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Research Article

Antimicrobial Drug Resistance in Sheep Farms in Greece: Assessment of the Current Situation and Investigation Towards Improving Surveillance and Control

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Abstract

The aim of this study was to assess the spread of antimicrobial resistance (AMR) in sheep farms in Greece and identify potential indicators to improve field surveillance.

Ninety-four samples of milk, drinking water, animal feed, bedding, and faeces were collected from 5 dairy sheep farms. The samples were processed for isolation of Staphylococcus aureus and Escherichia coli, and the assessment of AMR using conventional microbiology and the polymerase chain reaction.

Positivity to Escherichia coli and Staphylococcus aureus was 38.3% and 16%, respectively (36 and 15 of 94). Detection of Escherichia coli in animal feeds was significantly higher compared to the other types of test samples, and the presence of Staphylococcus aureus AMR in the certain types of samples increased probability of its detection in milk by 3.25 times. Investigation for associations between sample positivity with the use of antibiotics indicated that the higher the amount of antibiotics, the higher the proportion of Escherichia coli non-susceptible to at least two antimicrobial categories (AMR+), detected in milk. Escherichia coli isolates were significantly more likely to be AMR+ when the latter pathogen was resistant to ampicillin.

The results indicate that AMR is a common problem in the sampled farms, which is associated with high occurrence of mastitis and poor antibiotic stewardship for its treatment. Animal feeds and milk collected from the bulk milk tank proved suitable for the assessment of AMR, as did detection of ampicillin-resistant Escherichia coli. Isolation of AMR Staphylococcus aureus in animal feeds emerged as a promising indicator for monitoring mastitis.

Keywords: Antimicrobial Resistance; Staphylococcus aureus; Escherichia coli, Antibiotics; Dairy Sheep; Multidrug Resistance

Introduction

Antimicrobial drug resistance (AMR) is recognised by the World Health Organization as a global health and development threat that requires urgent multisectoral action. Misuse and overuse of antimicrobials are recognised as the primary causes of the emergence and spread of resistant strains, a problem that is exacerbated by poor water sanitation practices and inadequate disease prevention and control measures [1].

Despite public concern, antimicrobial drugs have been integrated into animal husbandry not only for therapeutic purposes but also as growth promoters [2]. In the latter case, the prolonged use of drugs at sub-therapeutic levels augments AMR and exerts a selection pressure which favours preservation of resistant genes in the environment [3,4]. Unfortunately, the need for increased productivity can hinder the efforts to minimise non-therapeutic use of drugs in animal production, particularly in highly competitive sectors, such as pig and poultry [2,5-7]. In this regard, decreasing the dependence of modern animal husbandry on antimicrobial drugs is a challenge, which requires accurate and cost-effective tools of farm surveillance and AMR monitoring [2].

For the reasons mentioned above, this study aimed to assess the presence of AMR in sheep farms in Greece, identify potential indicators of their spread and improve field surveillance. To this goal, *Staphylococcus aureus* and *Escherichia coli* were used as indicators of AMR in sheep farms, because they are recognised as common foodborne pathogens and are often used as epidemiological markers for monitoring AMR transfer between animals and humans [8].

Materials and Methods

Sample collection

Samples were collected from five (n = 5) dairy sheep farms, herein referred to as Farms A-E. These farms were selected based on location (different geographic regions and easily accessible locations), (Illustration 1), size [large (Farm A: 1520 sheep), medium (Farms C: 467 sheep, and D: 690 sheep), and small (Farms B: 240 sheep, and E: 270 sheep)], breeding practice [closed intensive (Farm D), semi-intensive (Farms A and C), and semi-extensive (Farms B and E)], and the availability of a reliable farm record, which was confirmed onsite through inspection and personal interview. Farm managers were asked to complete the Biocheck.

Illustration 1: The location of the study Farms (A-E).

UGent© biosafety questionnaire for cattle (https://biocheck.ugent.be), which was adapted to sheep, and included additionally, questions on management practices, measures of hygiene, use of antibiotics, and records of disease and vaccination (Table 1). Extensive sheep farms were not included in this investigation because none of those that consented to participate could satisfy the requirement for a reliable farm record.

Ninety-four (n = 94) samples consisting of milk (n = 45, 9 samples/farm), drinking water (n = 15, 3 samples/farm), animal feed (n = 14, 6 samples in Farm A, 2 in Farms B-E), floor bedding (n = 10, 2 samples/farm, 10 gr/sample), and faeces [n = 10 (samples collected from the floor), 2 samples/farm, 10 gr/sample] were collected aseptically from the Farms A-E, between June and August 2020 (Table 2). Milk samples were obtained from the bulk milk tank (n = 5, 1 sample/farm, 80 ml/sample) and from both udders of eight (n = 40, 8 samples/farm, 20 ml/sample) randomly selected, clinically healthy adult animals. The water samples were collected from the main water supply source (n = 5, 1 sample/farm, 20 ml/sample) and randomly selected watering bins (n = 10, 2 samples/farm, 20 ml/sample) within the farm. The samples of animal feed were obtained from the farms' storage [n = 9 (1 sample of every feed available on site), 10 gr/sample] and one randomly selected feeder (n = 5, 1 sample/farm, 10 gr/sample). The animal feeds used in

