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Molecular Epidemiology of *mcr* (1–5) and Other Critical Resistance Genes in Bacteria Isolated from Canine Otitis Externa in Ankara, Türkiye

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Abstract

Antimicrobial resistance in companion animals is a growing public health concern, yet data on last-resort resistance genes in clinical canine isolates remain scarce. This study characterizes the molecular distribution of critical resistance determinants, including *mcr* variants (1–5), in bacteria isolated from canine otitis externa in Ankara, Turkey. Using a combination of phenotypic disk diffusion and targeted quantitative polymerase chain reaction (qPCR), we identified *Enterobacter* spp., *Staphylococcus* spp., and *Pseudomonas aeruginosa* as the predominant pathogens. Notably, among Gram-negative isolates (n = 9), our results indicate preliminary evidence of mobile colistin resistance genes, particularly *mcr-3* in 44.4% (4/9), and *mcr-4* and *mcr-5* each in 33.3% (3/9), marking the first report of these variants in canine ear infections within an urban environment. The observed notable discrepancies between genotypic carriage and phenotypic expression suggest the possible presence of silent resistance reservoirs that traditional diagnostics may overlook. These findings underscore the urgent need for molecular-integrated surveillance in veterinary clinical practice to prevent the zoonotic spread of last-resort resistance genes and to safeguard both animal and public health under the 'One Health' framework.

Keywords: antimicrobial resistance; dog; ear swab; one health; otitis externa

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1. Introduction

Dogs commonly develop otitis externa, often requiring antibiotic treatment due to the underlying bacterial infections associated with them. This specific disease model is very useful in any research study relating to the emergence and development of antimicrobial resistance (AMR) due to the high occurrence of otitis externa and the regularity of treatment. Certain bacterial pathogens that can be considered primary in canine otitis externa cases include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus pseudointermedius* [1]. Even the variability of the frequency of bacterial genera cultured from cases of infection has been shown in various studies in different parts of the world. Variations in bacterial etiology and resistance profiles are primarily shaped by regional and local antimicrobial usage patterns [2–5].

Whereas *P. aeruginosa* is notoriously resistant to many classes of antibiotics including penicillins, β-lactams, cephalosporins, and trimethoprim-sulfamethoxazole, *S. pseudointermedius* generally shows moderate resistance to fluoroquinolones and gentamicin [3].

Together with the variability of bacterial resistance, these variations in AMR make treatment even more difficult and emphasize the need for cautious antibiotic use in veterinary medicine.

Companion animals, particularly dogs, are increasingly recognized as significant potential reservoirs for AMR. This risk is likely exacerbated by their close physical proximity to humans and the frequent administration of broad-spectrum antibiotics in veterinary practice [1,3,6]. However, due to incomplete recording of clinical cases, data on profiles of AMR in animals is rather scarce at the moment. In this context, since otitis externa is quite common in dogs and its treatment necessitates the use of antibiotics for bacterial infections, it becomes a very important condition through which resistance can be studied [4,7].

The most important among them is polymyxin resistance, and more specifically colistin resistance, because it is frequently the antimicrobial of last resort in human clinical practice against multidrug-resistant (MDR) bacteria. However, polymyxins are routinely employed in veterinary dermatology [8]. Specifically, they are common ingredients in topical ear drops used for managing canine otitis externa. This widespread application in pets creates a potential reservoir for resistance that directly threatens public health. Colistin resistance is plasmid-borne *mcr* gene-mediated; *mcr-1* was originally detected in China in 2015 and has since been found globally in human, animal, and food isolates [9]. A total of ten *mcr* types (*mcr-1* to *mcr-10*) have been identified [10,11]. The rapid global spread of these resistance genes from animals to humans, food, and the environment highlights the zoonotic risk, particularly as animals are estimated to play a crucial role in the transmission of these mobile genes due to shared environments [12]. Resistance to other last-resort antibiotics like vancomycin and imipenem (e.g., porin loss, efflux pumps) has also been observed [13]. The extensive use of antibiotics in animal treatment may facilitate the dissemination of resistance genes, posing a potential challenge to public health. Dispersion of resistant bacteria is more likely between human beings and animals sharing the same kind of environment [10]. Recent evidence has highlighted the emergence of last-resort resistance genes in urban settings, such as the identification of a transferable *mcr-1* gene in a healthy dog in South Korea [14].

