344). In 430 ewes (0.752 of ewes with subclinical mastitis), bacteriologically positive milk samples and concurrently increased CMT score plus neutrophil and lymphocyte proportion was detected in only one mammary gland and in 142 ewes in both mammary glands [0.248 of ewes with subclinical mastitis; i.e., prevalence in the population sampled was 0.064 (95% CI: 0.055–0.076)].

In total, 760 bacterial isolates were obtained from ewes with subclinical mastitis (i.e., 1.33 isolates per ewe and 1.06 isolates per sample). The most frequently isolated bacteria from ewes with subclinical mastitis were *Staphylococcus* spp. ($n = 531$; *Staphylococcus aureus* and coagulase-negative species), which accounted for 0.699 of isolates from ewes with subclinical mastitis. In total, 419 ewes were detected with staphylococcal subclinical mastitis; its prevalence in the population sampled was 0.191 (95% CI: 0.175–0.208). Within farms in the study, its prevalence varied from 0.000 to 0.750 (median value among farms: 0.158). Overall, staphylococcal subclinical mastitis accounted for 0.733 of all cases of subclinical mastitis.

Other frequently isolated bacteria were *Streptococcus* spp., *Corynebacterium* spp., *Escherichia coli*, *Micrococcus* spp., *Mannheimia haemolytica*, and *Trueperella*

pyogenes. The frequency of the various bacterial isolates from ewes with subclinical mastitis is shown in Table 1. Among CNS species, *Staphylococcus chromogenes* was the one most often identified. Other frequently identified species were *Staphylococcus epidermidis* and *Staphylococcus simulans*. The frequency of the various CNS isolates identified is shown in Table 2.

Among ewes sampled, in 219, mammary carriage was detected; its prevalence in the population sampled was 0.100 (95% CI: 0.088–0.113). Within farms into the study, its prevalence varied from 0.000 to 0.533 (median value among farms: 0.053). In total, 278 bacterial isolates were obtained from ewes with mammary carriage (i.e., 1.27 isolates per ewe). The most frequently isolated bacteria from ewes with mammary carriage were *Staphylococcus* spp. ($n = 177$), which accounted for 0.637 of the isolates from ewes with mammary carriage. Overall, staphylococcal mammary carriage accounted for 0.630 of all cases of mammary carriage; its prevalence in the population sampled was 0.063 (95% CI: 0.053–0.074). Details of bacteria identified are in Tables 1 and 2.

In 361 ewes, only increased CMT score in milk was detected; hence, its prevalence in the population sampled was 0.164 (95% CI: 0.149–0.180). Within farms into the study, its prevalence varied from 0.000 to 0.450 (median value among farms: 0.150). Of the above ewes, 122 (0.338) were in 5th to 8th month after lambing. Further, in 98 ewes of above ewes (0.271), increased

Table 2. Frequency of CNS species recovered from milk samples from ewes with subclinical mastitis or mammary carriage in Greece

Staphylococcal species	No. of isolates (frequency of isolation) ¹	
	Isolates from subclinical mastitis ²	Isolates from mammary carriage ²
<i>Staphylococcus chromogenes</i>	21 (0.230)	7 (0.292)
<i>Staphylococcus epidermidis</i>	18 (0.198)	4 (0.166)
<i>Staphylococcus simulans</i>	16 (0.176)	0 (0.000)
<i>Staphylococcus hominis</i>	8 (0.088)	0 (0.000)
<i>Staphylococcus xylosus</i>	7 (0.077)	5 (0.208)
<i>Staphylococcus lentus</i>	5 (0.055)	4 (0.166)
<i>Staphylococcus caprae</i>	3 (0.033)	1 (0.042)
<i>Staphylococcus haemolyticus</i>	3 (0.033)	1 (0.042)
<i>Staphylococcus capitis</i>	2 (0.022)	0 (0.000)
<i>Staphylococcus cohnii</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus equorum</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus hyicus</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus saprophyticus</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus schleiferi</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus sciuri</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus warneri</i>	1 (0.011)	1 (0.042)
<i>Staphylococcus warneri</i> or <i>saprophyticus</i>	1 (0.011)	0 (0.000)
<i>Staphylococcus vitulinus</i>	0 (0.000)	1 (0.042)
Total	91	24

¹The isolates were selected at random among those isolated in pure culture and identified to species level by using the Vitek 2 automated system.