Farm Record	Farm A	Farm B	Farm C	Farm D	Farm E
Location	Velestino (Magnesia Prefecture)	Kyparissia (Messenia Prefecture)	Megalopoli (Arkadia Prefecture)	Chiliomodi (Corinthia Prefecture)	Kranidi (Argolis Prefecture)
Animal stock	1520	240	467	690	270
Established (year)	2013	2000	2013	2017	2010
Animal restocking practice[1]	Internal	Internal	Internal	Internal/External	Internal
Breeding practice	Natural mating	Natural mating	Natural mating	Natural mating	Natural mating
Farm management practice[2]	Semi-intensive	Semi-extensive	Semi-intensive	Closed intensive	Semi-extensive
Method of milking	Milking machine	By hand	Milking machine	Milking machine	Milking machine
Udder disinfection before milking	No	No	Yes	Yes	No
Health problem	Neonatal diarrhoea, Mastitis	Mastitis	Mastitis	Mastitis	Mastitis
Routine vaccination for mastitis/neonatal diarrhoea	Yes/Yes	Yes/Yes	Yes/Yes	No/Yes	Yes/Yes
Drug susceptibility testing	No	No	No	No	No
Use of antibiotics					
mg[3]/year	1.860.000	110.000	62.500	40.000	45.000
mg[3]/year/animal	1.223,7	458,3	133,8	58,0	166,7
UI[4]/year	800.000.000	-	50.000.000	120.000.000	20.000.000
UI[4]/year/animal	526.315,8	-	107.066,4	173.913,0	74.074,1

Table 1: Location, size, management, and hygiene practice of Farms A-E, based on farm records.

[1]Animal restocking practice: restocking through internal breeding (internal) and purchase of animals when needed (external).

[2]Farm management practice: animals bred in confinement without access to pasture (closed intensive) or with access to confined pastures of exclusive use (semi-intensive), or to public pastures (semi-extensive).

[3]Streptomycin, tetracycline, amoxycillin, gentamicin, erythromycin.

[4]Penicillin G.

the study farms consisted mainly of own-produced forage mixed on site with supplements and concentrated feeds produced by licenced feed manufacturers.

After collection, samples were stored in isothermal containers on ice, and transferred to the laboratory within less than 5h. Upon arrival, the samples were aliquoted and stored at 4°C for 12-24h, before processing for the isolation of *Escherichia coli* and *Staphylococcus aureus*, and the assessment of drug resistance using conventional microbiology and the polymerase chain reaction (PCR).

Bacterial isolation

Sample processing for the isolation of *Staphylococcus aureus* and *Escherichia coli* was conducted based on standard procedures using 7% sheep Blood agar base (Oxoid Ltd., Basingstoke, UK) for milk samples, MacConkey agar (Oxoid Limited, UK) for milk, faeces and animal feed samples, Baird-Parker agar (Oxoid Limited, UK) for animal feed samples, and Chromogenic Coliform Agar (Thermo Fisher Scientific, USA) for filtrate of water samples [9]. Incubation of growth media was conducted aerobically at 37°C for 24h, except for Baird-Parker agar that was incubated for 48h.

Microbial identification

Colonies phenotypically consistent with *Staphylococcus aureus* (haemolytic on blood agar and black, surrounded by a clear zone on Baird-Parker agar) and *Escherichia coli* (bright pink on MacConkey agar and dark blue to violet on chromogenic coliform agar) were subcultured on Tryptone Bile Xglucuronide agar (TBX agar, Oxoid Limited, UK), (*Escherichia coli*) and Plate Count Agar (PCA, Oxoid Limited, UK) or Tryptone Soya Agar (TSA, Oxoid Limited, UK). Presumptive identification of the target pathogens relied on the oxidase and IMViC tests [10]. To complete identification, selected colonies (oxidase-negative, indole-positive, methyl red-positive, Vogues-Proskauer-negative, and citrate-negative) were tested with PCR. To this purpose, colonies were processed for DNA isolation using a commercially available kit, according to the manufacturer's instructions (Nucleospin® Tissue, Macheray-Nagel GmbH and Co. KG, Germany). The quality of the isolated DNA was assessed for purity and integrity with agarose gel electrophoresis followed by image analysis using a Bio-Rad ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories Inc., USA), whereas spectrophotometry was used to measure optical density at 260/280 nm via a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). Detection of *Staphylococcus aureus* and *Escherichia coli* DNA was conducted using PCR assays targeting the nuc [11] and rfbE genes [12], respectively.

For the confirmation of the specificity of the PCR amplification process, approximately 50% of the PCR-positive samples were submitted to sequence analysis, which was conducted on both strands using the Applied Biosystems BigDye Terminator Cycle Sequencing Kit and a PRISM 377 DNA Sequencer (Thermo Fisher Scientific Inc.). The results were compared against deposited sequences in the GenBank database using the Basic Local Alignment Search Tool from the National Center for Biotechnology Information [13].

Antimicrobial susceptibility testing

All *Staphylococcus aureus* (n = 15) and *Escherichia coli* (n = 36) isolates were grown on Mueller Hinton Agar II (MHA, NEOGEN Corporation, USA) at 35°C for 20h and were then tested for antimicrobial susceptibility using the disc diffusion method in line with the recommendations of the European Committee on Antimicrobial Susceptibility Testing [14]. In addition to the antimicrobials reported in the latter, *Staphylococcus aureus* was tested for susceptibility to ampicillin because of evidence of extended use of the certain antibiotic in the target farms.

In more detail, *Staphylococcus aureus* isolates were tested for susceptibility to penicillin G (1 unit), erythromycin (15 μg), cefoxitin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), tetracycline (30 μg), trimethoprim-sulphamethoxazole (25 μg), and ampicillin (10 μg). The isolates of *Escherichia coli* were tested for susceptibility to amoxicillin-clavulanic acid (30 μg), ampicillin (10 μg), cefotaxime (5 μg), cefoxitin (30 μg), ceftazidime (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), meropenem (10 μg), tetracycline (30 μg), and trimethoprim-sulphamethoxazole (25 μg).

DNA extracted from the *Staphylococcus aureus* isolates was tested with a multiplex PCR assay designed for the amplification of genomic regions associated with resistance to methicillin, as previously described [11,15,16].

