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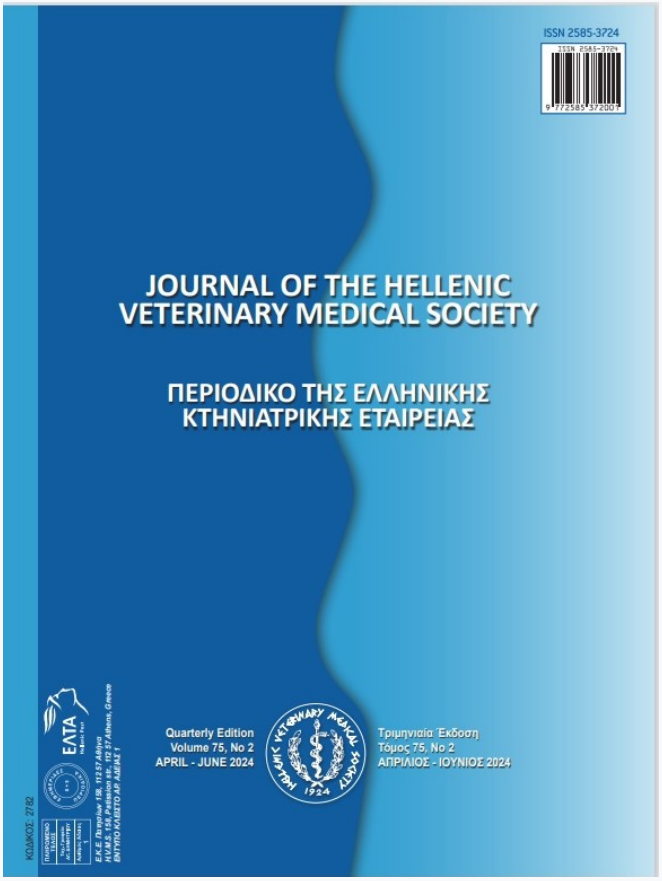

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Research article
Ερευνητικό άρθρο

Antimicrobial Resistance profile of *Staphylococcus aureus* from dairy cows with mastitis in the Region of Central Macedonia over a five-month period (March-July 2021)

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ABSTRACT: In Greece, a National Monitoring Plan for Antimicrobial Resistance in sick bovine animals was launched (2021). We aimed to study the antimicrobial resistance profiles of 118 *Staphylococcus aureus* isolates obtained from dairy cows >18 months with mastitis in the Region of Central Macedonia from 1st March to 31st July 2021, where 45% (Census 2020: 69.186/128.511) of dairy cows in Greece are concentrated. By using the disk diffusion method (according to the CLSI guidelines), we tested susceptibility of isolates to nine antimicrobials (penicillin, cefoxitin, cefquinome, gentamicin, tetracycline, marbofloxacin, erythromycin, clindamycin, enrofloxacin) selected following the marketing amounts of animal use in Greece. In the study, 62 (52.54%) of *S. aureus* isolates were resistant to penicillin ; 57/118 were 48,31% resistant to erythromycin, 56/118 were resistant 47,46% to clindamycin; 29/118 were 24,58% resistant to tetracycline and cefquinome both, 27/118 were 22,88% resistant to cefoxitin, 20/118 were 16,95% resistant to gentamicin, 3/118 were 2,54% resistant to marbofloxacin and, 2/118 were 1,69% resistant to enrofloxacin. In relation to monitoring the antimicrobial resistance of *Staphylococcus aureus* isolates responsible for mastitis in cattle (where a combination of penicillin and an aminoglycoside is the predominant therapeutic treatment in Greece), additional research is required for β-lactams (such as penicillin and cefoxitin). This investigation falls within the 'One Health' framework, addressing the judicious use of antibiotics in both human staphylococcal infections and veterinary medicine in Greece. However, it seems that several kinds of interventions at farm level led to a decrease in the quinolones use. The study offers a thorough understanding of the antimicrobial resistance patterns among *Staphylococcus aureus* isolates. The significant resistance rates to various antibiotics highlight the need for responsible antibiotic usage and continuous monitoring of resistance trends. These insights are crucial in developing effective strategies to monitor and combat the emergence and dissemination of resistant strains and ensuring better treatment and control of infections caused by this bacterium. The findings add valuable knowledge to our understanding of antibiotic resistance and its implications.