²Subclinical mastitis was considered in ewes in which a bacteriologically positive milk sample (>10 colonies of the same organism and no more than 2 different types of colonies) with concurrently increased California Mastitis Test (CMT) score (≥ 1) plus neutrophil and lymphocyte proportion ($\geq 65\%$ of all leukocytes) was detected. Mammary carriage was considered in ewes in which a bacteriologically positive milk sample (>10 colonies in pure culture) with no increased CMT score ($\leq$ trace) or neutrophil and lymphocyte proportion ($< 65\%$ of all leukocytes) was detected.

CMT score was detected in both glands of the animal; of these 98 animals, 42 (0.429) were in mo 5 to 8 after lambing.

Potential Predisposing Factors for Subclinical Mastitis

Management System. Farms into the study were dairy production flocks. At the start of each lactation period, lambs suckled their dams for 10 to 50–60 d after birth. Subclinical mastitis was detected more frequently in farms managed semi-intensively (prevalence: 0.296) or intensively (prevalence: 0.254) and less frequently in farms managed semi-extensively (0.196) or extensively (0.178); for staphylococcal mastitis, respective prevalence rates were 0.224, 0.179, 0.141 and 0.110 ($P < 0.001$ between all types of management system). Management system in flocks emerged from the multivariable mixed-effects model as the only significant factor for the prevalence of subclinical mastitis ($P = 0.008$).

Stage of Lactation Period. Subclinical mastitis occurred more frequently during the 2nd month of lactation period (prevalence: 0.305) or on 5th month and thereafter (prevalence: 0.292) and less frequently during 3rd and 4th months postpartum (0.246) or on 1st month postpartum (0.223); for staphylococcal mastitis, respective prevalence rates were 0.215, 0.227, 0.178, and 0.159 ($P < 0.005$ between all above periods). Also, it emerged that of the 26 *E. coli* isolates, 21 (0.808)

had been recovered during the first month of lactation period. From the multivariable mixed-effects model, stage of lactation period of ewes in flocks was not found significant for the prevalence of mastitis ($P = 0.151$).

Reproductive Control. Most farms into the study ($n = 81$, 0.730) did not practice reproductive control; the few farms that did practice reproductive control used intravaginal progestogen sponges ($n = 24$) or melatonin implants ($n = 6$). No association was evident between use of reproductive control and prevalence of subclinical mastitis ($P = 0.55$).

Sheep Breeds. Various breeds were recorded in visited flocks. Lacaune and Lacaune cross, as well as cross-breed animals, were the most numerous, seen in 38 and 23 farms, respectively. Highest prevalence of subclinical mastitis was recorded in farms with Friesarta (0.625), Assaf cross (0.600), Karagouniko cross (0.450), or Chios (0.318) breeds. Smallest prevalence of subclinical mastitis was recorded in farms with Assaf (0.100), Cephalonia (0.200), Crete (0.218), or Karagouniko (0.233) breeds.

Milking Practices. Differences on prevalence of subclinical mastitis or staphylococcal subclinical mastitis were seen between ewes that were milked (0.282 and 0.207, respectively), ewes that suckled lambs (0.214 and 0.167, respectively), or ewes in which milking and lamb suckling was applied concurrently (0.227 and 0.159, respectively; $P < 0.03$). Further, it emerged that of the 19 *M. haemolytica* isolates, 13 (0.684) had been

recovered in flocks, where ewes were suckling lambs at the time of sampling. In most farms into the study ($n = 74$; 0.667), machine-milking was performed. No difference on prevalence of subclinical mastitis was observed between farms where machine-milking (0.258) or hand-milking (0.285) was performed ($P = 0.49$); however, a difference on prevalence of staphylococcal subclinical mastitis was seen between farms where machine-milking was applied (0.179) and farms where hand-milking was practiced (0.228; $P = 0.035$). Machine-milking was applied mainly in farms with intensive (24 in 26 flocks) or semi-intensive (43 in 57 flocks) management ($P = 0.033$). Further, most *Streptococcus* ($n = 25$, 0.694) were isolated in flocks, where machine-milking was applied. Postmilking teat dipping was applied in only a few farms ($n = 12$; 0.108). In those, smaller subclinical mastitis or staphylococcal subclinical mastitis prevalence (0.196 and 0.128, respectively) was recorded than in farms where the practice was not applied (0.268 and 0.198, respectively; $P < 0.015$ for both comparisons). Odds ratio for prevalence of subclinical mastitis in farms, in which teat dipping was applied, was 0.665 (0.475–0.932, $P = 0.018$), compared with those in which the technique was not being practiced. From the multivariable mixed-effects model, application of post-milking teat dipping was not found to be significant for the prevalence of mastitis ($P = 0.054$).

