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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN CANINE STAPHYLOCOCCI FROM NORTH MACEDONIA

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ABSTRACT

Antimicrobial resistance (AMR) in *Staphylococcus* spp. is a growing problem in small animal practice, driven by the emergence of methicillin-resistant (MR) and multidrug-resistant (MDR) strains. This study analyzed 170 clinical *Staphylococcus* isolates from dogs in North Macedonia, using MALDI-TOF MS identification, disc diffusion susceptibility testing, and molecular detection of resistance genes (*mec*A, *mec*C, and *bla*Z). *Staphylococcus pseudintermedius* was identified as the most prevalent species (90%), followed by *S. aureus* (7.6%), *S. hemolyticus* (1.2%), *S. schleiferi* (0.6%), and *S. intermedius* (0.6%). Methicillin resistance was detected in 28.8% of the isolates by detecting *mec*A. Importantly, there was a significant discrepancy between phenotypic oxacillin resistance and *mec*A-positive isolates in *S. pseudintermedius*. Among the 49 *mec*A-negative but oxacillin-resistant isolates tested for *bla*Z, 65.3% were *bla*Z-positive, underscoring the critical role of beta-lactamase-mediated resistance. Overall, MDR was detected in 70.5% of isolates. High resistance was observed to multiple antibiotics, including penicillin G (73%) and clindamycin (61.8%), as well as critically important antibiotics (CIAs), such as fluoroquinolones, with resistance rates of 32.3% for enrofloxacin and 31.2% for marbofloxacin. Pradofloxacin showed the lowest resistance rate (22.3%). This study highlights the high prevalence of antimicrobial resistance in *Staphylococcus* spp. in dogs. Implementation of antimicrobial stewardship programs is critical to maintain the efficacy of key antimicrobials and ensure optimal treatment outcomes for companion animals in North Macedonia.

Key words: *Staphylococcus pseudintermedius*, companion animals, methicillin resistance, beta-lactam resistance, multi-drug resistance

INTRODUCTION

The emergence of antimicrobial resistance (AMR) in *Staphylococcus* spp. in companion animals is a cause for concern and poses a significant threat to public health (1). *Staphylococcus* infections are commonly diagnosed in small animal clinical practice with most dog isolates showing resistance to at least one antibiotic (2). In addition, the emergence and spread of methicillin-resistant strains has led to the development of multidrug-resistant (MDR) bacteria (3), defined as resistance to at least one drug from three or more antibiotic classes (3, 4). These MDR strains exhibit resistance to nearly all antimicrobials approved for veterinary use, posing a significant challenge in small animal practice. In 2021, EFSA identified *S. pseudintermedius* as one of the top three antimicrobial-resistant bacteria in the EU that pose a risk to the health of dogs and cats (5). Although *S. pseudintermedius* is a commensal bacterium, as an opportunistic pathogen, it can be responsible for many infections, including skin infections, otitis externa, urinary,

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respiratory, and reproductive infections (5). In addition to *S. pseudintermedius*, other clinically important Staphylococci can also be isolated and cause infections, such as *Staphylococcus aureus, Staphylococcus schleiferi, Staphylococcus intermedius, Staphylococcus hemolyticus* and *Staphylococcus hyicus* (2, 4, 6).

Methicillin resistance (MR) is one of the most important public health concerns in human medicine due to the high prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections that are now increasingly observed in isolates from dogs (10). In addition, methicillin-resistant *S. pseudintermedius* (MRSP) has been identified as one of the most important bacterial pathogens for companion animals in the European Union and is sporadically associated with human infections (11, 12, 13). *Staphylococcus* species acquire methicillin resistance mainly through the *mec*A gene, which encodes an altered penicillin-binding protein (PBP2a). The altered protein has a lower affinity for beta-lactam antibiotics, making them ineffective against methicillin-resistant strains. Methicillin-resistant strains of *Staphylococcus* spp. commonly exhibit resistance to oxacillin or cefoxitin, which serve as phenotypic indicators of methicillin resistance (7). While the *mec*A gene is the gold standard for MR identification, other mechanisms such as altered penicillin-binding proteins, β-lactamase hyperproduction, or the presence of less common methicillin-resistance genes may be responsible for the observed oxacillin/cefoxitin resistance in these isolates (8). However, another gene, *mec*C, has also been identified and contributes to this resistance. The *mec*C gene codes for an alternative penicillin-binding protein called PBP2c, which, like PBP2a produced by *mec*A, has a low affinity for beta-lactam antibiotics. In addition, the *bla*Z gene, which encodes for beta-lactamase production, is also crucial as it contributes to resistance against beta-lactam antibiotics and further complicates treatment options.