Since regional and environmental characteristics shape patterns of pathogens and resistance, local data generation is crucial for effective treatment regimen design and security of public health. The purpose of this study is therefore to determine the bacterial etiology of otitis externa in dogs, their pattern of resistance and resistance gene pattern to provide an evaluation of the risk of transmission to human health. The study was conducted within the "One Health" framework. This article presents in detail the techniques employed in the analysis of canine otitis externa, from collection of the sample to microbiological examination, AMR typing, and genetic resistance profiling.

2. Materials and Methods

2.1. Sample Collection

Ear swabs were collected from 25 dogs with otitis externa (50 samples) at clinics in Ankara, Turkey. Data on age, sex, breed, and weight were recorded with owner consent. Two gel-based and one standard swab were taken per dog; gels supported anaerobic growth. Genetic samples were stored in RNALater at −80 °C (DiaRex RL-0342, Diagen, Ankara, Türkiye), while bacteriological samples were transported at +4 °C on the same day. Genetic samples remained frozen until batch transfer.

2.2. Bacteriological Examination

Ear swabs were cultured on nutrient agar, 5% sheep blood agar, and MacConkey agar, then incubated at 37 °C for 24 h. Purified colonies were identified using Gram

staining, catalase, and oxidase tests. *Staphylococcus* spp. were characterized as catalase-positive, oxidase-negative spheres, and *Streptococcus* spp. as negative in both tests. Among Gram-negative rods, *Pseudomonas* spp. were identified by green pigment on nutrient agar, and *E. coli* by typical colony characteristics on MacConkey and EMB agars [15]. In cases where mixed bacterial growth was observed or different species were isolated from the bilateral ear swabs of a single dog, the predominant microorganism for that patient was determined based on the highest relative colony density (heaviest growth) observed on the primary culture plates. In cases of polymicrobial growth, the predominant microorganism was defined as the species exhibiting the highest semi-quantitative colony-forming unit (CFU) count on primary culture media. While this selection focused on the primary clinical driver of the infection, we acknowledge the inherent limitation of excluding secondary pathogens that may harbor distinct resistance profiles. The selection of the predominant microorganism was a deliberate methodological choice to establish a standardized baseline for correlating specific genetic determinants with their corresponding phenotypic expressions. This approach focused on the primary clinical driver of the infection to ensure that the detected resistance genes could be more accurately attributed to the pathogen most likely responsible for the clinical presentation. To ensure culture purity, all isolates were subcultured at least twice on selective media prior to DNA extraction. Colony morphology, Gram staining results, and biochemical test outcomes were recorded for each subculture to confirm monoculture status. We acknowledge, however, that in the absence of 16S rRNA gene sequencing-based confirmation, the possibility of low-level contamination cannot be entirely excluded, particularly for the findings observed in polymicrobial samples. This represents a limitation of the current study and underscores the need for WGS-based validation of these results.

2.3. Antibiotic Susceptibility Tests

Bacterial susceptibility to antibiotics was determined by the Kirby-Bauer Disk Diffusion method, as recommended by The Clinical and Laboratory Standards Institute [16]. Forty antibacterial disks (shown in Table 1) were utilized. Agar plates were incubated at 37 °C for 18–24 h, and inhibition zone diameters were measured according to CLSI standards [16,17]. Selective media, including blood agar and mannitol-salt agar for Gram-positive bacteria and MacConkey agar for Gram-negative bacteria, were employed. While intermediate susceptibility results hold certain clinical relevance, this study exclusively reported 'resistant' outcomes. The primary rationale for this exclusion was to strictly highlight the isolates posing the highest risk of definitive therapeutic failure in clinical veterinary practice, thereby maintaining a sharp focus on multidrug resistance and the efficacy of 'last-resort' antibiotics within the One Health context. Colistin susceptibility testing was initially performed using the disk diffusion method; however, in accordance with current CLSI and EUCAST recommendations, this method is not considered reliable for polymyxins. Therefore, phenotypic colistin susceptibility results were excluded from further analysis and interpretation. Metronidazole was included in the initial panel; however, as all isolates in this study were aerobic or facultative bacteria, the results were excluded from further analysis due to intrinsic resistance.