Mastitis Preventive Measures at End of Lactation Period. In only a few farms ($n = 14$), administration of antimicrobial agents (complete or selective) was practiced at end of a lactation period. However, no differences in prevalence of subclinical mastitis or staphylococcal subclinical mastitis were evident between farms where antimicrobial agents were (0.265 and 0.194, respectively) or were not (0.260 and 0.190, respectively) used; similar findings were observed even when only farms with ewes at the first month of lactation period were taken into account (for farms where antimicrobial agents were administered: 0.193 and 0.143; for farms where antimicrobial agents were not administered: 0.228 and 0.163, respectively). In other farms ($n = 26$), ewes that had previously developed mastitis were culled at the end of the lactation period. In general, application of any of the above measures did not appear to be associated with prevalence of mastitis ($P > 0.55$ for all comparisons). No difference was evident among farms in the various management systems in intramammary administration of antimicrobial agents at the end of the lactation period or in anti-mastitis vaccinations ($P > 0.5$).

Anti-Mastitis Vaccination. Anti-mastitis vaccination was performed in 28 farms by using immunological products licensed in the European Union. Only anti-staphylococcal mastitis vaccines were em-

ployed. In 4 farms, inappropriate vaccination schedules (i.e., other than the one licensed) were undertaken. Prevalence of subclinical mastitis and staphylococcal subclinical mastitis in farms in which vaccination was applied correctly (0.221 and 0.167, respectively), were smaller than in farms in which no vaccination was applied (0.272 and 0.197, respectively). Odds ratio for prevalence of subclinical mastitis in farms, in which vaccination was applied, was 0.758 (0.596–0.966, $P = 0.025$), compared with those in which vaccination was not practiced. Finally, when possible effects of vaccination were evaluated in models, which already included other potentially predisposing factors, no interaction of vaccination with any factor was evident ($P > 0.5$). Postmilking teat dipping and vaccination were applied concurrently in 8 farms; in these, prevalence of subclinical mastitis and staphylococcal subclinical mastitis was found to be smaller (0.181 and 0.123, respectively) than in other farms (0.266 and 0.196, respectively; $P = 0.019$ and $P = 0.025$); prevalence was also smaller than in farms in which only one of these practices was being applied. The odds ratio for prevalence of subclinical mastitis in farms in which postmilking teat dipping and vaccination were applied concurrently was 0.608 (0.399–0.925, $P = 0.020$) compared with those in which neither practice was applied.

The findings regarding the various potential predisposing factors for subclinical or staphylococcal subclinical mastitis, with $P \leq 0.2$ in the univariable models, are summarized in Table 3. Results of the multivariable mixed-effects analysis are in Table 4.

Significance of Mammary Carriage

A tendency was observed for an association between prevalence of subclinical mastitis and mammary carriage in the same farm ($r = 0.128$, $P = 0.09$), which was stronger between prevalence of staphylococcal subclinical mastitis and staphylococcal mammary carriage ($r = 0.143$, $P = 0.067$).

Prevalence of mammary carriage was smaller in farms in which postmilking teat dipping was applied (0.097) than in farms in which it was not (0.119). Similarly, in farms in which anti-mastitis vaccination was applied, prevalence of mammary carriage and staphylococcal mammary carriage (0.088 and 0.050, respectively) was smaller than in farms in which it was not (0.101 and 0.066, respectively; for all above comparisons: $0.05 < P < 0.10$).