While research on AMR in companion animals has been extensive in Western Europe, including established monitoring systems and stewardship guidelines, studies and systematic data collection from the Balkan region remains limited (9, 10). In particular, data on the prevalence of AMR in *Staphylococcus* species isolated from dogs in North Macedonia is scarce. A single study by Cvetkovikj et al. (11) reported a significant prevalence of MRSP and MRSA among canine isolates in the country, highlighting the need for further investigation. To address the gap, this study aimed to evaluate phenotypic resistance profiles and examine the presence of key resistance genes (*mec*A, *mec*C, and *bla*Z) in 170 *Staphylococcus* species isolates obtained from canine clinical samples. The findings will contribute to the regional data on AMR trends and support the development of effective antimicrobial stewardship strategies.

MATERIAL AND METHODS

Strain collection

A total of 170 *Staphylococcus* isolates from clinical samples from 170 dogs were analyzed over a five–year-period (2019-2024). All samples were collected by private veterinarians and submitted to the laboratory for bacteriologic diagnosis according to the clinical manifestations observed. Any data on previous antimicrobial treatment, breed, age, or sex were not available. The research was conducted as part of the Project FVMS-IPR-4, “Antimicrobial resistance in bacteria isolated from companion animals in the Republic of North Macedonia”, approved by the Faculty of Veterinary Medicine in Skopje (Decision No. 0202-359/11 from 31.3.2023).

The study had two phases. In the first, prospective phase, 48 isolates from clinical samples were analyzed for routine culture and bacteriology testing from April 2023 to May 2024. To ensure comprehensive data, in the second, retrospective phase, an additional 122 isolates from dogs were retrieved from the microbial strain collection of the Laboratory of Microbiology at the Faculty of Veterinary Medicine in Skopje (FVMS). These additional isolates were from clinical samples submitted between October 2019 and March 2023 for routine culture and bacteriology testing. All historical isolates were stored at -80 °C in 20% glycerol and tryptic soy broth (TSB; Oxoid, UK) and recultured before analysis. Importantly, no animals were specifically selected for participation in this study.

Samples included swabs from skin, nose, ears, eyes, vagina, and wounds/abscess swabs, milk, and urine samples. When appropriate, sampling sites were grouped into broader categories, such as combining skin samples with those from wounds and abscesses into a single category labeled "skin/soft tissue samples”. All samples were cultured on 5% sheep blood agar (C-pharm, Croatia) and incubated at 37 °C for 24 h under aerobic conditions.

Bacterial identification

Bacterial species were identified by culture morphology and MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). For identification, a Direct Transfer Procedure (DT) was used. The addition of formic acid in the DT was used when necessary to ensure a reliable log (score). Measurements were performed using Flex Control 3.4 software, and results with a log (score) ≥2.0 were considered reliable and verified for species-level identification. Quality control was conducted using the reference strain *Staphylococcus aureus* ATCC 29213 to ensure accurate identification.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested using the disc-diffusion method (Kirby-Bauer) with a panel of 12 antibiotics representing eight classes (Table 1). Oxacillin was used to screen for methicillin resistance in *S. pseudintermedius*, and cefoxitin was used as a surrogate test as an indicator of methicillin resistance in coagulase-negative staphylococci (CoNS) and *S. aureus*. Antibiotic susceptibility interpretations followed CLSI guidelines: VETO1S-Ed6 (12) for canine-specific breakpoints and M100 standards (13) where species-specific breakpoints were unavailable (Table 1). To analyze the phenotypic resistance profiles, intermediate susceptibility results were categorized as resistant to account for their potential clinical significance.

Table 1. Antibiotics used for disc diffusion in *Staphylococcus* spp.

Antimicrobial class	Antibiotic	Abbreviation	Concentration	CLSI Standard
Penicillin β-lactam	Oxacillin (screening)	OXA	1 µg	CLSIVET01
	Cefoxitin (screening)	FOX	30 µg	CLSIVET01
	Penicillin G	PG	10 µg	M100
Tetracyclines	Tetracycline	TET	30 µg	CLSIVET01
Macrolides	Erythromycin	ERY	15 µg	M100
Lincosamides	Clindamycin	CD	2 µg	CLSIVET01
Aminoglycosides	Gentamicin	GM	10 µg	M100
Fluoroquinolones	Marbofloxacin	MAR	5 µg	CLSIVET01
	Enrofloxacin	ENR	5 µg	CLSIVET01
	Pradofloxacin	PRD	5 µg	CLSIVET01
Amphenicols	Chloramphenicol	C	30 µg	M100
Folate-pathway inhibitors	Trimethoprim/ sulfamethoxazole	SXT	1.25/23.75 µg	M100

Molecular detection of resistance genes

Bacterial DNA was extracted using the boiling method technique. One colony of a pure bacterial isolate was suspended with PBS solution (200 µl) and incubated in a thermoblock for 30 min at 95 °C.

