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TREND OF ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS* CHROMOGENES ISOLATED FROM RAW MILK SAMPLES OF DAIRY COWS IN ROMANIA BETWEEN 2018 AND 2023

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Abstract

Staphylococcus chromogenes (St. chromogenes) continues to be one of the primary causative agents of mastitis in dairy cattle. This retrospective study aimed to present the trends of antimicrobial resistance in St. chromogenes isolated from raw milk samples of dairy cows with subclinical and clinical mastitis over a six-year period. From January 2018 to December 2023, a total of 79 St. chromogenes isolates were evaluated for antimicrobial resistance against 22 antibiotics. The current study revealed a significant increase in resistance to various antimicrobial agents. For example, amoxicillin resistance increased from 23.33% to 50.00%, marbofloxacin from 12.50% to 25.00%, doxycycline from 13.33% to 75.00%, oxytetracycline from 13.33% to 50.00%, and streptomycin from 16.67% to 50.00%. The increasing trend of antimicrobial resistance underscores the need for robust infection control strategies and judicious antibiotic use in dairy farms. To summarise, this study can serve as an essential resource for evaluating treatment protocols and mitigating the further spread of resistance.

Key words*: cow, mastitis, trends, drug resistance, Romania.*

INTRODUCTION

Bovine milk production plays a significant role in the global economy, serving as a crucial income source for producers of all sizes, including small, medium, and large-scale operations (Bojovic & McGregor, 2023; Crippa et al., 2024; Jafri et al., 2024).

Nevertheless, infections of the mammary gland, primarily caused by bacteria, pose a significant challenge to animal welfare, productivity, and the economy, particularly within dairy farming, resulting in substantial losses for the dairy sector (Antók et al., 2019; Crippa et al., 2024; Dhital et al., 2023).

Bovine mastitis and intramammary infections have been associated with more than twenty types of Non-aureus staphylococci (NAS), including *St. chromogenes* (Beuckelaere et al., 2021; Persson Waller et al., 2023; Phophi et al, 2019). Many studies have reported that *St. chromogenes* was isolated from animals exhibiting mild, moderate, or severe symptoms of subclinical and clinical mastitis as well as chronic infections (Getahun et al., 2024; Király et al., 2024; Persson Waller et al., 2023; Zigo et al., 2022). *St. chromogenes* is considered a host-adapted species, capable of overcoming the udder's physical defences and forming biofilms (Bochniarz et al., 2016; Crippa et al., 2024). Additionally, this microorganism is highly adapted to the bovine mammary gland, where it may serve as a microbial reservoir and a potential source of infections (Crippa et al., 2024). The highest representation of virulence factors (production of hemolysis, gelatinase, and biofilm; the ability to hydrolyse DNA; resistance to antibiotics) was found in NAS, such as *St. chromogenes* (Antók et al., 2019; Bochniarz et al., 2016; Zigo et al., 2022). Resistance to antibiotics represents a significant risk to both animal and human health (Naranjo-Lucena & Slowey, 2023).

The escalating issue of antibiotic resistance is already a critical challenge, reducing the effectiveness of antimicrobial agents and hindering treatment options for both veterinary and human medicine (Naranjo-Lucena & Slowey, 2023; Sipahi et al., 2023). Therefore, the main objective of this study is to evaluate the

antibiotic resistance of *St. chromogenes* isolated from Romanian dairy cattle. This analysis aims to elucidate local antimicrobial resistance trends, thus contributing valuable information on the evolutionary dynamics of antibiotic resistance in the context of bovine intramammary infections.

MATERIALS AND METHODS

The present study was conducted on 79 *St. chromogenes* bacterial isolates, obtained from raw milk samples of dairy cows with subclinical and clinical mastitis, collected over a six-year period. This investigation was carried out at the Synevovet Laboratory in Bucharest, Romania, with the analysis period spanning from January 2018 to December 2023.

The samples were cultured on Columbia agar and Cled agar and then incubated at 35-37°C for 20-24 h in aerobic conditions. After incubation, *Staphylococcus* species were selected based on their colony morphology and then were identified using MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) technology.

The isolates' antibiotic resistance profiles were determined by a disk diffusion method on Mueller-Hinton agar plates. The sizes of the inhibition zones around the antibiotic discs were measured using the ADAGIO automated system (Bio-Rad). The resistance profiles were interpreted based on the standards set by the Clinical and Laboratory Standards Institute (CLSI) for the following antimicrobials: amoxicillin, ampicillin, amoxicillin-clavulanic acid, erythromycin, streptomycin, ceftiofur, doxycycline, oxytetracycline, tetracycline, penicillin, penicillin-novobiocin, gentamycin, pirlymicin, trimethoprim-sulfamethoxazole, kanamycin-cephalexin, penicillin G-framycetin, cefquinome, neomycin, cefoperazone, enrofloxacin, marbofloxacin, and cloxacillin.