Use of Ancillary Tests for Diagnosis of Mastitis

In most farms ($n = 89$), no routine diagnostic laboratory examinations were performed to assist in diagnosis

Table 3. Summary of findings regarding the various potential predisposing factors for subclinical or staphylococcal subclinical mastitis (factors with $P \leq 0.2$ in the univariable models are presented)¹

Variable	Subclinical mastitis			Staphylococcal subclinical mastitis		
	Prevalence	OR (95% CI)	P-value	Prevalence	OR (95% CI)	P-value
Management system practiced in flocks ²			<0.001			<0.001
Intensive (n = 26)	0.254	1.576 (0.944–2.627)	0.082	0.179	1.763 (0.950–3.273)	0.072
Semi-intensive (n = 57)	0.296	1.946 (1.193–3.172)	0.008	0.224	3.083 (1.709–5.561)	<0.001
Semi-extensive (n = 22)	0.196	1.125 (0.664–1.907)	0.662	0.141	1.321 (0.699–2.497)	0.352
Extensive (n = 6)	0.178	Reference		0.110	Reference	
Stage of lactation period of ewes in flock			0.004			0.001
1st month pp (n = 39)	0.223	Reference		0.159	Reference	
2nd month pp (n = 22)	0.305	1.533 (1.170–2.009)	0.002	0.215	1.448 (1.068–1.964)	0.017
3rd and 4th months pp (n = 23)	0.246	1.138 (0.871–1.488)	0.343	0.178	1.144 (0.845–1.548)	0.384
5th month pp and thereafter (n = 27)	0.292	1.439 (1.118–1.852)	0.048	0.227	1.546 (1.1169–2.046)	0.002
Suckling lambs or milking		0.004				0.028
Ewes suckling lambs (n = 13)	0.214	Reference		0.167	1.058 (0.707–1.582)	0.786
Milking ewes (n = 70)	0.282	1.438 (1.041–1.985)	0.028	0.207	1.383 (1.063–1.799)	0.016
Ewes suckling lambs and milking concurrently (n = 28)	0.227	1.337 (1.061–1.686)	0.014	0.159	Reference	
Application of postmilking teat dipping			0.014			0.007
Application of postmilking teat dipping (n = 12)	0.196	Reference		0.128	Reference	
No application of postmilking teat dipping (n = 99)	0.268	1.504 (1.073–2.107)	0.018	0.198	1.689 (1.133–2.517)	0.010
Anti-mastitis vaccination			0.028			0.217
Vaccination (n = 24)	0.221	Reference		0.167	Reference	
No vaccination (n = 83)	0.272	1.319 (1.036–1.679)	0.025	0.197	1.224 (0.935–1.601)	0.142

¹pp = postpartum; OR = odds ratio; odds ratios calculated against the lowest prevalence associations of the variables.

²Management systems classified as per European Food Safety Authority (2014).

of mastitis. In these, prevalence of mastitis was smaller (0.251) than in farms where ancillary tests were performed (0.296; $P = 0.22$).

DISCUSSION

The paper describes an extensive field investigation of subclinical mastitis in ewes, one of the largest ever on a worldwide basis. Sheep farms in all regions of Greece were included into the study; that way, conditions prevailing throughout the country were taken into account

and factors of regional importance weighed less. Application of consistent methodologies for examination and sampling of animals, as well as performance of all tasks always by the same investigator, contributed to minimize possible bias in selection of farms.

Prevalence of Subclinical Mastitis

Present Results. Overall prevalence of subclinical mastitis has been calculated to be 0.260. In this study, the primary interest was to report the prevalence of mas-

Table 4. Results of multivariable analysis for prevalence of subclinical mastitis in ewes in Greece (mixed effects logistic regression with farm as random effect)

Variable	Odds ratio (95% CI) ¹	P-value
Management system practiced in flocks		
Intensive	2.002 (0.896–4.470)	0.008
Semi-intensive	2.481 (1.145–5.352)	0.090
Semi-extensive	1.292 (0.574–2.906)	0.021
Extensive	Reference	0.536
Stage of lactation period of ewes in flock		
1st month postpartum	Reference	0.151
2nd month postpartum	1.682 (1.084–2.612)	0.020
3rd and 4th months postpartum	1.159 (0.753–1.783)	0.502
5th month postpartum and thereafter	1.225 (0.808–1.859)	0.339
Application of postmilking teat dipping		
Application of postmilking teat dipping	Reference	0.054
No application of postmilking teat dipping	1.695 (0.989–2.905)	0.055

¹Odds ratios calculated against the lowest prevalence associations of the variables.