Conventional PCR was used to detect the *mec*A and *mec*C genes (14) in all isolates to identify methicillin resistance. Additionally, the *blaZ* gene (15) was tested in a subset of *S. pseudintermedius* isolates showing discordance between *mec*A results and oxacillin resistance. Specifically, 49 *mec*A-negative but oxacillin-resistant *S. pseudintermedius* isolates were analyzed for the presence of *blaZ*, as a determinant of beta-lactamase-mediated resistance. The primers and reaction conditions were the same as previously described (Table 2).

Table 2. Primers used for detection of resistant genes (supplementary material)

Antibiotic-resistance genes	Primers	Sequence (5' – 3')	Amplicon size (bp)	References
***mec*A**	*mec*A f	TGGCTCAGGTACTGCTATCCAC	776	(14)
	*mec*A r	AGTTCTGCAGTACCGGATTTGC		
***mec*C**	*mec*LGA251 f	GCTCCTAATGCTAATGCA	304	(14)
	*mec*LGA251 r	TAAGCAATAATGACTACC		
***bla*Z**	*bla*Z3	TGA CCA CTT TTA TCA GCA ACC	700	(15)
	*bla*Z2	GCC ATT TCA ACA CCT TCT TTC		

Statistical analysis

Associations between resistance patterns, bacterial species (*S. pseudintermedius*, *S. aureus,* CoNS), and the presence of methicillin resistance genes (*mec*A, *mec*C, and *bla*Z) were analyzed using Fisher's Exact Test for contingency tables with expected cell frequencies below 5 and Pearson's Chi-square test for larger tables. Resistance and susceptibility rates were calculated for all isolates and specific subgroups (e.g., methicillin-resistant strains), with 95% confidence intervals determined using the Wilson Score Interval method. The data was organized and analyzed in Microsoft Excel, and the statistical significance was defined as $p<0.05$.

RESULTS

The study identified a total of 170 *Staphylococcus* isolates. The majority (153/170, 90%, 95% CI: 84.7–93.6%) were identified as *Staphylococcus pseudintermedius*, followed by *Staphylococcus aureus* (13/170, 7.6%, 95% CI: 4.2–12.7%), *Staphylococcus hemolyticus* (2/170, 1.2%, 95% CI: 0.2–4.3%), *Staphylococcus schleiferi* (1/170, 0.6%, 95% CI: 0.03–3.50%), and *Staphylococcus intermedius* (1/170, 0.6%, 95% CI: 0.03–3.50%).

The distribution of isolates across sample sites showed skin and soft tissue infections (SSTIs) as the most common source (61/170, 34.6%), followed by ear infections (41/170, 24.1%) and vaginal samples (25/170, 14.7%). Less frequent sources included ocular (21/170, 12.3%), nasal (19/170, 11.2%), milk (2/170, 1.2%), and urine samples (1/170, 0.6%) (Table 3).

Table 3. Distribution of *Staphylococcus* species regarding the site of infection

		Sample number (%)						
Species	**Nº %**	**SSTi**	**Ear**	**Nose**	**Eye**	**Vagina**	**Milk**	**Urine**
Staphylococcus pseudintermedius	153 (90.0%)	53 (34.6%)	41 (26.8%)	13 (8.5%)	20 (13.1%)	24 (15.7%)	2 (1.3%)	0
Staphylococcus aureus	13 (7.6%)	5 (38.4%)	0	6 (46.1%)	0	1 (7.1%)	0	1 (7.1%)
Staphylococcus schleiferi	1 (0.6%)	0	0	0	1 (100%)	0	0	0
Staphylococcus intermedius	1 (0.6%)	1 (100%)	0	0	0	0	0	0
Staphylococcus hemolyticus	2 (1.2%)	2 (10.0%)	0	0	0	0	0	0
Total	**170**	61 (36.0%)	41 (24.1%)	19 (11.2%)	21 (12.3%)	25 (14.7%)	2 (1.2%)	1 (0.6%)

Table 3 presents the prevalence and distribution of *Staphylococcus* species from clinical samples in dogs. The columns show sample sites, while the rows list the species. Each cell shows the count and percentage of isolates from each species at specific sites, relative to the total for that species. The 'Total' row summarizes the overall counts and percentages for each site out of 170 samples, highlighting common infection locations

Antimicrobial resistance analysis revealed a significant variation across antibiotic classes (χ^2=237.24, p<0.0001). Beta-lactams demonstrated the highest resistance rates, with 73% (124/170, 95% CI: 66.3–79.6%) of isolates resistant to penicillin G. Oxacillin/cefoxitin resistance was observed in 56.5% (96/170, 95% CI: 49.4–63.4%) of isolates. Tetracyclines showed 70% (119/170, 95% CI: 63.1–76.9%) resistance to tetracycline. Among macrolides and lincosamides, clindamycin resistance was 61.8% (105/170, 95% CI: 54.5–69.1%), while erythromycin resistance was 62.3% (106/170, 95% CI: 55.1–69.6%). Fluoroquinolones showed varied resistance: enrofloxacin resistance was 32.3% (55/170, 95% CI: 25.2–39.4%), marbofloxacin resistance was 31.2% (53/170, 95% CI: 24.2–38.2%), and the pradofloxacin resistance was the lowest at 22.3% (38/170, 95% CI: 16.0–28.6%). Among folate-pathway inhibitors, resistance to trimethoprim/sulfamethoxazole was 45.3% (77/170, 95% CI: 38.1–52.5%), while chloramphenicol resistance was 32.3% (55/170, 95% CI: 25.2–39.4%). (Table 4).