Then, the strains were categorized as resistant (R), intermediate (I), and susceptible (S). Data collection and processing were performed using Microsoft Office Excel (Microsoft, USA). Figures were also generated using Microsoft Office. Statistical analyses for this study were conducted using a linear regression (parametric approach) from SAS with *p*-values less than 0.05 considered statistically significant.

RESULTS AND DISCUSSIONS

In our study, the 79 strains of *St. chromogenes* showed a varied overall resistance to the tested antibiotics as seen in Table 1.

Table 1. Antimicrobial susceptibility of *St. chromogenes* isolates tested between 2018 and 2023

Antimicrobials	Total	Susceptible No. (%)	Intermediate No. (%)	Resistant No. (%)
β-lactamase				
Amoxicillin (A)	79	57 (72.15)	0 (0)	22 (27.85)
Ampicillin (AM)	79	58 (73.42)	0 (0)	21 (26.58)
Amoxicillin-clavulanic acid (AMC)	79	79 (100%)	0 (0)	0 (0)
Cefoperazone (CFP)	79	73 (92.41)	2 (2.53)	4(5.06)
Cefquinome (CEF)	79	74 (93.67)	0 (0)	5 (6.33)
Ceftiofur (EFT)	79	79 (100)	0 (0)	0 (0)
Penicillin (P)	79	58 (73.42)	0 (0)	21 (26.58)
Penicillin G-framycetin (PFY)	79	79 (100%)	0 (0)	0 (0)
Penicillin-novobiocin (PNV)	79	78 (97.73)	0 (0)	1 (1.27)
Cloxacillin	79	77 (97.47)	0 (0)	2 (2.53)
Quinolones				
Enrofloxacin (ENR)	79	79 (100%)	0 (0)	0 (0)
Marbofloxacin (MAR)	79	77 (97.47)	0 (0)	2 (2.53)
Macrolide				
Erythromycin	74	64 (86.49)	0 (0)	10 (13.51)
Tetracyclines				
Doxycycline (DO)	79	62(78.48)	0(0)	17(21.52)
Tetracycline (TE)	79	49 (66.22)	2(2.70)	23(31.08)
Oxytetracycline (OT)	79	60 (75.95)	0 (0)	19 (24.05)
Aminoglycosides				
Gentamicin (CM)	79	78 (98.73)	0 (0)	1 (1.27)
Streptomycin (S)	79	57 (72.15)	5 (6.33)	17 (21.52)
Neomycin (N)	79	79 (100)	0 (0)	0 (0)
Kanamycin-cephalexin (KCL)	79	79 (100%)	0 (0)	0 (0)
Sulphonamides				
Sulphamethoxazole-Trimethoprim (SxT)	79	78 (98.73)	0 (0)	1 (1.27)
Lincosamide				
Pirlimycin	77	72 (93.51)	0 (0)	5 (6.49)

These results demonstrate a concerning level of antimicrobial resistance, with tetracycline exhibiting the highest resistance rate at 31.08%, followed by amoxicillin (27.85%), then penicillin, and ampicillin, both with the same level of resistance (26.58%). Additionally, significant resistance was observed against oxytetracycline (24.05%), doxycycline (21.52%), and streptomycin (21.52%). The observed antimicrobial resistance in *St. chromogenes* isolates from bovine milk with mastitis may result from a combination of factors, including the overuse or misuse of antibiotics, inadequate treatment regimens, and poor farm hygiene practices (Antók et al., 2019). Our findings regarding the resistance of *St. chromogenes* are consistent with those reported in a similar study conducted globally, further

supporting the reliability and reproducibility of these results. Los Santos et. al presented the penicillin resistance percentages of *Staphylococcus* spp. isolates in South America. In their study, the penicillin resistance was detected at 25% for *St. chromogenes*, this value being similar to our results (De Los Santos et al., 2022). A total of 142 confirmed coagulase-negative staphylococcal isolates were used in a study conducted in South Africa. Ninety-three percent (93/100) of *St. chromogenes* were resistant to at least one antimicrobial. *St. chromogenes* exhibited a high prevalence of resistance to penicillin (87%), ampicillin (87%), erythromycin (69%), and streptomycin (54%) compared to our study, where the resistance rates to these antibiotics were much lower, specifically 26.58%, 26.58%, 13.51%, and 21.52%, respectively (Phophi et al., 2019). Getahunet al. also studied the antibioresistance of coagulase-negative staphylococci from bovine milk in Gondar City and found that all the *St. chromogenes* exhibited 100% resistance to amoxicillin, and ampicillin. In contrast, our study shows a much lower resistance to these antibiotics, with amoxicillin resistance at 27.85% and ampicillin resistance at 26.58%. The stark contrast in resistance rates between the two studies may be attributed to differences in geographic location, sample characteristics, testing methodologies, or antibiotic usage patterns (Getahun et al., 2024).