titis within farms and not to extrapolate to the entire population of ewes in Greece. For this, it was possible to estimate the overall prevalence of subclinical mastitis in the farms used, by declaring the farms to be strata; by using this approach, estimated overall proportion of ewes with subclinical mastitis in the study farms combined has been calculated to be 0.250. The results confirm subclinical mastitis as a frequent problem in sheep farms, the importance of which is further underlined by its financial significance. In dairy flocks, significance of subclinical mastitis becomes easily understood, as it has a well-documented detrimental effect on milk yield (causing up to 50% reduction; Saratsis et al., 1999; Giadinis et al., 2012) and composition (leading to changes adversely affecting cheese-making properties) (Fragkou et al., 2008; Marti De Olives et al., 2013; Abdelgawad et al., 2016). The importance of subclinical mastitis in meat-type production flocks is not straightforward; in such flocks, it can lead to growth retardation and suboptimal BW of lambs (Fthenakis and Jones, 1990b; Arsenaault et al., 2008). Further, subclinical mastitis can often develop to clinical mastitis (Fthenakis and Jones, 1990c), which has been singled out as the most important welfare problem of ewes across all production types and management systems (European Food Safety Authority, 2014). The study has also indicated variations between farms; within-farm prevalence varied from 0% to 85%. This large range is consistent with the multifactorial nature of mastitis. In view of that, results according to management system applied in farms have been produced and differences were found to be significant; more intensive management has been associated with increased prevalence of subclinical mastitis. The finding that management system emerged of high significance in the multivariable analysis, further supports this presentation.

Comparison with Previous Findings. Investigations into frequency of subclinical mastitis are of interest, because laboratory examination is required for accurate diagnosis; hence, it cannot be seen under clinical conditions and its financial losses cannot be clearly appreciated. In Greece, in a field investigation performed 25 yr ago, smaller prevalence of subclinical mastitis (up to 17%) has been recorded (Fthenakis, 1994). A significant change in the sheep industry in Greece during the last 25 yr has been its increasing intensification, with involvement of high-yielding breeds (e.g., Lacaune, Friesarta; Zygoiannis, 2006; Gelasakis et al., 2012). Further, practices consistent with intensification (e.g., increased stocking rate) have been associated with increased bacterial presence in milk (Albenzio et al., 2004; Caroprese, 2008) and hence may also contribute in the increased prevalence of subclinical mastitis in farms under intensive or semi-intensive management.

Although specific measures for prevention of mastitis have not been found to be widely implemented, the findings nevertheless indicate a clear increase in their application; at the time of the previous investigation (Fthenakis, 1994), such measures were virtually nonexistent in sheep farms in Greece. In France, Bergonier et al. (2003) reported that postmilking teat dipping was being performed only in a minority of sheep farms (20%), whereas administration of antimicrobial agents at end of lactation period was more widely practiced (70% of farms). It is noteworthy that in a recent study, sheep farmers in Greece considered administration of antimicrobial agents at end of lactation period of little significance for the welfare of their animals (Gougoulis et al., 2017), which is consistent with the current small prevalence of the practice. The results are also of significance to other countries in the region, where similar production type farms exist, especially given that, for example, in France, no relevant recent studies have been found (Gelasakis et al., 2015). In Italy, relevant reports involved work in limited number of flocks (1 to 15), with widely varying results (10 to 50% frequency of bacterial isolation; Gelasakis et al., 2015). In Turkey, prevalence of subclinical mastitis in one study (16 flocks) was found to be 18.5% (Ergun et al., 2009). In Spain, in an older study (Las Heras et al., 1999), prevalence of subclinical mastitis was found to be 34%. Of course, a direct comparison of results of field studies is always difficult, as different designs and techniques would have been applied, which might account for differences in results between studies. The significance of definition criteria is evidenced when a recent study by Dore et al. (2016) is considered; these authors published a meta-analysis of findings among Italian state veterinary laboratories, taking into account isolation of one colony of primary pathogens (e.g., *S. aureus*) and 5 colonies of CNS (from 10 μ L) to indicate bacteriologically positive samples (i.e., broader criteria than the ones in the present study) and reported 45% frequency of bacteriologically positive samples (i.e., greater than in the present study; Dore et al., 2016). Moreover, Las Heras et al. (1999) took into account isolation of 2 colonies of bacteria to indicate bacteriologically positive samples (again broader criteria than the ones in the present study) and reported 34% average prevalence of subclinical mastitis.