The *Staphylococcus aureus* and *Escherichia coli* isolates were characterised as AMR or multidrug resistant (MDR) as proposed by others [17]. In brief, AMR is defined as non-susceptibility to at least one antimicrobial to which the test pathogen is typically susceptible, whilst MDR, as non-susceptibility to at least one agent from three antimicrobial categories. In addition to AMR and MDR, the statistical analysis that was conducted includes reference to AMR+, which was determined as AMR isolates exhibiting resistance to 2 categories of antibiotics.

Statistical analysis

The investigation for associations between the study parameters was conducted using the binary logistic regression modelling with the test-result represented as dichotomous variable that acquires the values yes/no for samples reacting positively or negatively to the tested pathogens [18]. Nine (n = 9) binary logistic regression models were implemented to investigate for potential associations of an equal number of binary variables, namely, positivity to both target pathogens (total positivity), AMR positivity, and AMR+/MDR positivity to *Staphylococcus aureus* or *Escherichia coli,* with farm size, management/breeding practice, method of milking, AMR, and type of sample being the selected explanatory variables (Tables 1 and 2). Modelling the binary categorical responses relied on the assumption that the dependent variable $y_i = (y_i, y_i,)^t$ follows a binomial distribution; thereof, its association with the set of the *m* predictor variables $\{X_1, X_2,...,X_m\}$ is determined by the following equation,

$$\log\left(\frac{p_i}{p_{i^*}}\right) = \boldsymbol{\beta}^T \mathbf{X} + \boldsymbol{\varepsilon}$$

Where p_i denotes the probability of category i, which is the reference category of the response variable of positivity (i = yes, *i** = no), X denotes the matrix of covariates, $\boldsymbol{\beta}$ the vector of parameter estimates corresponding to $\boldsymbol{X}$, and $\boldsymbol{\varepsilon}$ the error-term. Upon fitting the various logistic regression models, covariate selection was performed using a backward elimination stepwise approach.

For the investigation for associations, a logistic regression analysis was conducted between animal feed and milk, using one model for each of the tested pathogens (*Staphylococcus aureus* and *Escherichia coli*). In both cases, AMR positivity/negativity in milk was defined as the dependent, and AMR positivity/negativity in animal feeds as the independent variable.

The regression modelling analysis was conducted using the R Statistical Software [19].

Association between detection of the test pathogens and the use of antibiotics (total amount in mg/UI per farm/year/animal) was inferred through the Pearson correlation coefficient analysis, using the SPSS statistical package (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). The same approach was followed in the investigation for potential associations between the use of antibiotics and the detection of AMR or AMR+/MDR isolates of *Staphylococcus aureus* and *Escherichia coli* in milk (proportion of AMR or AMR+/MDR isolates of the total number of AMR isolates detected in each farm), (Tables 1, 3, and 4).

Type of sample	Farm A					Farm B					Farm C					Farm D					Farm E					Total positive Farms A-E				
	T	Sa	Ec	B	Sa+Ec	T	Sa	Ec	B	Sa+Ec	T	Sa	Ec	B	Sa+Ec	T	Sa	Ec	B	Sa+Ec	T	Sa	Ec	B	Sa+Ec	T	Sa	Ec	B	Sa+Ec
Milk (udder)	8	4	2	-	6	8	2	0	-	2	8	1	2	1	2	8	1	0	-	1	8	1	1	-	2	40	9 22.5%	5 12.5%	1	13 32.5%
Milk (tank)	1	1	1	1	1	1	1	0	-	1	1	0	1	-	1	1	1	1	1	1	1	0	1	-	1	5	3 60%	4 80%	2	5 100%
Water (central supply)	1	0	0	-	0	1	0	0	-	0	1	0	0	-	0	1	0	0	-	0	1	0	0	-	0	5	0 0%	0 0%	-	0 0%
Water (watering bin)	2	0	2	-	2	2	0	2	-	2	2	0	2	-	2	2	0	0	-	0	2	0	0	-	0	10	0 0%	6 60%	-	6 60%
Animal feed (storage)	5	0	3	-	3	1	0	1*	-	1	1	1	1	1	1	1	0	1*	-	1	1	0	0	-	0	9	1 11.1%	6 66.7%	1	6 66.7%
Animal feed (feeder)	1	0	1*	-	1	1	1	1	1	1	1	0	1*	-	1	1	1	1	1	1	1	0	1	-	1	5	2 40%	5 100%	2	5 100%
Bedding	2	0	0	-	0	2	0	0	-	0	2	0	0	-	0	2	0	0	-	0	2	0	0	-	0	10	0 0%	0 0%	-	0 0%
Faeces	2	0	2	-	2	2	0	2	-	2	2	0	2	-	2	2	0	2	-	2	2	0	2	-	2	10	0 0%	10 100%	-	10 100%
Total per farm	22	5 22.7%	1 50%	1	15 68.2%	18	4 22.2%	6 33.3%	1	9 50%	18	2 11.1%	9 50%	2	9 50%	18	3 16.7%	5 27.8%	2	6 33.3%	18	1 5.6%	5 27.8%	0	6 33.3%	94	15 16%	36 38.3%	6 6.4%	45 47.9%

Table 2: The total number (T) of samples tested in Farms A-E, and the respective level of positivity to either or both (B) of *Staphylococcus aureus* (Sa) and *Escherichia coli* (Ec).