Table 4. Antimicrobial susceptibility of 170 *Staphylococcus* spp. isolates from dogs

Antimicrobials	Number (and Percentage) of Isolates			CI (%)
	Susceptible	Intermediate	Resistant	
PG	46 (27.0%)	0	124 (73.0%)	65.8-79.1
TET	44 (25.9%)	7 (4.1%)	119 (70.0%)	62.7-76.4
ERY	62 (36.5%)	2 (1.2%)	106 (62.3%)	54.9-69. 3
CD	60 (35.3%)	5 (2.9%)	105 (61.8%)	54.3-68.7
GM	106 (62.4%)	7 (4.1%)	57 (33.5%)	26.9-40.9
MAR	111 (65.3%)	6 (3.5%)	53 (31.2%)	24.7-38.5
ENR	103 (60.6%)	12 (7.0%)	55 (32.4%)	25.8-39.7
PRD	121 (71.2%)	11 (6.5%)	38 (22.3%)	16.7-29.2
C	115 (67.6%)	0	55 (32.4%)	25.8-39.7
SXT	90 (52.9%)	3 (1.8%)	77 (45.3%)	38.0-52.8

Legend: PG=Penicillin, TET=Tetracycline, ERY=Erythromycin, CD=Clindamycin, GM=Gentamicin, MAR=Marbofloxacin, ENR=Enrofloxacin, PRD=Pradofloxacin, C=Chloramphenicol, SXT=Trimethoprim/sulfamethoxazole. The 95% Confidence Intervals (CI) for resistance rates are provided to indicate the statistical precision of the estimates

Table 5. Heatmap of antimicrobial resistance in *S.pseudintermedius* and *S.aureus*

Organism	Resistance (and percentage)											
	Total	OXA/ FOX	PG	T	E	CD	GM	MAR	ENR	PRD	C	SXT
MRSP	44	37	40	40	40	40	23	29	28	18	13	34
		84.1%	90.9%	90.9%	90.9%	90.9%	52.3%	65.9%	63.6%	40.9%	29.5%	77.3%
*MRSP	49	49	35	33	29	30	18	6	13	5	16	18
		100%	71.4%	67.3%	59.2%	61.2%	36.7%	12.2%	26.5%	10.2%	32.7%	36.7%
MSSP	67	0	46	36	30	29	9	9	12	6	22	14
			68.7%	53.7%	44.8%	43.3%	13.4%	13.4%	17.9%	8.9%	32.8%	20.9%
MRSA	4	3	4	4	4	4	3	1	2	2	1	2
		75%	100%	100%	100%	100%	75%	25%	50%	50%	25%	50%
*MRSA	4	4	4	4	4	4	4	1	2	1	0	1
		100%	100%	100%	100%	100%	100%	25%	50%	25%		25%
MSSA	5	0	4	5	2	1	1	2	2	1	2	2
			80%	100%	40%	20%	20%	40%	40%	20%	40%	40%

This heatmap provides a comprehensive visualization of antibiotic resistance percentages in *S.pseudintermedius* and *S.aureus*, including **MRSP**-Methicillin-resistant *S. pseudintermedius* confirmed with *mec*A positive; ***MRSP**-Methicillin-resistant *S.pseudintermedius* defined by oxacillin resistance, *mec*A negative; **MSSP**–Methicillin-susceptible *S.pseudintermedius* (*mec*A negative, oxacilin susceptible); **MRSA**–Methicillin-resistant *Staphylococcus aureus* confirmed with *mec*A positive; ***MRSA**–Methicillin-resistant *S.aureus* confirmed with cefoxitin screening, *mec*A negative; **MSSA**–Methicillin-susceptible *S.aureus* (*mec*A negative, cefoxitin susceptible). The heatmap employs a continuous color gradient, where increasing resistance percentages correspond to progressively darker shades. No fixed intervals were used, allowing a smooth representation of data distribution

The antibiotic resistance patterns of *S. pseudintermedius* and *S.aureus* were analyzed and are summarized in Table 5.