Keywords: Antimicrobial Resistance; *Staphylococcus aureus*; mastitis; prudent use of antibiotics; One Health

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INTRODUCTION

In Europe, the rise of antimicrobial resistance presents a substantial concern for public health, resulting in a yearly toll of over 30,000 human deaths[1]. To combat this issue, the European Union (EU) is dedicated to implement a comprehensive One Health surveillance approach, in line with the EU One Health Action Plan against Antimicrobial Resistance (AMR) [2]. In the context of humans, the European Centre for Disease Prevention and Control (ECDC) has launched the 'European Antimicrobial Resistance Surveillance Network (EARS-Net)' initiative. This program focuses on monitoring AMR in bacteria implicated on invasive or isolated from invasive infections found in hospitalized patients[3].

After conducting a review of AMR surveillance in the veterinary sector, experts from 14 (fourteen) EU/EEA (European Union/European Economic Area) countries[4], many of them were engaged in the existing monitoring programs, collaborated to establish the foundational methodology for the EARS-Vet. The aim was to create a comprehensive and unified approach to monitor and address AMR in veterinary medicine across Europe.

The proposed scope for EARS-Vet, which was developed through a consensus-driven and bottom-up approach, considers the existing surveillance activities in most participating countries[5]. This scope encompasses 6 (six) animal hosts, namely cattle, swine, chickens, turkeys, cats, and dogs, and focuses on monitoring 11 (eleven) bacterial species.

In 2021, Greece, as part of an initiative, launched a Pilot Project (Study) aimed at laying the groundwork for the Greek National Monitoring Plan for Antimicrobial Resistance in diseased animals, specifically focusing on bovine and swine. The primary objectives of the study were to conduct analyses pertaining to specific bacteria and antibiotics, identify potential hurdles, and develop recommendations to enhance future data collection to combat antimicrobial resistance effectively.

This particular paper discusses the results that interested to study the antimicrobial resistance (AMR) profile of *Staphylococcus aureus* bacteria isolated from dairy cows experiencing mastitis recovered from 10 farms in the Administrative Region of Central Macedonia. This region is recognized as the most densely populated area for dairy cows in Greece. The study, which spanned a thorough and extensive five-month period from March to July 2021, aimed to provide a comprehensive understanding of the situation. The research seeks to derive valuable insights into the present trends of resistance by analyzing the collected data. These findings will be instrumental in devising focused and efficient strategies to manage and prevent the emergence of resistance in the livestock population of the region, as well as in Greece, in the years to come.

MATERIALS AND METHODS

Scope/Materials

The main goal of this initiative was to establish a robust national-level communication network that would enable seamless collaboration between dairy cow farms, sample testing laboratories, and the Administration (Regional and Central level). The primary focus of this network was to accurately measure the resistance of *S. aureus* strains isolated from the milk of cows suffering from mastitis. To understand the distribution of dairy cows across Greece's 13 Administrative Regions, we diligently collected and analyzed data, which is presented in Table 1. This data provided valuable insights into the concentration of dairy cows in each Regional Unit (RE), allowing us to devise customized strategies for addressing specific regional needs.

A total of 118 *S. aureus* isolates from dairy cows older than 24 months that had mastitis. This survey took place in the Administrative Region of Central Macedonia between 1st March and 31st July 2021, an area where 45% (Census 2020: 69,186 out of 128,511) of Greece's dairy cows are concentrated.

Methods

During the milk collection process, samples were obtained from dairy cows by combining samples from all udder quarters. Before sampling, tubes were labeled with relevant information. Udders were cleaned, and teat ends disinfected with 70% alcohol-soaked cotton swabs. After discarding initial milk streams, three to four streams (1-2 mL each) from each udder quarter were collected into sterile containers. Additional steps included observing clinical signs of mastitis, dipping teats in a disinfectant, and thorough drying. Teat ends were vigorously scrubbed with alcohol-moistened cotton, ensuring cleanliness. The collection process started with the closest teat and moved to the far side. Caps were removed without touching inner surfaces, and one to three milk streams were collected per teat.