*Two isolates of *Escherichia coli.*

Farm	Type of sample	Antibiotic*								MDR
		CIP	TE	SXT	CN	AM	P	E	FOX	
A	Milk (udder)					+	+			
	Milk (udder)						+			
	Milk (udder)		+			+	+			
	Milk (udder)						+			
	Milk (tank)						+			
	Total Farm A	0 of 5 0%	1 of 5 20%	0 of 5 0%	0 of 5 0%	2 of 5 40%	5 of 5 100%	0 of 5 0%	0 of 5 0%	0 of 5 0%
B	Milk (udder)						+			
	Milk (udder)		+			+	+			
	Milk (tank)						+			
	Feed (feeder)		+	+			+	+		+
	Total Farm B	0 of 4 0%	2 of 4 50%	1 of 4 25%	0 of 4 0%	1 of 4 25%	4 of 4 100%	1 of 4 25%	0 of 4 0%	1 of 4 25%
C	Milk (udder)			+		+	+	+		+
	Feed (storage)		+	+	+	+	+	+	+	+
	Total Farm C	0 of 2 0%	1 of 2 50%	2 of 2 100%	1 of 2 50%	2 of 2 100%	2 of 2 100%	2 of 2 100%	1 of 2 50%	2 of 2 100%
D	Milk (udder)		+	+			+	+		+
	Milk (tank)		+				+	+		
	Feed (feeder)		+	+			+	+		+
	Total Farm D	0 of 3 0%	3 of 3 100%	2 of 3 66.7%	0 of 3 0%	0 of 3 0%	3 of 3 100%	3 of 3 100%	0 of 3 0%	2 of 3 66.7%
E	Milk (udder)						+			
	Total Farm E	0 of 1 0%	0 of 1 0%	0 of 1 0%	0 of 1 0%	0 of 1 0%	1 of 1 100%	0 of 1 0%	0 of 1 0%	0 of 1 0%
Antibiotic resistance										Total
Farms A-E		0 of 15 0%	7 of 15 46.7%	5 of 15 33.3%	1 of 15 6.7%	5 of 15 33.3%	15 of 15 100%	6 of 15 40%	1 of 15 6.7%	5 of 15 33.3%

Table 3: Antimicrobial resistant (AMR) and multidrug resistant (MDR) *Staphylococcus aureus* isolates per antibiotic, type of sample and farm.

*CIP: Ciprofloxacin; TE: Tetracycline; SXT: Trimethroprin - Sulfamethoxazole (Cotrimoxazole); CN: Gentamicin; AM: Ampicillin; P: Penicillin G (Benzylpenicillin); E: Erythromycin; FOX: Cefoxitin.

Farm	Type of sample CAZ	Antibiotic*										MDR
		FOX	MEM	CTX	AMC	CIP	CN	TE	AM	SXT		
A	Milk (udder)	+				+		+	+	+		+
	Milk (udder)	+				+		+	+	+		+
	Milk (tank)					+		+	+	+		+
	Water (watering bin)									+		
	Water (watering bin)									+		
	Faeces					+			+	+		+
	Faeces					+				+		
	Feed (feeder)		+						+	+		+
	Feed (storage)									+		
	Total Farm A	2 of 9 22.2%	1 of 9 11.1%	0 of 9 0%	0 of 9 0%	5 of 9 55.6%	0 of 9 0%	3 of 9 33.3%	5 of 9 55.6%	9 of 9 100%	0 of 9 0%	5 of 9 55.6%
B	Water (watering bin)									+		
	Water (watering bin)									+		
	Faeces									+		
	Faeces	+								+		
	Feed (feeder)					+			+	+		+
	Feed (storage)	+			+					+		+
	Total Farm B	2 of 6 33.3%	0 of 6 0%	0 of 6 0%	1 of 6 16.7%	1 of 6 16.7%	0 of 6 0%	0 of 6 0%	1 of 6 16.7%	6 of 6 100%	0 of 6 0%	2 of 6 33.3%
C	Milk (tank)									+		
	Water (watering bin)									+		
	Water (watering bin)									+		
	Faeces	+								+		
	Faeces	+								+		
	Feed (feeder)		+			+				+		+
	Feed (storage)	+	+		+					+		
	Total Farm C	3 of 7 42.9%	2 of 7 28.6%	0 of 7 0%	1 of 7 14.3%	1 of 7 14.3%	0 of 7 0%	0 of 7 0%	0 of 7 0%	7 of 7 100%	0 of 7 0%	1 of 7 14.3%
D	Milk (tank)									+		
	Faeces									+		
	Faeces									+		
	Feed (feeder)	+			+	+	+	+	+	+	+	+
	Feed (storage)	+	+		+	+	+	+		+		+
	Total Farm D	2 of 5 40%	1 of 5 20%	0 of 5 0%	2 of 5 40%	2 of 5 40%	2 of 5 40%	2 of 5 40%	1 of 5 20%	5 of 5 100%	1 of 5 20%	2 of 5 40%

E	Milk (udder)									+		
	Milk (tank)		+		+	+				+	+	+
	Faeces									+		
	Faeces									+		
	Total Farm E	0 of 4 0%	1 of 4 25%	0 of 4 0%	1 of 4 25%	1 of 4 25%	0 of 4 0%	0 of 4 0%	0 of 4 0%	4 of 4 100%	1 of 4 25%	1 of 4 25%

Antibiotic resistance											Total	
Farms A-E	9 of 31 29%	5 of 31 16.1%	0 of 31 0%	5 of 31 16.1%	10 of 31 32.3%	2 of 31 6.5%	5 of 31 16.1%	7 of 31 22.6%	31 of 31 100%	2 of 31 6.5%	11 of 31 35.5%	

Table 4: Antimicrobial resistant (AMR) and multidrug resistant (MDR) *Escherichia coli* isolates per antibiotic, type sample and farm.

*CAZ: Ceftazidime; FOX: Cefoxitin; MEM: Meropenem; CTX: Cefotaxime; AMC: Amoxicillin - Clavulanic Acid; CIP: Ciprofloxacin; CN: Gentamicin; TE: Tetracycline; AM: Ampicillin; SXT: Trimethoprim - Sulfamethoxazole (Cotrimoxazole).