Multidrug resistance (MDR) prevalence across Staphylococcus species

Multidrug Resistance was observed in 70.5% of the isolates. Multidrug Resistance was observed in 69.2% of *S. pseudintermedius* isolates (106/153, 95% CI: 61.97%–76.59%) and 85% of *S. aureus* isolates (11/13, 95% CI: 54.55%–98.08%). Fisher's Exact Test revealed no statistically significant difference in MDR prevalence between species (p=0.134), likely due to the limited sample size of *S. aureus* isolates. Odds ratio analysis suggests that *S. aureus* isolates may be approximately 2.44 times more likely to exhibit MDR than *S. pseudintermedius* isolates (OR: 2.44). However, further validation with larger sample sizes is required.

Table 6. Prevalence of oxacillin or cefoxitin resistance, *mec*A positive isolates and MDR in *Staphylococcus* spp.

Species (n)	Oxacillin (cefoxitin) R (%)	*mec*A + (%)	MDR isolates
Staphylococcus pseudintermedius	86/153 (56.2%)	44/153 (28.7%)	106/153 (69.2%)
Staphylococcus aureus	7/13 (53.8%)	4/13 (30.8%)	11 (85%)
Staphylococcus schleiferi	0	0	0
Staphylococcus intermedius	1/1 (100%)	1/1 (100%)	1 (100%)
Staphylococcus haemolyticus	2 (100%)	0	2 (100%)
Total	96/170 (56.5%)	49/170 (28.8%)	120/170 (70.5%)

Oxacillin/Cefoxitin resistance

The *mec*A gene was detected in 28.8% of all *Staphylococcus* isolates (49/170, 95% CI: 23.0%–35.2%). Among the species, *mec*A was identified in 28.7% of *Staphylococcus pseudintermedius* isolates (44/153, 95% CI: 22.4%–35.9%) and 30.8% of *S. aureus* isolates (4/13, 95% CI: 12.8%–58.6%). The *mec*C gene was not detected in any of the 170 isolates analyzed in this study.

Among the 153 *Staphylococcus pseudintermedius* isolates, phenotypic oxacillin resistance was observed in 86 isolates (56.2%; 95% CI: 48.1%–63.1%), which was nearly double from the prevalence of *mec*A. Fisher's Exact Test revealed a statistically significant difference between the prevalence of *mec*A and oxacillin resistance ($p<0.001$).

In *Staphylococcus aureus* (n=13), the discrepancy between *mec*A prevalence (30.8%) and phenotypic cefoxitin resistance (53.8%) was not statistically significant ($p=0.108$), likely due to the small sample size.

Among the less commonly isolated species, the single *S. intermedius* isolate was oxacillin-resistant and *mec*A-positive. Both *Staphylococcus haemolyticus* isolates (2/2; 100%, 95% CI: 34.2%–100%) exhibited phenotypic cefoxitin resistance but lacked the *mec*A and *mec*C genes. The *S. schleiferi* isolate showed no resistance to the tested antimicrobials and did not carry the *mec*A or *mec*C genes.

Resistant profiles in Staphylococcus pseudintermedius

Among the 153 *S. pseudintermedius* isolates, 145 (94.8%; 95% CI: 89.9%–97.8%) exhibited resistance to at least one antibiotic, while 8 isolates (5.2%; 95% CI: 2.2%–10.1%) showed no resistance to the antibiotics tested. Phenotypic oxacillin resistance was observed in 86 isolates (56.2%; 95% CI: 48.2%–63.8%), whereas *mec*A was detected in 44 isolates (28.7%; 95% CI: 22.4%–35.9%). Multidrug resistance was identified in 106 isolates (69.3%; 95% CI: 61.7%–76.1%). Among the 49 *S. pseudintermedius* isolates that were *mec*A-negative but oxacillin-resistant, 32 isolates (65.3%; 95% CI: 51.0%–77.4%) were *bla*Z-positive, while 17 isolates (34.7%; 95% CI: 22.6%–49.0%) were *bla*Z-negative. All *bla*Z-positive isolates were resistant to oxacillin, and 27 were resistant to penicillin G (84.3%; 95% CI: 61.4%–89.6%).