Composite samples were obtained by collecting 1-2 mL of milk from each quarter in the same tube. For samples taken post-milking, teats were dipped in a germicidal disinfectant. Samples were stored on ice or refrigerated, and those for later culture were frozen immediately (within 48 hours).

Upon receiving the samples (the total number of samples are at an animal level), we initiated the culturing process to isolate *Staphylococcus aureus*. This process involves diluting 1 ml of milk with 9 ml of MRD (Maximum Recovery Medium) diluent. Next, the resulting dilution was plated onto both blood agar and Mannitol Salt agar plates. After incubation period of 24-48 hours at 37°C, we chose three colonies exhibiting mannitol positivity and three colonies displaying hemolytic activity on blood agar. The selected isolated colonies were submitted to Gram staining and catalase testing. Colonies that tested positive in these initial tests were then subject to coagulase tests using the tube and slide methods with coagulase plasma containing EDTA (Ethylenediaminetetraacetic acid). Once the colonies were confirmed, we proceeded with Kirby-Bauer susceptibility testing[6]. This involved applying various antibiotics on Mueller Hinton agar plates and measuring the resulting inhibition zones around the colonies. The obtained results were then compared with the CLSI (Clinical and Laboratory Standards Institute) standards (VET01, 5th edition, 2019) to determine the sensitivity, intermediate, or resistance rates of the coagulase positive staphylococci to each antibiotic[7].

RESULTS

According to the study's results, a total of 118 Staphylococcus coagulase positive (CPS) isolates were analyzed to assess their antibiotic susceptibility profile against various molecules . The findings indicated the following levels of resistance (see Table 2): The higher resistance profiles were ascribed to penicillin, erythromycin, and clindamycin, recording values of 52.54%, 48.31%, and 47.46%, respectively. In contrast, lower resistance levels were observed for tetracyclines, cefquinome, and cefoxitin. Specifically, 24.58% of the isolates exhibited resistance to both tetracycline and cefquinome, while approximately 22.88% displayed resistance to cefoxitin.

Moreover, it has been observed that 5 out of the 118 strains, accounting for 4.24%, exhibited multidrug resistance (MDR). The phenotypes of multidrug resistance profiles of isolated strains in this study are as follows (Table 1):

Table 1. Resistance profiles of the isolates

No	Resistance Profile	Related Isolates
1	FOX-CLID-E	1
2	FOX-TE-E	1
3	TE-CLID-E	2
4	FOX-GEN-TE-CLID-E	1

Antimicrobial Agents: *Clindamycin (CLID) [Category: Lincosamides], Tetracycline (TE) [Category: Tetracyclines], Erythromycin (E) [Category: Macrolides], Gentamicin (GEN) [Category: Aminoglycosides], Ciprofloxacin (FOX) [Category: Fluoroquinolones].*

Table 2. Greece's Dairy Cows Sampling Plan (2021)

ADMINISTRATIVE REGION OF WESTERN GREECE		Percentage of Animals in total (%)	Number of Samples in total
TOTAL	5.980	5	18
ADMINISTRATIVE REGION OF ATTICA			
TOTAL	1.900	1	5
ADMINISTRATIVE REGION PELOPONNESE			
TOTAL	1.081	1	2
ADMINISTRATIVE REGION OF EPIRUS			
TOTAL	5.035	4	15
ADMINISTRATIVE REGION OF CENTRAL GREECE			
TOTAL	4.755	4	14
ADMINISTRATIVE REGION OF WESTERN MACEDONIA			
TOTAL	8.791	7	26
ADMINISTRATIVE REGION EASTERN MACEDONIA & THRACE			
TOTAL	17.825	14	53
ADMINISTRATIVE REGION NORTH AEGEAN			
TOTAL	665	1	2
ADMINISTRATIVE REGION OF IONIAN ISLANDS			
TOTAL	228	1	1
ADMINISTRATIVE REGION OF CENTRAL MACEDONIA			
TOTAL	59.186	45	177
ADMINISTRATIVE REGION OF CRETE			
TOTAL	262	0	1
ADMINISTRATIVE REGION OF THESSALY			
TOTAL	18.632	14	55
ADMINISTRATIVE REGION OF SOUTH AEGEAN			
TOTAL	5.171	3	15
GRAND TOTAL	128.511	100	384