Table 1. Antibiotic disks and concentrations selected to determine the susceptibility of bacteria isolated from external ear infections of dogs by disk diffusion method.

Antibiotic	Symbol/Concentration	Antibiotic	Symbol/Concentration
Aminosidin	AN-60 µg	Florfenicol	FFC-30 µg
Amoxicillin	AML-20 µg	Fosfomycin	FF-30 µg
Amoxicillin/clavulanic acid	AMC-30 µg	Imipenem	IMP-10 µg
Ampicillin/cloxacillin	APX 30 µg	Lincomycin	L-15 µg
Ampicillin/sulbactam	SAM-20 µg	Marbofloxacin	MAR-5 µg
Cefazolin	CZ-30 µg	Methicillin	ME-5 µg
Cefepime	FEP-10 µg	Metronidazole	MET-10 µg
Cefoperazone	CEP-75 µg	Neomycin	N-30 µg
Cefquinome	CEQ-30 µg	Oxytetracycline	T-30 µg
Ceftriaxone	CRO-30 µg	Penicillin G	P-10 U
Cephalexin	CL-30 µg	Spectinomycin	SPT-25 µg
Chloramphenicol	C-30 µg	Spiramycin	SP-30 µg
Ciprofloxacin	CIP-10 µg	Streptomycin	S-25 µg
Clarithromycin	CLR-15 µg	Sulfadiazine	SD-300 µg
Clindamycin	DA-10 µg	Sulphamethoxazole	SMZ-100 µg
Cloxacillin	CX-30 µg	Tetracycline	TE-30 µg
Colistin	CT-25 µg	Trimethoprim	TMP-30 µg
Danofloxacin	DAN-5 µg	Trimethoprim/sulphamethoxazole	SXT-25 µg
Doxycycline	DO-30 µg	Tulathromycin	TUL-30 µg
Erythromycin	E-15 µg	Vancomycin	VA-10 µg

2.4. Identification of Resistance Genes

Resistance genes were selected according to AMEG (Antimicrobial Advice Ad Hoc Expert Group) classification by the European Medicines Agency [18]. Target genes included *ampC* (cefepime; restricted to Gram-negative bacteria), *mcr-1–5* (colistin; restricted to Gram-negative bacteria), bla_{IMP} (imipenem), *gyrA*, *gyrB* (ciprofloxacin), and *OXA-1* (amoxicillin-clavulanic acid). The penA gene, encoding a penicillin-binding protein specific to *Neisseria* spp., was excluded from the panel as it is not applicable to the bacterial species isolated in this study.

DNA was extracted from ear swab samples using the DiaRex® Genomic DNA Extraction Kit (Cat. No: SD-0323, Ankara, Turkey) following the manufacturer's instructions, with minor modifications for swab samples [19]. In brief, 250 µL lysis solution was added to 200 µL of ear swab sample, followed by glass and zircon beads for mechanical disruption using a homogeniser. After homogenization, 25 µL Proteinase K was added and in-cubated at 56 °C for 60 min. Post-incubation, the sample was centrifuged (5000 *g*, 5 min), and the supernatant transferred. Next, 200 µL lysis buffer was added, incubated at 70 °C for 10 min, followed by the addition of 250 µL ethanol. The lysate was then passed through a column, washed, and DNA was eluted in 100 µL buffer after centrifugation (8000 *g*, 1 min).

Extracted DNA was analyzed using real-time qPCR (ROCHE LightCycler® 480, Roche Diagnostics GmbH, Mannheim, Germany). Primers were selected based on previous studies [9,10,20–22]. The qPCR protocol included an initial denaturation at 95 °C, followed by 40 cycles of denaturation (95 °C), annealing (55 °C), and extension (72 °C). Each gene was tested in separate monoplex reactions, and gene presence was assessed post-amplification (Table 2).