Results

Staphylococcus aureus and *Escherichia coli* were detected respectively in 16% (15 of 94) and 38.3% (36 of 94) of the samples tested. The presence of both bacteria was demonstrated in 6.4% (6 of 94) of the test samples. The sequence analysis conducted on the PCR products of the assays incorporated to the identification of the target pathogens was in all cases confirmatory of the specificity of the amplification process.

The percentage of samples positive to *Staphylococcus aureus* or *Escherichia coli* per farm varied between 33.3% (Farms D and E) and 68.2% (Farm A). The level of positivity for each pathogen ranged from 5.6% (Farm E) to 22.7% (Farm A), and from 27.8% (Farms D and E) to 50% (Farm A) for *Staphylococcus aureus* and *Escherichia coli*, respectively. The highest percentage of positivity was recorded in Farm A, in animal feeds (66.7%, 4 of 6 positive to *Escherichia coli*) and milk (55.6%, 5 of 9 positive to *Staphylococcus aureus*), (Table 2, Figure 1).

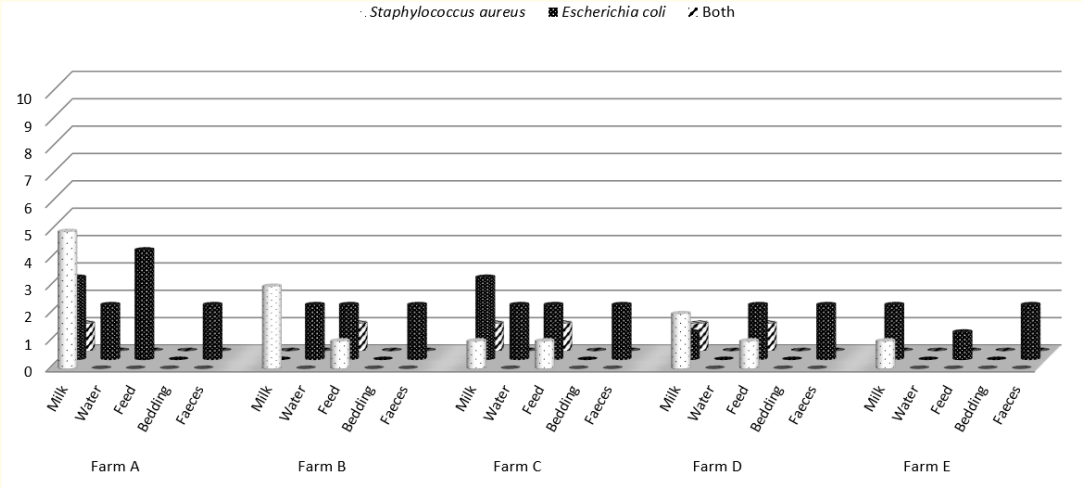

Figure 1: Number of samples positive to Staphylococcus aureus and/or Escherichia coli per type of sample and farm.

In line with the relevant criteria [14], all *Staphylococcus aureus* isolates (100%, 15 of 15) and 86.1% (31 of 36) of the *Escherichia coli* were characterised as AMR, exhibiting resistance to ampicillin and penicillin G, respectively. Based on the outcome of the relevant multiplex PCR assay, none of the AMR isolates of *Staphylococcus aureus* were identified as carriers of methicillin resistance genes. Notably, 5 of these isolates (33.3%, 5 of 15) were non-susceptible to ampicillin (10 μg). Sixteen (n = 16) of the 46 (34,8%) AMR isolates of both target pathogens were characterised as MDR [5 of 15 (33.3%) AMR isolates of *Staphylococcus aureus* and 11 of 31 (35.5%) of *Escherichia coli*], (Tables 3 and 4).

Higher proportion of *Staphylococcus aureus* AMR isolates was recorded in animal feeds compared to the other sample-types tested, with all isolates (100%) being resistant to tetracycline, trimethoprim – sulfamethoxazole, penicillin G and erythromycin and 33.3% of them to gentamicin, ampicillin and cefoxitin (Figure 2). All *Staphylococcus aureus* milk isolates (100%) were resistant to penicillin G, 33.3% of them were resistant to tetracycline and ampicillin, 25% to erythromycin, and 16.7% to trimethoprim – sulfamethoxazole.