Regarding susceptibility, according to Bochniarz et al. who examined 38 isolates of *St. chromogenes* from 335 samples of milk from cows with subclinical coagulase-negative staphylococci mastitis, 84.2% of the isolates were susceptible to amoxicillin with clavulanic acid, 81.6% to ampicillin, and 73.7% to penicillin. Our study shows higher susceptibility to amoxicillin with clavulanic acid (100%) compared to their study (84.2%), suggesting that *St. chromogenes* in our region may be more sensitive to this combination antibiotic. However, the susceptibility to ampicillin in our study (73.42%) is slightly lower than in their study (81.6%), indicating that our isolates might have a higher level of ampicillin resistance. The results for penicillin susceptibility are almost identical (73.7% vs. 73.42%), indicating similar resistance levels in both regions (Bochniarz et al., 2016).

The presence of antibiotic-resistant bacteria in bovine mastitis and the potential transmission to humans through unpasteurized dairy products pose significant public health risks (Ghimpețeanu et al., 2022). The frequent use of antibiotics by farmers to treat mastitis contributes to the development of resistant strains, leading to financial losses and diminishing the effectiveness of prevention and management strategies (Khasapane et al., 2024; Neculai-Valeanu et al., 2024).

Moreover, this study highlighted concerning trends in antimicrobial resistance among *St. chromogenes* isolated from bovine with intramammary infections by analysing milk samples collected over a six-year period. Our research indicates a notable level of resistance among *St. chromogenes* strains against tetracyclines, with doxycycline resistance frequencies recorded as 37.50% in 2022, and 75.00% in 2023. Similarly, for oxytetracycline, the resistance rose from 13.33% in 2018 to 50.00% in 2023. The resistance rates for tetracycline displayed variations over the study investigation. Specifically, in 2019, the resistance percentage for tetracycline was recorded as 84.62%, higher than the rates of 14.29% in 2021, 42.86% in 2022 and 75.00% in 2023. Among aminoglycosides, streptomycin resistance increased from 12.50% in 2020 to 50.00% in 2022. Resistance to β-lactamase, particularly amoxicillin, increased from 23.33% in 2018 to 37.50 % in 2022, and 50.00% in 2023. The resistance percentages for ampicillin peaked in 2022, with 37.50% compared to 14.29% in 2021, and 25.00% in 2022, 2023, respectively. For cefoperazone, the maximum resistance percentage was observed in 2018 at 10.00%, followed by a decrease to 6.67% in 2019. Examination of milk samples indicated a rise in resistance for quinolones, notably marbofloxacin, from 12.50% in 2022 to 25.00% in 2023. Additionally, our results indicated a rise in resistance rates for pirlimycin between 2018 and 2019, from 6.67% to 15.38%, followed by a decrease to 12.50% in 2022 as presented in Table 2.

The analysis of antibiotic resistance trends for *St. chromogenes* between 2018 and 2023 revealed statistically significant findings as seen in Figure 1. The resistance of this pathogen to oxytetracycline rose significantly, with a

p-value of 0.0028, suggesting a notable and significant trend. Similarly, resistance to amoxicillin also exhibited significant upward trends, with a p-value of 0.0177, indicating statistical significance. Additionally, resistance to marbofloxacin, doxycycline, and streptomycin demonstrated significant increasing trends, with p-values of 0.0406, 0.0481 and 0.0443, respectively, confirming statistical significance. In contrast, cefoperazone and penicillin resistance in this pathogen showed a significant decrease, with p-values of 0.0344 and 0.0464, respectively, suggesting that the reduction in resistance was statistically significant and unlikely to be due to random variation.

Our results, which reflect trends in antimicrobial resistance of *St. chromogenes* strains, indicate an increase in resistance rates by the end of 2023.

Future strategies should focus on strengthening surveillance of antimicrobial resistance, ensuring rigorous adaptation of control measures to prevent intramammary infections on dairy farms, and directing research efforts toward developing innovative treatment protocols to maintain the efficacy of existing antibiotic therapies.