Table 2. Primers used to produce real-time qPCR standards.

Target Gen	Primer Name	Primer Sequence (5′ → 3′)
mcr-1	colistin_mcr_1-F	GTCCGTTTGTTCTTGTGG
	colistin_mcr_1-R	GTCTGTAGGGCATTTTGG
mcr-2	colistin_mcr_2-F	GTATTCTGTGCCGTGTATG
	colistin_mcr_2-R	GTATTGTTGGTTGCTGATTT
mcr-3	colistin_mcr_3-F	GCCTCATTTTGATTGGTTTC
	colistin_mcr_3-R	TAAGTTTGGTTTCGCCATTT
mcr-4	colistin_mcr_4-F	CCCGAACACTAAACCTAAC
	colistin_mcr_4-R	AAACATACAGGGTAGAGACA
mcr-5	colistin_mcr_5-F	ACTGATTCTGCTTGCTGT
	colistin_mcr_5-R	TCATTACCGCTTGTTTCC
bla_{IMP}	Imipenem-F	GAGTGGCTTAATTCTCRATC
	Imipenem-R	AACTAYCCAATAYRTAAC
gyrA	gyrA-F	TGTCCGAGATGGCCTGAAGC
	gyrA-R	TACCGTCATAGTTATCCACG
gyrB	gyrB-F	TCGGCGTCGTTGTTGTCATA
	gyrB-R	GCGGTGGGTTTCAAAATCTG
ampC	Cefepime_ampC-F	TTCTTGTCTACTTTTATCCCC
	Cefepime_ampC-R	ACTGCTATTTACGGCTTTTT
OXA-1	Amoxicillin + clavulanic acid_OXA1-F	TTTTCTGTTGTTTGGGTTTC
	Amoxicillin + clavulanic acid_OXA1-R	CTATGGTGTTTTCTATGGCT

For primer validation, conventional PCR was also performed. Primers were designed using Primer3 software based on NCBI FASTA files. PCR was run in 20 µL reactions containing polymerase, Diagen PCR mix, genomic DNA, and Biomers primers. Cycling conditions included 95 °C for 10 min, followed by 40 cycles (95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min), and a final extension at 72 °C for 10 min.

2.5. Statistical Analysis

Descriptive statistics were used to summarize the prevalence of bacterial species and resistance genes. For key resistance determinants (*mcr* variants), 95% confidence intervals (CIs) were calculated using the Wilson score method to estimate the precision of the prevalence rates. All calculations were performed using SPSS software (version 20.0, IBM, Armonk, NY, USA).

3. Results

3.1. Isolates Detected and Resistance Genes of the Isolates

The dog breeds (age, weight and sex) with otitis externa included in the study, the dominant bacterial strain isolated from each dog, and the resistance genes detected in this bacterial strain are shown in Table 3. The distribution of resistance genes across all isolates is illustrated as a heatmap (Figure 1). Dominant bacteria isolated according to bacteriological analysis results: *Staphylococcus aureus* (28%), *S. epidermidis* (24%), *Enterobacter* spp. (20%), *P. aeruginosa* (16%) and *Streptococcus* spp. (12%) (Figure 2).

Table 3. Dog breeds sampled, characteristics, dominant bacterial species, and detected genetic determinants [1].