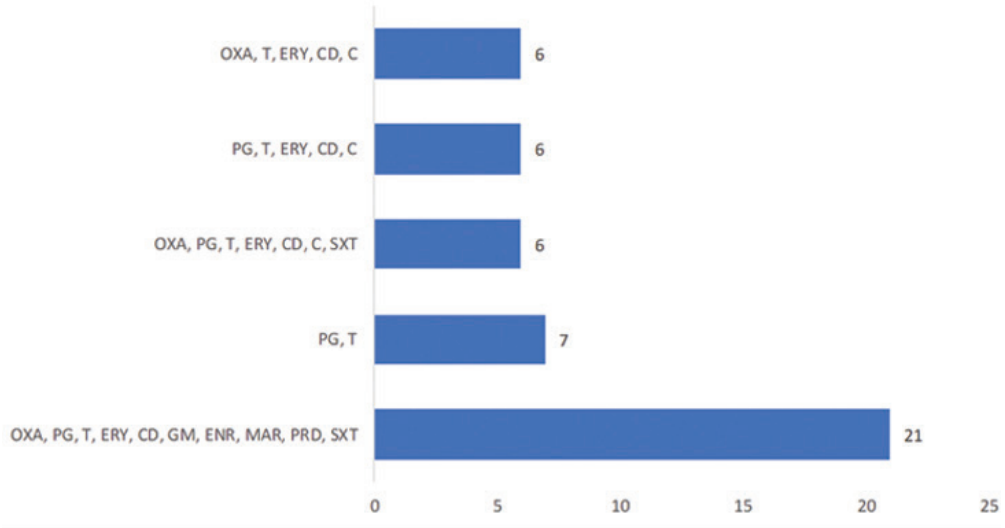

Figure 1. The most prevalent resistance profiles in *S. pseudintermedius*

The analysis revealed 68 unique resistance profiles. Overall, the most frequent resistance profile (n=21) included OXA, PG, T, ERY, CD, GM, ENR, MAR, PRD, and SXT (Fig. 1). This profile was observed in 13 *mec*A-positive isolates (59.1%; 95% CI: 38.8%–76.7%) and 8 *mec*A-negative isolates (40.9%; 95% CI: 23.3%–61.2%) (Fig. 2). Further analysis revealed that all 8 *mec*A-negative isolates were *bla*Z-positive. One of the most frequent resistance profiles, observed in 6 isolates (12.2%; 95% CI: 5.7%–23.5%), included OXA, PG, T, ERY, CD, C, and SXT. Among these, 5 isolates were *bla*Z-positive (83.3%; 95% CI: 43.6%–97.0%), and 1 isolate was *mec*A-positive (16.7%; 95% CI: 3.0%–56.4%). A total resistance profile covering OXA, PG, T, ERY, CD, GM, ENR, MAR, PRD, C, and SXT was identified in 3 isolates, with 2 being *mec*A-positive (66.7%; 95% CI: 20.8%–93.9%) and 1 being *bla*Z-positive (33.3%; 95% CI: 6.1%–79.2%). (Fig. 2).

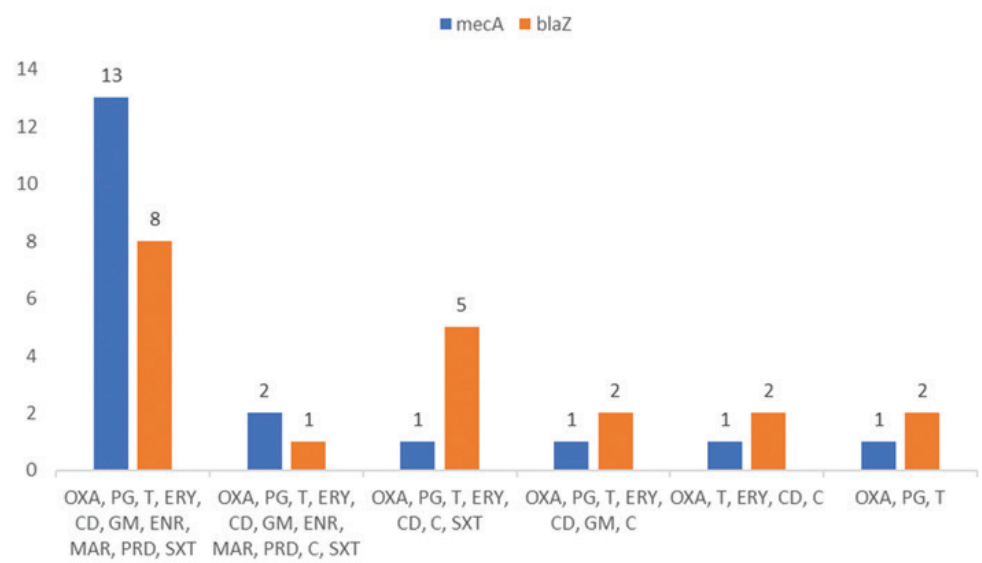

Figure 2. Overlapping resistance patterns between *mec*A-positive and *bla*Z-positive isolates. The bar chart above visualizes the distribution of resistance profiles between *mec*A-positive and *bla*Z-positive isolates. Each bar shows the number of isolates for a specific resistance profile, highlighting the differences and similarities between the two groups

Resistant profiles in Staphylococcus aureus

All *Staphylococcus aureus* isolates exhibited resistance to at least one antibiotic. Of these, 4 isolates were *mec*A-positive (4/13, 36.4%, 95% CI: 14.9%–64.8%), and 7 were *mec*A-negative (7/13, 63.6%, 95% CI: 35.2%–85.1%).