Etiology of Subclinical Mastitis

Significance of Causative Bacteria. Staphylococci (*S. aureus* and coagulase-negative species) have been identified as the primary etiological agents of subclinical mastitis. These results are broadly similar to those reported before, although in previous stud-

ies frequency of identification of *S. chromogenes* has been smaller, in contrast to our results, in which that species have been identified as the most frequent coagulase-negative staphylococci. *Staphylococcus aureus* has traditionally been considered as a cause of clinical mastitis and less frequently of subclinical (Fthenakis, 1994; Las Heras et al., 1999). *Staphylococcus aureus* isolation might also indicate an early stage of clinical mastitis, although it may also be the result of effective host defenses, which allowed only limited invasion that might have resulted in subclinical mastitis alone. The frequent isolation of *Streptococcus* from ewes in machine-milked flocks indicates a potential predisposing role for increased prevalence of subclinical mastitis specifically caused by that organism and can be aligned to the findings of Marogna et al. (2010) who reported an association between machine-milking of ewes and increased frequency of clinical mastitis of streptococcal etiology. The frequent isolation of *M. haemolytica* from ewes suckling lambs provides field corroboration of experimental results that we have published before (Gougoulis et al., 2008b; Fragkou et al., 2011), indicating transmission of the organism from the tonsils of lambs to the teat of ewes during suckling. *Mannheimia haemolytica* is the most frequent causal agent of mastitis in meat production flocks (Gelasakis et al., 2015), which obviously results from transmission of the organism during suckling. Further, frequent mastitis caused by *E. coli* during the first month postpartum might have resulted from uterine fluid contamination (Ioannidi et al., 2015). Environmental sources can also contribute to coliform infections, as, during that period, sheep are housed for longer periods and stocking densities are increased, which, in association with incorrect ventilation and high relative humidity, may lead in mastitis (Bergonier et al., 2003; Caroprese, 2008). Other organisms isolated have been identified as sporadic etiological agents of mastitis during lactation period in ewes. The significance of the various bacteria, such as *Corynebacterium* (Tel and Bozkaya, 2012; Dore et al., 2016), *Micrococcus* (Keisler et al., 1992; Dore et al., 2016), *Trueperella* (Saratsis et al., 1998; Ribeiro et al., 2015), and *Enterococcus* (Sanciu et al., 2013), has been discussed in case reports from around the world.

Significance of Mammary Carriage. The term mammary carriage (or carrier state; Verhoeven et al., 2014) is used to describe the presence of bacteria in the mammary gland (identified by isolation in milk samples) with no increased somatic cell numbers (i.e., in the absence of inflammation). In most cases, that refers to bacterial flora present in the teat duct or teat cistern (Fragkou et al., 2007a; Mavrogianni et al., 2007). Occasionally, the term may also refer to results of sampling a ewe at the early stage of infection before

an inflammatory reaction is elicited (Fthenakis and Jones, 1990c). The same outcome would also occur in cases of contamination of milk samples during collection; nevertheless, with experienced staff and strict maintenance of aseptic sampling conditions, that is minimal. For example, Rovai et al. (2014), in a field investigation, found that only 2% of milk samples from ewes were contaminated. In the present study, some association of mammary carriage with prevalence of subclinical mastitis has been found and was stronger for staphylococci. This may indicate that mammary carriage organisms might become pathogenic under the effect of various factors, which decrease defensive efficacy of hosts or promote pathogenicity of bacteria; that was documented with CNS in a previous experimental study (Fragkou et al., 2007a).

Significance of Bacteriologically Negative–Cytologically Positive Samples. Observation of CMT-positive samples, which were not bacteriologically positive, may be indicative of receding cases of subclinical mastitis, in which bacterial numbers were reduced or had fully disappeared, as the result of effective defenses of the host animals. In fact, in previous experimental studies of subclinical mastitis, increased cell content without bacterial isolation has been often recorded subsequent to d 20 postchallenge (Fthenakis and Jones, 1990b,c; Gougoulis et al., 2008a). Systemic and local efficient defenses in ewes contribute to achieving “self-cure,” especially when bacterial isolates have reduced pathogenicity. The importance of effective defense mechanisms in self-cure in staphylococcal mastitis has been underlined by Ali-Vehmas et al. (1997), who reported that subsequently to selenium supplementation (which contributes to improved cellular defense mechanisms) led to increased self-cure and halving the prevalence rate of mammary glands producing bacteriologically positive milk samples (Ali-Vehmas et al., 1997). Of course, infection by low virulence strains can also result in transient bacterial isolation followed by longer-lasting increased SCC (Fthenakis and Jones, 1990c). Further, one should also take into account that CMT-positive samples in ewes after the middle of a lactation period (particularly in ewes with bilaterally increased scores) may possibly reflect a “natural” increase in cellular content in milk of ewes, as the lactation period advances, a finding that has been previously reported by Fthenakis (1996).