No.	Dog Breed	Age-Weight-Sex	Dominant Microorganism	Detected Resistance Genes/Determinants [2]	Phenotypic Resistance Profile
1	Belgian Sheepdog	4 years old-35 kg-female	*Pseudomonas aeruginosa*	*mcr-4*, *gyrA*	Ciprofloxacin, Sulfonamides
2	Beagle	3 years old-17 kg-male	*Pseudomonas aeruginosa*	*mcr-4*, *ampC*, *gyrA*	Ciprofloxacin, Beta-lactams
3	Yorkshire Terrier	5 years old-3 kg-male	*Staphylococcus aureus*	–	Beta-lactams, Sulfonamides
4	Basset Hound	10 years old-36 kg-male	*Staphylococcus epidermidis*	*gyrA*, *gyrB*, *OXA-1*	Ciprofloxacin, Amoxicillin-Clav, Beta-lactams
5	Golden Retriever	10 years old-33 kg-male	*Pseudomonas aeruginosa*	*mcr-3*, *ampC*, *gyrA*, *gyrB*	Ciprofloxacin, Beta-lactams
6	German Shepherd	7 years old-30 kg-male	*Enterobacter* spp.	*mcr-4*, *ampC*, *gyrA*, *gyrB*	Imipenem, Ciprofloxacin
7	Pug	4 years old-8 kg-female	*Enterobacter* spp.	*mcr-1*, *mcr-5*	Imipenem, Sulfonamides
8	Akita	1 year old-36 kg-male	*Streptococcus* spp.	*gyrA*, *gyrB*, *OXA-1*	Ciprofloxacin, Beta-lactams
9	English Cocker Spaniel	7 years old-10 kg-male	*Staphylococcus epidermidis*	*gyrA*, *gyrB*, *OXA-1*	Ciprofloxacin
10	Golden Retriever	13 years old-28 kg-female	*Staphylococcus aureus*	*gyrA*, *gyrB*	Ciprofloxacin, Beta-lactams
11	Maltese Terrier	4 years old-2.5 kg-female	*Enterobacter* spp.	*mcr-3*, *gyrA*, *OXA-1*	Imipenem, Ciprofloxacin
12	English Cocker Spaniel	11 years old-9 kg-male	*Staphylococcus epidermidis*	*gyrA*	Ciprofloxacin, Beta-lactams
13	Bernese Mountain Dog	6 years old-50 kg-female	*Staphylococcus aureus*	*OXA-1*, *gyrA*	Ciprofloxacin, Amoxicillin-Clav
14	Chihuahua	7 years old-1.5 kg-male	*Staphylococcus aureus*	*gyrA*, *gyrB*	Ciprofloxacin, Beta-lactams, Sulfonamides
15	Golden Retriever	3 years old-28 kg-male	*Staphylococcus epidermidis*	*gyrA*	Ciprofloxacin
16	Golden Retriever	3 years old-33 kg-female	*Staphylococcus aureus*	*gyrA*	Ciprofloxacin
17	Cavalier King Charles Spaniel	10 years old-15 kg-male	*Enterobacter* spp.	*mcr-1*, *ampC*	Imipenem, Colistin, Beta-lactams
18	Yorkshire Terrier	7 years old-2 kg-male	*Staphylococcus aureus*	*gyrA*	Ciprofloxacin
19	Golden Retriever	3 years old-25 kg-female	*Streptococcus* spp.	*OXA-1*	Beta-lactams
20	Golden Retriever	10 years old-33 kg-female	*Pseudomonas aeruginosa*	*mcr-3*, *mcr-5*, *gyrA*, *gyrB*	Ciprofloxacin, Beta-lactams
21	Hybrid Dog	2 years old-23 kg-female	*Staphylococcus epidermidis*	–	Beta-lactams
22	Golden Retriever	13 years old-40 kg-female	*Staphylococcus aureus*	*gyrA*	Ciprofloxacin, Beta-lactams
23	Kangal Shepherd Dog	1 year old-45 kg-male	*Enterobacter* spp.	*mcr-3*, *mcr-5*, *gyrA*	Imipenem, Ciprofloxacin
24	Golden Retriever	9 years old-35 kg-female	*Streptococcus* spp.	*gyrA*, *OXA-1*	Ciprofloxacin
25	English Cocker Spaniel	5 years old-11 kg-female	*Staphylococcus epidermidis*	*gyrA*	Ciprofloxacin, Beta-lactams

[1] Colistin susceptibility was not assessed phenotypically due to methodological limitations; only the genotypic (mcr) presence was reported. [2] The presence of gyrA and gyrB refers to the detection of target gene sequences; phenotypic resistance is associated with specific mutations within these regions.