The most common resistance profiles identified were FOX, PG, T, ERY, CD, GM (2 isolates: 1 *mec*A-positive, 50.0%, 95% CI: 9.5%–90.5%; 1 *mec*A-negative, 50.0%, 95% CI: 9.5%–90.5%) and FOX, PG, T, ERY, CD, GM, ENR, MAR, PRD, C, SXT (2 isolates: 1 *mec*A-positive, 50.0%, 95% CI: 9.5%–90.5%; 1 *mec*A-negative, 50.0%, 95% CI: 9.5%–90.5%).

Unique resistance profiles were observed, including PG, T, ERY, ENR (1 isolate, 100%, 95% CI: 21.7%–100%) and FOX, PG, T, ERY, CD, ENR, PRD (1 isolate, 100%, 95% CI: 21.7%–100%), reflecting significant diversity in resistance mechanisms among *mec*A-negative isolates. A Chi-square test comparing resistance profiles between *mec*A-positive and *mec*A-negative isolates indicated a statistically significant difference (χ^2=10.54, p=0.034).

Both *Staphylococcus hemolyticus* isolates exhibited an identical resistance profile: FOX, PG, T, ERY, CD, GM, and C. *Staphylococcus intermedius* displayed the resistance profile: OXA, PG, T, ERY, CD, and SXT.

DISCUSSION

This study provides the first detailed analyses of AMR profiles and genetic determinants in *Staphylococcus* spp. isolates from canine clinical samples in North Macedonia. These findings address a critical gap in AMR surveillance within the Balkan region offering valuable insights into local resistance trends and their alignment with broader European patterns. The results demonstrate a high prevalence of MDR and MR among the isolates, underscoring the significant challenges posed by resistant strains. Specifically, 70.5% of isolates exhibited MDR, reflecting resistance to multiple antibiotic classes, including critically important antimicrobials (CIAs) for human medicine. The presence of *mec*A was confirmed in 28.8% of isolates, highlighting the growing threat of methicillin-resistant strains in companion animals.

Our study revealed a high prevalence of MRSP with 28.7% of isolates *mec*A-positive. These rates are significantly higher than those reported in Denmark, where oxacillin resistance ranges between 6-8%, and in Norway, which reports a 4.4% prevalence based on *mec*A detection (5). In the Balkan region, data on AMR is limited, with the prevalence of the *mec*A gene reported as 26.3% in Serbia (16) and 24.4% in Bosnia and Herzegovina (17). In contrast, Croatia reported a much lower prevalence, with only 7.5% of *S. pseudintermedius* isolates resistant to oxacillin (18). Additionally, Bulgaria reported a high prevalence of MDR in *Staphylococcus* spp. at 59.3% (19). Similarly, a study in Romania (20) reported that 82.8% of isolates exhibited MDR phenotypes, reflecting the widespread resistance challenges in the region. Our findings further emphasize the significant concern of MDR strains, with 69.3% of *S. pseudintermedius* and 85% of *S. aureus* isolates exhibiting resistance to multiple antimicrobial classes. The observed diversity in resistance rates across Europe highlights the multifaceted nature of AMR in Staphylococci isolated from dogs. This heterogenicity results from a combination of factors, including variability in antimicrobial prescribing practices and stewardship initiatives (10) and diversity in clinical bacteriology diagnostic methodologies, including AMR mechanisms screening methods (21). This evidence substantiates the necessity for cooperation in AMR surveillance, standardization of microbiological diagnostic methodologies, and implementation of antimicrobial stewardship initiatives to combat AMR effectively.

Methicillin resistance in *S. pseudintermedius* (MRSP) often presents complex mechanisms that extend beyond the commonly studied *mec*A and *mec*C genes. Interestingly, 27.6% of *S. pseudintermedius* (*MRSP) isolates showed phenotypic oxacillin resistance despite testing negative for the *mec*A and *mec*C genes. These findings are consistent with the study of Bertelloni et al. (6), which reported a similar discrepancy, with 44% of isolates phenotypically resistant to oxacillin and only 24% positive for *mec*A. Such discrepancies highlight the complexity of methicillin resistance mechanisms in *Staphylococcus* spp., including the potential involvement of alternative genetic determinants. Furthermore, our study highlights the critical role of beta-lactamase-mediated resistance, with *bla*Z detected in 65.3% of *MRSP isolates. This suggests that hyperproduction of beta-lactamase may contribute to oxacillin resistance in *mec*A-negative isolates (22, 23, 24). This

finding aligns with the study of Arede et al. (25) who demonstrated the significant role of *bla*Z in resistance expression in MRSA. This finding emphasizes the importance of extending diagnostic testing to include *bla*Z detection, especially in regions where *mec*A-negative oxacillin resistance is prevalent.