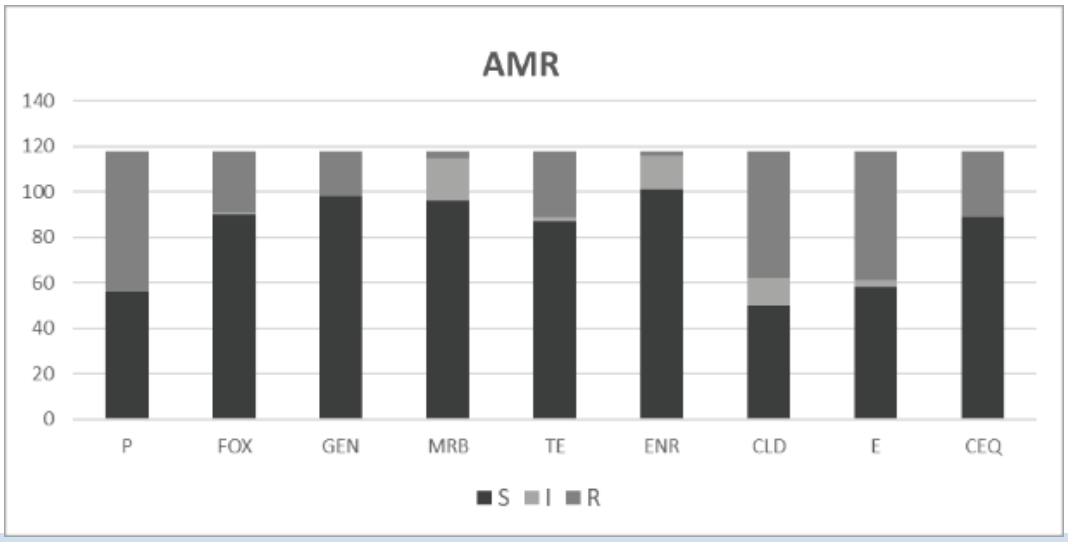

Table 3. Antimicrobial Resistance profiles in 118 dairy cows in the Region of Central Macedonia *(S: Sensitive, I:Intermediate, R:Resiatant)*

Antimicrobial Agents: *Ampicillin (P),Ciprofloxacin (FOX), Gentamicin (GEN), Marbofloxacin (MRB), Tetracycline (TE), Enrofloxacin (ENR), Clindamycin (CLID), Erythromycin (E), Cefquinome (CEQ).*

DISCUSSION

This work aimed to establish a consensus among experts regarding the combinations of animal species, bacterial species, specimens, production types, and antimicrobials to be monitored in the Greek AMR Pilot Project, with a focus on the One Health perspective. The project was based on the national veterinary AMR monitoring scope, which guided discussions among Greek AMR experts by identifying relevant and feasible monitoring targets for the country. This approach recognized that some combinations of animal species, production types, age categories, bacterial species, specimens, and antimicrobials might be challenging to assess accurately due to the lack of previous experience in the country.

Several studies have been conducted to establish a connection between the physical traits and genetic characteristics of *S. aureus* and its manifestation as clinical or subclinical mastitis in cows [12]. The selection of *S. aureus*, a major bacterial pathogen linked to veterinary antimicrobial usage in Greece, for monitoring in dairy cows was particularly important for public health. Monitoring AMR in dairy cows over 24 months of age was considered essential, as both production type and age can significantly influence AMR levels within the same animal species. While it may not have been feasible to widely collect this information, production type and age category could often be inferred from the bacterial species and/or specimen. Stakeholders were encouraged to report such data to the effort, regardless of the type of isolate.