3.2. Antibiogram Results (40 Antibiotics)

It is important to explicitly state that all resistance rates and percentages presented in the following sections were calculated based on the 25 predominant bacterial isolates (i.e., exactly one characterized isolate per dog), rather than the total number of collected ear swabs (n = 50) or the total number of mixed colonies initially cultured. High resistance rates (80–96%) were observed across the aminoglycoside class, with the notable exception of gentamicin, which exhibited 84% susceptibility. Regarding cephalosporins, while all isolates were susceptible to cefepime and ceftriaxone, susceptibility to cefazolin and cefquinome was markedly low (4–8%). Almost all samples demonstrated resistance to beta-lactams such as amoxicillin, penicillin G, and ampicillin-cloxacillin, whereas ampicillin-sulbactam retained 36% susceptibility. In the quinolone group, marbofloxacin showed relatively high efficacy (76% susceptibility) compared to danofloxacin (44%). Susceptibility rates for other antibiotic classes were generally low, including phenicols (16–24%), macrolides (8–40%), and tetracyclines (12–20%). Additionally, fosfomycin showed 40% susceptibility. Notably, all samples were found to be resistant to sulfadiazine and sulfamethoxazole individually, although the trimethoprim-sulfamethoxazole combination displayed 28% susceptibility.

All isolates exhibited intrinsic resistance to metronidazole, consistent with their aerobic or facultative nature; therefore, these results were not considered in the comparative resistance analysis.

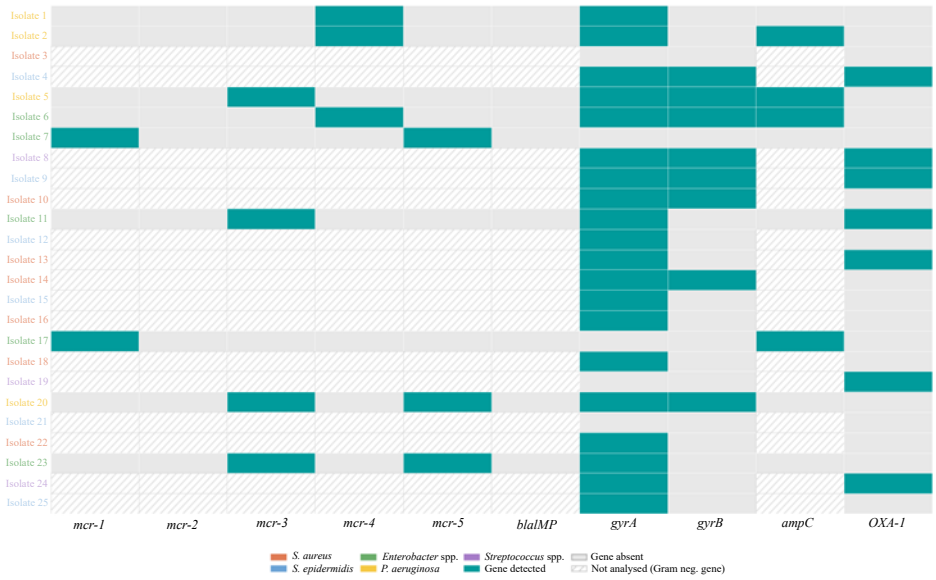


Figure 1. Heatmap of resistance gene distribution across 25 canine otitis externa isolates. Teal cells indicate gene detection; gray cells indicate absence. Isolate labels on the y-axis are color-coded by bacterial species (see legend).