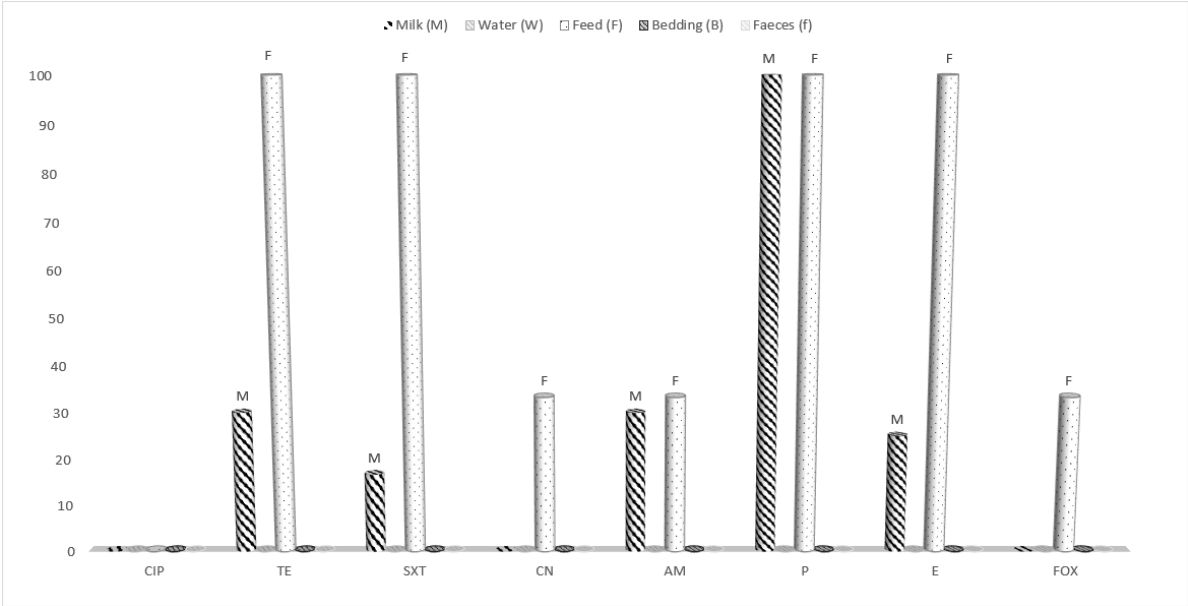

Figure 2: Percentage (%) of AMR Staphylococcus aureus isolates per type of sample, of those detected in Farms A-E.
* CIP: Ciprofloxacin; TE: Tetracycline; SXT: Trimethoprim - Sulfamethoxazole (Cotrimoxazole); CN: Gentamicin; AM: Ampicillin; P: Penicillin G (Benzylpenicillin); E: Erythromycin; FOX: Cefoxitin.

The relevant results recorded in connection with *Escherichia coli* indicate that all (100%) isolates detected in milk samples (7 of 7), water (6 of 6), animal feeds (8 of 8) and faeces (10 of 10) were ampicillin-resistant. Disregarding ampicillin-resistance, which as mentioned above was a common feature of all *Escherichia coli* isolates, the sample types for which the proportion of *Escherichia coli* AMR was comparatively higher, were milk [ceftazidime (28.6%, 2 of 7), cefoxitin (14.3%, 1 of 7), cefotaxime (14.3%, 1 of 7), amoxycillin – clavulanic acid (57.1%, 4 of 7), gentamycin (42.9%, 3 of 7), tetracycline (42.9%, 3 of 7), trimethoprim – sulfamethoxazole (14.3%, 1 of 7)], animal feeds [ceftazidime (50%, 4 of 8), cefoxitin (50%, 4 of 8), cefotaxime (50%, 4 of 8), amoxycillin – clavulanic acid (50%, 4 of 8), ciprofloxacin (25%, 2 of 8), gentamycin (25%, 2 of 8), tetracycline (37.5%, 3 of 8), trimethoprim – sulfamethoxazole (12.5%, 1 of 8)], and faeces [ceftazidime (30%, 3 of 10), amoxycillin – clavulanic acid (20%, 2 of 10), tetracycline (10%, 1 of 10)], (Figure 3).

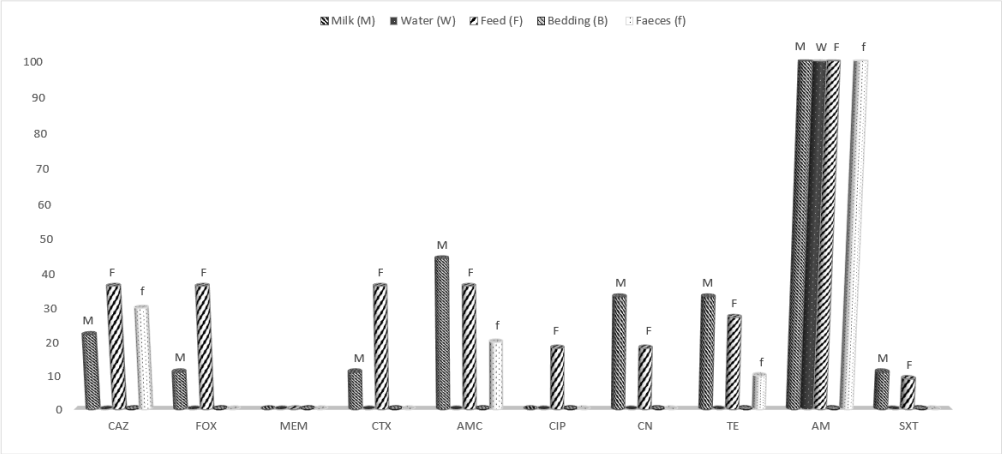

Figure 3: Percentage (%) of AMR Escherichia coli isolates per type of sample, of those detected in Farms A-E. *CAZ: Ceftazidime; FOX: Cefoxitin; MEM: Meropenem; CTX: Cefotaxime; AMC: Amoxicillin - Clavulanic acid; CIP: Ciprofloxacin; CN: Gentamicin; TE: Tetracycline; AM: Ampicillin; SXT: Trimethroprin - Sulfamethoxazole (Cotrimoxazole).

Staphylococcus aureus and *Escherichia coli* were detected in milk collected from the udder in 22.5% (9 of 40) and 12.5% (5 of 40) of the tested samples, respectively. The percentages of milk positivity collected from the farms' bulk milk tanks were 60% (3 of 5) and 80% (4 of 5). A comparison between farms with regards to udder milk-positivity to *Staphylococcus aureus* indicates lower percentage for Farms D and E (12.5%, 1 of 8) and higher for Farm A (50%, 4 of 8). With regards to *Escherichia coli*, Farms B and D ranked first (0%, 0 of 8), Farm E second (12.5%, 1 of 8), and the Farms A and C last (25% 2 of 8), (Table 2), (Figure 1).

The analysis conducted in milk samples did not provide positive results for AMR *Escherichia coli* in Farm B; all other farms were positive, with 3 AMR isolates detected in Farms A, 1 in Farms C and D, and 2 in Farm E. MDR *Escherichia coli* isolates were detected in samples of milk collected from Farm A (1 isolate resistant to 4, and 2 isolates resistant to 5 antimicrobial drugs) and Farm E, in which the MDR isolate was detected in the bulk milk tank and exhibited resistance to 5 antimicrobials.