Potential Predisposing Factors for Subclinical Mastitis

Management System Practiced in Flocks. In the multivariable analysis, flock management emerged as the only significant factor affecting prevalence of

subclinical mastitis. This finding may reflect that the management system would include various aspects of flock husbandry and organization [e.g., milking routine, animal breed, housing facilities, and nutrition, which have been considered to affect development of mastitis in ewes (Gelasakis et al., 2015; Fthenakis et al., 2017)]. Management system encompasses a variety of factors, which, to a varying extent for each one, contribute to subclinical mastitis or its control. The findings confirm the multifactorial nature of subclinical mastitis and indicate that its control should rely on many approaches.

Stage of Lactation Period. Differences in prevalence of subclinical mastitis were found between the various stages of lactation period. During the first month postpartum, which coincided with the period of lamb suckling, prevalence of mastitis was smaller than average. Suckling of lambs has been associated with transmission of bacteria; the possibility to transmit *M. haemolytica* from tonsils of lambs to teat duct has been discussed here above. Later, a sharp increase was recorded during the 2nd month, when lambs were taken away from their dams and full-scale milking started. In a previous study in Greece, the same period had been identified as the one with the highest incidence risk of clinical mastitis (Fthenakis and Jones, 1990a). One may suggest that the abrupt change stressed the mammary gland, on the one hand increasing IMI and on the other impeding local defenses, ultimately resulting in increased mastitis prevalence, as discussed by Mavrogianni et al. (2007). Subsequently, the gland returns to small rate of infections and mastitis, followed by a new increase toward the end of the lactation period, as chances of infection become perennial and frequency of mastitis increases (Fthenakis, 1994).

Milking Practices. Marco Melero (1994) was the first to identify transmission of staphylococci from hands of milkers to teats of ewes during milking. Mavrogianni et al. (2007) showed that hand-milking resulted in increased rate of bacterial colonization within the teat duct. Possibly, there were greater probabilities for organisms to colonize and cause mastitis, as in hand-milked animals postmilking teat dipping was not practiced frequently; any impediment of the defense mechanisms (local or systemic) may shift the balance and allow the bacteria to multiply, invade the mammary gland, and cause mastitis. Machine-milking has also been associated with transmission of staphylococci to teats (Bergonier and Berthelot, 2003). However, in the present study, farms in which machine-milking was applied were found with smaller mastitis prevalence, likely because of the application of accompanying mastitis preventing measures, specifically postmilking teat dipping, which reduces bacterial populations at the teat. The potential effects of postmilking teat dipping

in ewes have not been studied as extensively as in cows; there is only anecdotal evidence regarding effects of teat dipping in ewes (Paape et al., 2001; Bergonier et al., 2003). Present findings indicate that the practice is not widely applied. The results also provide some evidence ($P = 0.054$ in the multivariable model) regarding the beneficial role of the practice in reducing mastitis frequency in ewes. It is noteworthy that post-milking teat dipping was the only management factor, among those examined, that has been included in the multivariable model; omission of the practice has been found that it might result in up to 2.9 times higher prevalence of subclinical mastitis in respective flocks. However, one should be aware that wrong application of the practice can support mastitis development; for example, use of dense solution of a product would cause teat chapping, which can predispose ewes to mastitis (Mavrogianni et al., 2006; Fragkou et al., 2007b), or contaminated products can result in mastitis caused by infrequent pathogens (e.g., *Serratia marcescens*; Tzora and Fthenakis, 1998).

Mastitis Preventive Measures at End of Lactation Period. Intramammary administration of antimicrobial agents at end of lactation period is considered to contribute significantly in reducing incidence of mastitis during the dry period, as well as in the first days postpartum (Petridis and Fthenakis, 2014), findings that have not been confirmed at the present study. Reasons that might have accounted for this discrepancy could be application of an inappropriate treatment regimen by farmers or presence of resistant bacterial strains in the farms, which have been documented to occur frequently in Greece (Fthenakis, 1998).