Beyond resistance to β-lactams, MR isolates show co-resistance to multiple antibiotic classes, including tetracycline, macrolides, lincosamides, and fluoroquinolones (6, 26). This study identified a significant correlation between MR and resistance to aminoglycosides, fluoroquinolones, lincosamides, and macrolides, emphasizing the frequent occurrence of co-resistance in MR strains (27, 28). A concerning finding in this study is the widespread multidrug resistance exhibited by isolates. In particular, *S. pseudintermedius* showed remarkable resistance patterns: three isolates (3.2%) were resistant to all eight antibiotic classes tested, while twenty-one isolates (13.7%) were resistant to seven: oxacillin, penicillin G, tetracycline, erythromycin, clindamycin, gentamicin, enrofloxacin, marbofloxacin, pradofloxacin, and trimethoprim-sulfamethoxazole. This is consistent with the pattern reported by Morais et al. (27), where 30.4% of isolates showed resistance to beta-lactams, erythromycin, clindamycin, tetracyclines, gentamicin, fluoroquinolones and trimethoprim-sulfamethoxazole.

Additionally, two isolates of *S. aureus* (18.2%) were resistant to all antimicrobials tested, highlighting the robust adaptability of *S. aureus* as a species. Furthermore, the high prevalence of multidrug resistance (MDR) in *S. aureus*, with 84.6% exhibiting resistance to multiple drug classes, raises serious concerns regarding treatment options and highlights the critical need for effective antimicrobial stewardship.

The high rate of resistance to clindamycin, a lincosamide classified as category C (29), is a significant concern in veterinary medicine. Clindamycin is widely recommended as one of the first-line antibiotic for treating of skin infections in dogs (4, 30). However, this study found a resistance rate of 61.8% in *Staphylococcus* isolates, which significantly compromises its efficacy as a primary therapeutic option. The increasing resistance to clindamycin not only reduces its clinical use, but also limits available treatment options for common infections, potentially leading to the overuse of broader-spectrum or critically important antibiotics, such as fluoroquinolones.

Equally concerning are the resistance rates to fluoroquinolones, with 31.2% for marbofloxacin and 32.3% for enrofloxacin. As CIAs, fluoroquinolones play a crucial role in the treatment of serious infections in veterinary and human medicine. Their widespread use in small animal practice in our country (31) likely contributes to the observed resistance and emphasizes the need to limit their use to cases where alternatives have failed and susceptibility data demonstrate their efficacy. In contrast, pradofloxacin had the lowest resistance rate among the antibiotics tested, with only 22.3% of isolates showing resistance. This result is in line with our previous study (31), which highlighted the limited use of pradofloxacin in small animal practice. These lower resistance levels are likely due to the fact that pradofloxacin has only recently been introduced in the country and was approved in 2021 (32).

These findings emphasize the need to revise empirical treatment protocols and prioritize antimicrobial susceptibility testing (AST) to guide therapy. Our prior study (31) revealed that veterinarians primarily rely on "scientific literature" (45.61%) and "personal experience" (43.86%) when selecting antimicrobials for treatment. While these factors contribute to informed decision-making, they may also lead to variability in prescribing practices, particularly in regions lacking robust local resistance data (10). To address these challenges, promoting AST and providing veterinarians with region-specific resistance data are critical steps toward optimizing antimicrobial use and reducing resistance (28).

While this study provides valuable insights into the prevalence and resistance patterns of MR and MDR *S. pseudintermedius* and *S. aureus* in North Macedonia, several limitations must be acknowledged. First, the study population was derived from diagnostic samples, which may overrepresent resistant strains, as veterinarians tend to submit samples primarily from treatment failures or recurrent infections (31). Furthermore, the lack of differentiation between first infections and those previously treated with antibiotics introduces variability in the dataset, potentially biasing the results (33). Despite these limitations, the findings highlight the urgent need for antimicrobial stewardship. Future research should include molecular tools like MLST to better understand resistance mechanisms and transmission dynamics in North Macedonia.

CONCLUSION

In conclusion, this study reveals a significant prevalence of methicillin resistance and multidrug resistance in *Staphylococcus* spp. from canine clinical samples in North Macedonia. The notable resistance of *Staphylococcus pseudintermedius* and *Staphylococcus aureus* to critical antimicrobials raises concerns about treatment efficacy. The detection of *blaZ* in *mec*A-negative isolates underscores the complexity of resistance mechanisms and highlights the need for molecular diagnostics in routine antimicrobial resistance surveillance. These findings emphasize the urgent need for antimicrobial stewardship and targeted AMR strategies to address the spread of resistant strains while understanding their clonal distribution to inform effective control measures.

CONFLICT OF INTEREST

The authors declare that they have no financial or non-financial conflict of interest regarding authorship and publication of this article.

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AUTHORS’ CONTRIBUTION

IC conceived the study and supervised the manuscript's writing. IS drafted the original manuscript, analyzed the data, and interpreted the results. ZPH performed the PCR analysis. IM, MJP, and MRM participated in reviewing and editing the manuscript. AC was involved in manuscript writing and reviewing. All authors have reviewed and approved the final version of the manuscript.

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