AMR *Staphylococcus aureus* was detected in milk samples collected from all target farms (5 AMR detected in Farm A, 3 in Farm B, 1 in Farms C and E, and 2 in Farm D). The AMR isolate of *Staphylococcus aureus* detected in milk collected from Farm C and one of the two isolates of the same category detected in Farm D were MDR (Table 3).

Positive results were recorded in the samples of feed, in connection with the presence of the targeted pathogens (Table 2) and the detection of AMR isolates (Tables 3 and 4).

Logistic regression analysis provided evidence of statistically significant differences or associations between the following explanatory variables: type of sample, farm, and non-susceptibility to ampicillin. With regards to sample type, positivity of animal feeds to *Escherichia coli* was found to be significantly higher compared to the others at 1% level of statistical significance (b = 3.602; p-value<0.01). Detection of both *Escherichia coli* and *Staphylococcus aureus* was significantly higher in samples of faeces (b = 3.421; p-value<0.01), animal feed (b = 3.217; p-value<0.01), and milk (b = 2.011; p-value<0.1) compared to water (beta coefficient not statistically significant) and bedding (reference category).

Resistance of *Escherichia coli* to ampicillin was found to increase the likelihood of the relevant isolates being AMR+ at 1% level of statistical significance (b = 4.01; p-value<0.001).

Comparison between farms indicated that the level of positivity of Farm E was lower than that of the other farms at the 10% significance level in connection with the detection of both target pathogens (b = -1.168; p-value<0.1) and AMR+ isolates (b = -1.926; p-value<0.1).

The two models of logistic regression analysis that were used for the investigation of associations between animal feeds and milk indicated that the presence of AMR *Staphylococcus aureus* in animal feeds is associated with AMR *Staphylococcus aureus* in milk [odds ratio = 3.25; 95% confidence interval for OR = (0.519 - 20.37)]. The relevant analysis conducted with regards to *Escherichia coli* did not provide evidence of statistically significant associations [odds ratio = 0.063, 95% confidence interval for OR = (0.006 - 0.649)].

Associations between sample positivity to the target pathogens and the use of antibiotics per animal (total amount in μg per farm/year/animal) were statistically significant for *Escherichia coli* AMR+ isolates in milk but not for *Staphylococcus aureus* (p-value>0.1). In connection with the former pathogen, positive correlation was confirmed between the use of antibiotics and the proportion of AMR+ isolates, indicating that the higher the amount of antibiotics (total amount in μg per farm/year/animal), the higher the proportion of the certain category of isolates of *Escherichia coli* detected in milk (r = 0.897; p-value<0.05). This finding was consistent with the respective negative correlation that was also confirmed at statistically significant level, indicating that the higher the amount of antibiotics used in the farm (total amount in μg per farm/year/animal), the lower the proportion of AMR isolates of *Escherichia coli* detected in milk, implying higher proportion of the AMR+ isolates (r = -0.897; p-value<0.05).

Discussion

To the best of our knowledge, this is the first study on the spread of AMR in dairy sheep farms and the identification of markers that could be used to improve AMR surveillance in practice. Admittedly, the large number of variables that influence the spread of pathogens and AMR development in animal farms renders the possibility of drawing safe conclusions in studies such as the one presented here, a rather challenging goal. The current evidence is that many factors associated with the pathogens, such as bacterial adaptation and horizontal transmission of gene transfer, or the farms, including hygiene practices and use of antimicrobials, are responsible for the occurrence, persistence, and transmission of AMR [20]. In this regard, careful selection of the study parameters is necessary to acquire evidence of practical value. Thereof, this study focused to five dairy sheep farms and two indicators of AMR burden, namely, *Staphylococcus aureus* and *Escherichia coli,* and resulted in observations that are reported here with reservation, even in connection with those that were confirmed at statistically significant level, due to the complex and dynamic environment in which they were recorded. Nevertheless, the small amount of relevant information available in the literature in connection particularly with AMR in sheep highlights the importance of these findings.

In further elaborating on the rationale of the sample plan, it should be mentioned that the samples of animal feed and water collected from the farms' storage and main water source respectively, were used in this study to assess the introduction of infection and AMR strains inside the test farms. For these types of samples, it was considered necessary to collect one sample from every feed and water supply source available in the farm, which accounts for the different number of study samples of the certain types. In terms of assessing the spread of infection and AMR strains within farms, the selected sample types covered a much broader spectrum i.e., milk, bedding, faeces, water from the farms' watering bins and feed from the feeders. Samples of udder milk were collected from randomly selected clinically healthy individuals, whose number was not proportional to the farm's size, since the relevant study indicators were also assessed in samples collected from the bulk milk tank. The credibility of this approach was confirmed by the consistency of the results recorded in the samples collected from the individual animals and the bulk milk tanks, which indicates that the proposed sampling plan, though not proportional with regards to the number of individuals tested, is representative (Table 2). This was also confirmed in connection with the number of samples included in the sample plan, based on the high level of positivity that was recorded for most of the test parameters, and the fact that this was inversely proportional to the size of the study farms, a finding which is referred to in more detail below.

Comparison between farms indicated lower level of total positivity (b = -1.926; p-value<0.1) and detection of AMR+ in Farm E, which is a small, semi-extensive sheep farm established in 2010 in

the Argolis Prefecture of south Greece (Table 1). Though this observation was not confirmed at statistically significant level in connection with other study parameters, comparison between farms indicates a trend, which is consistent with Farm E being superior to the others in terms of hygiene. More specifically, positivity per type of sample to either or both target pathogens was comparatively lower in Farm E (Figure 1), as was the number of AMR *Staphylococcus aureus* isolates (1 in Farm E *versus* 5, 4, 2 and 3 in Farms A-D, respectively) and the MDR *Escherichia coli* isolates detected in animal feed samples (0 in Farm E *versus* 1, 2, 1 and 2 in Farms A-D, respectively), (Tables 2-4).