Anti-Mastitis Vaccination. Anti-mastitis vaccination referred to only vaccination against staphylococci. This strategy can be considered as correct given that these bacteria account for the larger proportion of causal agents of mastitis (Lacasta et al., 2015; Fthenakis and Calvo Gonzalez-Valerio, 2017). Vaccines active against other pathogens (e.g., the J5 vaccine against *E. coli* mastitis or experimental vaccines against *Pseudomonas aeruginosa* or *Streptococcus uberis*) can be effective, but of little benefit for general use in flocks (Fthenakis and Calvo Gonzalez-Valerio, 2017). Vaccination against mastitis has been considered effective against staphylococcal mastitis (Vasileiou et al., 2016, 2017). In the present study, a smaller prevalence of staphylococcal mastitis was found among farms with vaccinated ewes and vaccination has been found to be associated with reduced odds of subclinical mastitis prevalence. Further, vaccination was found to be associated with reduced prevalence of staphylococcal mammary carriage; similar findings have been found in an extensive field trial of anti-staphylococcal mastitis vaccine in

Greece (Vasileiou et al., 2017). Given the association found between staphylococcal mammary carriage and mastitis and the development of clinical mastitis, reduction of mammary colonization by potential pathogens might be a pathway through which protective effects of vaccination might take place. No differences were evident according to management system in frequency of vaccination of ewes against mastitis, in contrast to postmilking teat dipping, which was being applied primarily in intensively managed flocks. Further, vaccination was performed more frequently than postmilking teat dipping or administration of antimicrobial agents at the end of lactation. That would indicate that antimastitis vaccination might be integrated into the health management of the flock conveniently, possibly because farmers prefer the practice and can incorporate it into the general routine of flocks more easily.

CONCLUSIONS

The results confirmed the significance of subclinical mastitis as a frequent problem of sheep. Staphylococci were the primary etiological agents. Management system practiced at farms was identified as the most important factor predisposing animals to subclinical mastitis. Animal-related factors (e.g., stage of lactation period) may also play a role in development of mastitis. Application of targeted health management methods (e.g., postmilking teat dipping) may be associated with reduced prevalence of subclinical mastitis.

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APPENDIX

Table A1. Information collected by means of interview of farmers in 111 sheep farms¹ visited across Greece

Question	Possible answers	No. of farms
Production type in flock	Breeding	0
	Dairy	0
	Dairy and meat	111
	Meat	0
	Wool and hide	0
Management system in flock ²	Intensive	26
	Semi-intensive	57
	Semi-extensive	22
	Extensive	6
	Shepherding	0
	Mixed	0
Stage of lactation period in ewes in flock (i.e., at time of the visit)	Month postpartum	
	1st	38
	2nd	20
	3rd	10
	4th	14
	5th	4
	6th	9
	7th	5
	8th	9
	1st and 2nd	1
	2nd and 4th	1
	Suckling lambs	13
	Milking	70
	Suckling lambs and milking concurrently	28
Use of reproductive control	Yes	30
	No	81
Method of reproductive control employed (in flocks that applied such control)	Use of progestogen sponges	24
	Use of melatonin implants	6
Milking technique applied (in dairy/dairy and meat production flocks)	Machine-milking	74
	Hand-milking	37
Application of postmilking teat dipping (in dairy/dairy and meat production flocks)	Yes	12
	No	99
Intramammary administration of antimicrobial agents at end of lactation period	Yes	14
	No	97
Type of intramammary administration of antimicrobial agents at end of lactation period (in flocks that applied such procedure)	Selective	4
	Complete	10
Culling of ewes with history of mastitis during preceding lactation period	Yes	26
	No	85
Application of mastitis preventive measures at end of a lactation period ³	No measures applied	74
	Culling of ewes	23
	Administration of antimicrobial agents	11
	Culling of ewes and administration of antimicrobial agents	3
Application of anti-mastitis vaccination	Yes	28
	No	83
Mammary pathogens against which vaccination was performed (in flocks that applied such procedure)	Staphylococci	28
	Other	0
Use of licensed vaccine or autogenous vaccine (in flocks that applied such procedure)	Licensed vaccine	28
	Autogenous vaccine	0
Vaccination schedule followed (in flocks that applied such procedure)	Licensed schedule	24
	Own schedule	4

¹Mean number ($\pm$ SE) of ewes in flock = 323.5 ± 21.5 .

²Management systems classified as per the European Food Safety Authority (2014).

³Information deduced by the investigators after taking into account results of interviews.