Table 2. Antibiotic resistance profile of *St. chromogenes* isolates from raw milk (n=79)

Antimicrobials	***St. chromogenes* % of resistant isolates (no. of tested isolates)**						
	2018	**2019**	**2020**	**2021**	**2022**	**2023**	***p*-value**
β-lactamase							
Amoxicillin (A)	23.33 (30)	26.67 (15)	25.00 (8)	28.57 (14)	37.50 (8)	50.00 (4)	0.0177*
Ampicillin (AM)	26.67 (30)	33.33(15)	37.50(8)	14.29 (14)	25.00 (8)	25.00 (4)	0.4602
Amoxicillin-clavulanic acid (AMC)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Cefoperazone (CFP)	10.00 (30)	6.67 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.0344*
Cefquinome (CEF)	6.67 (30)	13.33 (15)	0 (8)	0 (14)	0 (8)	25.00(4)	0.6000
Ceftiofur (EFT)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Penicillin (P)	26.67 (30)	40.00 (15)	37.50(8)	21.43(14)	0 (8)	0 (4)	0.0464*
Penicillin G-framycetin (PFY)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Penicillin-novobiocin (PNV)	0 (30)	6.67 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.4411
Cloxacillin	6.67 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.1583
Quinolones							
Enrofloxacin (ENR)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Marbofloxacin (MAR)	0 (30)	0 (15)	0 (8)	0 (14)	12.50 (8)	25.00 (4)	0.0406*
Macrolide							
Erythromycin	19.23 (26)	14.29 (14)	0 (8)	0 (14)	37.50 (8)	0 (4)	0.8596
Tetracyclines							
Doxycycline (DO)	13.33 (30)	13.33 (15)	0 (8)	35.71 (14)	37.50 (8)	75.00 (4)	0.0481*
Tetracycline (TE)	14.29 (28)	84.62 (13)	0 (8)	14.29 (14)	42.86 (70	75.00 (4)	0.5719
Oxytetracycline (OT)	13.33 (30)	20.00 (15)	25.00 (8)	28.57 (14)	50.00 (8)	50.00 (4)	0.0028*
Aminoglycosides							
Gentamicin (CM)	3.33 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.1583
Streptomycin (S)	16.67 (30)	20.00 (15)	12.50 (8)	21.43 (14)	37.50 (8)	50.00 (4)	0.0443*
Neomycin (N)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Kanamicină-cefalexin (KCL)	0 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0
Sulphonamides							
Sulphamethoxazole-Trimethoprim (SxT)	3.33 (30)	0 (15)	0 (8)	0 (14)	0 (8)	0 (4)	0.1583
Lincosamide							
Pirlimycin	6.67 (30)	15.38 (13)	0 (8)	0 (14)	12.50 (8)	0 (4)	0.6000

*statistically significant differences.

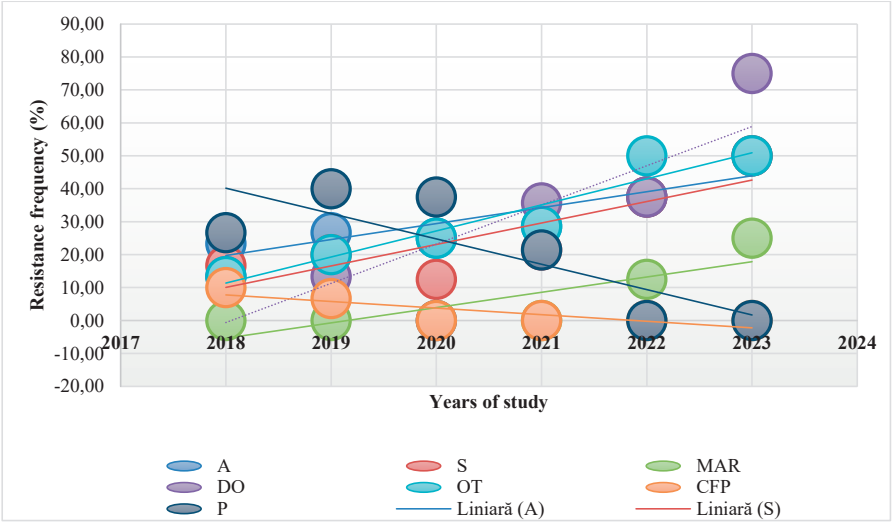

Figure 1. Antibiotic resistance of *St. chromogenes* across 2018 to 2023. The trend of increasing resistance to oxytetracycline, amoxicillin, marbofloxacin, doxycycline, and streptomycin is noticeable. On the other hand, the trend of decreasing resistance to cefoperazone and penicillin is visible

CONCLUSIONS

The observed resistance of *St. chromogenes* to multiple commonly used antibiotics, including tetracycline (31.08%), amoxicillin (27.85%), penicillin (26.58%), ampicillin (26.58%), and others, underscores the growing challenge of managing mastitis infections. A concerning increase in antimicrobial resistance has been noted, particularly for oxytetracycline. This trend emphasizes the need for ongoing surveillance, prudent antibiotic use, and the exploration of alternative treatment options to effectively address resistant strains and improve herd health management. In order to accurately monitor and address the changing dynamics of bacterial resistance, it is crucial to conduct thorough and continuous surveillance of antimicrobial sensitivity and resistance trends over time, as intended in the current study.

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