As already mentioned, the higher level of positivity of animal feeds to *Escherichia coli* compared to the other sample types was confirmed, at statistically significant level ($b = 3.602$; p-value<0.01). *Staphylococcus aureus* was not detected in any of the samples of feed that were tested, whereas *Escherichia coli* was detected in all the samples of this type, in Farms B-D. MDR *Escherichia coli* isolates were detected in the feed samples collected from the Farms A-D (Table 4). Faecal or environmental contamination of animal feeds in the feeder could of course justify detection of *Escherichia coli*. However, considering that the feed was stored appropriately, as confirmed in all the study farms through on-site inspection, it is likely that it is already contaminated with MDR *Escherichia coli* when delivered to them.

All the farms (100%, 4 of 4) in which AMR *Escherichia coli* isolates were detected in the feed samples collected from the storage were also positive to AMR of the same pathogen in the feeders, and *vice-versa*; Farm E, which was negative to AMR *Escherichia coli* in feed storage samples, was also negative to the same parameter assessed in samples of feed collected from feeders. An analysis of the antimicrobial susceptibility profile of the isolates of *Escherichia coli* detected in the samples of feed from the storage and the feeder documents that these were not identical, and it is thereof unlikely that the isolates detected in the feeders derive from the animal feeds.

As expected, the number of AMR *Staphylococcus aureus* isolates detected in animal feeds was smaller compared to those of *Escherichia coli*. However, AMR *Staphylococcus aureus* was also detected in samples collected from the feeder (Farms B and D) and the feed storage (Farm C). It is worth noting that all *Staphylococcus aureus* isolates detected in the feeds were MDR (Table 3). Furthermore, the presence of AMR isolates of *Staphylococcus aureus* in animal feeds was found to increase the probability of milk positivity to AMR *Staphylococcus aureus* by 3.25 times. Clearly, this does not imply causal association between AMR *Staphylococcus aureus* positivity of feeds and milk but indicates that this parameter maybe a valuable indicator for monitoring mastitis. It is worth noting that mastitis is very common in the study farms and, as already mentioned, this problem is often addressed with administration of ampicillin, which may explain why 33.3% (5 of 15) of the AMR *Staphylococcus aureus* isolates are strongly resistant (ampicillin 10 μg) to this antibiotic. Based on the above, it would be logical to assume that the contamination of animal feeds with AMR *Staphylococcus aureus,* combined with the occurrence of mastitis and the misuse of ampicillin for its treatment, are parameters involved in a vicious circle that exacerbates the problem of mastitis in the farms and renders its control difficult.

Although the high level of AMR and MDR positivity of the animal feeds generates concern about the spread of genetic determinants conferring drug resistance into the farm and their potential impact on public health, it is not an uncommon finding [8]. The contamination of animal feeds with AMR strains of many bacterial pathogens has been repeatedly reported in the past [3,4,21,22]. This problem seems to be associated with the production line of animal feeds, which is prone to contamination that is very difficult to be prevented in practice. Therefore, the improvement of animal feed safety can be achieved mainly by promoting good manufacturing practices, including postproduction decontamination and use of suitable facilities for storage [4].

To the contrary of *Escherichia coli,* which was expectedly detected in all the tested samples of faeces, none of them yielded *Staphylococcus aureus*. Faecal isolates of *Escherichia coli* exhibited multidrug resistance less frequently, when compared to isolates obtained from milk, bedding, water, and animal feed (Table 4). Other studies conducted in swine, poultry, and cattle indicate that the concentration of antibiotic residues in faeces can be correlated with the use of antimicrobial drugs in the respective farms [8,23,24]. However, the results recorded in this study suggest that the presence of AMR and MDR isolates of *Staphylococcus aureus* and *Escherichia coli* in sheep milk is probably a more sensitive indicator of their spread within the farm, compared to faeces.

The outcome of the analysis conducted on samples of water and bedding was in most cases negative, which indicates that the certain types of samples do not contribute significantly to the introduction of pathogens into the study farms. It is worth noting that all the samples that were collected from the farms' central water source were also negative. However, *Escherichia coli* was detected in samples collected from the watering bins of Farms A and C, which indicates faecal contamination.

The use of antibiotics in the study farms is rather alarming, in terms both of extend and practice, since to the contrary of the applicable regulations, it does not rely on drug susceptibility testing. The amount of antibiotics used in the certain farms was associated at statistically significant level with detection of both target pathogens ($r = 0.911$; p-value<0.1) and AMR+ isolates of *Escherichia coli* in milk ($r = 0.897$; p-value<0.05).

The overall context of the use of antibiotics in the study animal population indicates a regulatory gap and renders the adoption of strict measures by the competent authorities, necessary towards strengthening antimicrobial stewardship. Investigating the use of antibiotics within the study farms indicates that this is in most cases associated with mastitis and/or neonatal diarrhoea (Table 1). Interestingly, the analysis of the information gathered in connection with disease management, provided evidence of poor practice, such as improper use of disinfectants, use of unsuitable vaccines or vaccination schemes, and inadequate administration of antiparasitic agents. Unfortunately, appropriate veterinary support is not a service which is prioritised by the farm managers for financial reasons. In the absence of adequate monitoring by the relevant competent authorities, the latter results in poor hygiene and management practices, exacerbating misuse of antibiotics.

Conclusion

AMR is a common problem in the study farms, which is depicted primarily in samples of milk collected from the bulk milk tank and animal feed. Both sample types proved suitable for a cost-effective assessment of AMR, as did detection of ampicillin-resistant *Escherichia coli*. Isolation of AMR *Staphylococcus aureus* in animal feed emerged as a promising indicator for monitoring mastitis. This study suggests that the use of antimicrobials in the test farms can be reduced considerably with appropriate veterinary support and measures aiming to discourage their misuse.

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Declarations of Interest

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