The Pilot Project's scope included antimicrobials relevant to both animals and humans, covering all categories of the Antimicrobial Advice ad hoc Expert Group of EMA[6]. The selection was based on their potential effects on public health due to increased AMR when used in animals, while also considering their necessity in veterinary medicine.

Unlike most national monitoring systems that rely on passive data collection of AST results from routine veterinary diagnostic laboratories, the Greek Pilot Project adopted an active data collection approach. This meant that external funding would be required for the country's participation in this initiative. In the meantime, establishing a platform for the collection and sharing of such data would be necessary.

It is essential to note that the scope of the Pilot Project should be continuously reviewed and expanded to accommodate changes in the epidemiology of AMR, involve more combinations of bacteria/species/antimicrobials, and adapt to the evolving feasibility of monitoring specific combinations.

Recent studies have reported a higher resistance rate to penicillin and a lower resistance rate to tetracycline among bovine isolates in Greek dairy herds. This suggests that the frequent use of antimicrobials in bovines has led to higher selective pressure, resulting in increased resistance to penicillin and less re-

sistance to tetracycline[7]. Our survey also confirmed this trend, as approximately 52.54% (62 out of 118 isolates) coagulase positive staphylococci isolates were resistant to penicillin, while 24.58% (29 out of 118 isolates) showed resistance to tetracycline.

Based on other studies[80] related to the antimicrobial resistance patterns of *Staphylococcus aureus* in mastitis-affected lactating cows, it was found that among the *S. aureus* isolates, over 58.70% (54 out of 92 isolates) showed resistance to erythromycin. Interestingly, these results agree with those of our study where 57 out of 118 isolates (48.31%) exhibited erythromycin resistance. Additionally, in the same study, approximately 27.17% (25 out of 92 isolates) of the samples demonstrated resistance to erythromycin, which again had a striking resemblance to the results of our Project's research. On the other hand, a significant number of isolates (59 out of 92) in the recent study displayed resistance to gentamicin, accounting for 64.3%. This resistance rate was notably higher compared to the findings in our study, where only 20 out of 118 isolates (16.95%) were resistant to gentamicin[10].

In our Pilot Project's study, we found that the lowest resistance was seen in enrofloxacin, with only 2 out of 118 isolates exhibiting resistance, making up 1.69% of the samples. These findings corroborate with results from separate studies carried out in Southern European nations, where instances of enrofloxacin resistance have also been identified in Italy[9] and Portugal[10]. On the other hand, we observed a higher prevalence of cefquinome resistance in our study, with 29 out of 118 isolates (24.58%) showing resistance. This differs from other studies that reported even higher rates of antimicrobial resistance (AMR) for cefquinome, reaching up to 88.89%[12]. Similarly, our investigation revealed significant resistance to clindamycin, with 56 out of 118 isolates (47.46%) demonstrating resistance, while other studies reported a lower percentage of 23.7% (42 out of 177)[11]. For marbofloxacin, only 3 out of 118 isolates (2.54%) were resistant in our study, whereas most other studies did not report any resistance to marbofloxacin [12]. These varying resistance patterns emphasize the importance of monitoring AMR in different regions and populations to develop effective antimicrobial stewardship and control strategies.

CONCLUSION

Regarding to the surveillance on the antimicrobial resistance of the *Staphylococcus aureus* isolates, that cause mastitis in cattle (penicillin in combination with an aminoglycoside, is the most commonly applied therapeutic treatment scheme against mastitis in Greece), to the β-lactams (penicillin, cefoxitin) it is needed further investigation, under the 'One Health' approach in respect to the prudent use of antibiotics in staphylococcal infections in humans and in the veterinary medicine in Greece. However, it seems that several kinds of interventions at farm level led to the protection of quinolones.

Carefully collecting samples from different regions and ensuring they represent various units within each region would allow us to assess the network's performance in various scenarios. This approach would also help us identify potential challenges and opportunities that may arise in different geographical contexts. Conducting this comprehensive evaluation will provide valuable insights to make necessary improvements to the network before implementing it nationwide.

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CONFLICT OF INTEREST

None declared

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