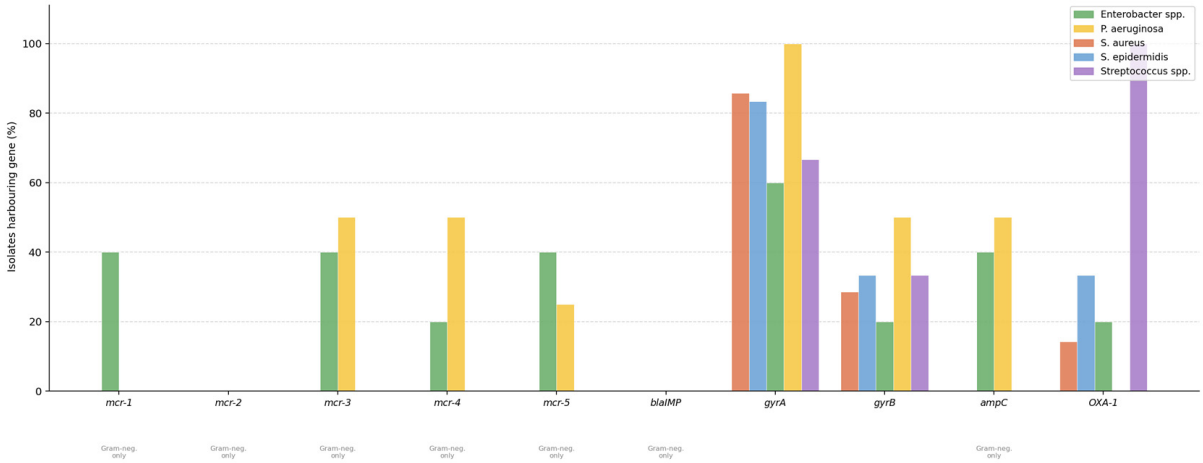


Figure 2. Prevalence of resistance genes by bacterial species in canine otitis externa isolates (n = 25). Bar height represents the percentage of isolates within each species harboring the respective gene. Gram-negative-specific genes (*mcr* variants, *ampC*, bla_{IMP}) are shown only for Gram-negative species.

3.3. Antimicrobial Resistance Distribution

Although *mcr* genes were detected in multiple isolates, phenotypic susceptibility to colistin was not evaluated due to methodological limitations. In contrast, all isolates remained susceptible to cefepime and ceftriaxone. For imipenem, resistance was observed in all *Enterobacter* spp. (5/5) and 75% of *P. aeruginosa* (3/4), consistent with intrinsic resistance mechanisms and/or carbapenemase activity. Phenotypic non-susceptibility was also recorded in 71% of *S. aureus* (5/7), 60% of *Streptococcus* spp. (3/5), and 33% of *S. epidermidis* (2/6); however, these findings in Gram-positive isolates should be interpreted with caution, as CLSI disk diffusion breakpoints for imipenem are not standardized for *Staphylococcus* or *Streptococcus* spp. in veterinary settings, and the clinical relevance of such profiles in these species remains uncertain. The bla_{IMP} gene was not detected in any sample, suggesting

the involvement of alternative resistance mechanisms or phenotypic misclassification in Gram-negative isolates, and questioning the biological significance of the observed non-susceptibility in Gram-positive strains. It should be noted that *P. aeruginosa* and *Enterobacter* spp. exhibit intrinsic resistance to amoxicillin-clavulanic acid and were therefore excluded from susceptibility interpretation for this agent; similarly, *Streptococcus* spp. are inherently susceptible to this agent and no acquired resistance was recorded. Among the interpretable species, resistance to amoxicillin-clavulanic acid was observed in 17% of *S. epidermidis* and 14% of *S. aureus*, with the *OXA-1* gene identified in seven samples. Finally, ciprofloxacin resistance was found in 60% of *Enterobacter* spp., 50% of *P. aeruginosa*, 33% of both *Streptococcus* spp. and *S. epidermidis*, and 14% of *S. aureus*, with *gyrA* and/or *gyrB* genes detected in all resistant isolates.

3.4. Concordance Between Resistance Genes and Phenotypic Resistance

Analysis of resistance genes by PCR revealed notable discrepancies between genotype and phenotype. In some instances, resistance was observed in the absence of the targeted genes, whereas in others, isolates remained susceptible despite harboring resistance determinants. For example, among the interpretable species (*Staphylococcus* spp.), one of the two *OXA-1*-carrying isolates (*S. epidermidis*) remained susceptible to amoxicillin-clavulanic acid.

4. Discussion

The present study provides an evaluation of both phenotypic AMR and the underlying genotypic profiles in bacterial isolates from canine otitis externa. Our investigation yielded three core findings within the One Health framework. First, we observed notable rates of multidrug resistance within this small cohort among the predominant pathogens (e.g., *Staphylococcus* spp., *Pseudomonas aeruginosa*, and *Enterobacter* spp.), particularly against commonly prescribed veterinary antibiotics. Second, we detected multiple resistance genes associated with 'last-resort' human therapeutics—such as *mcr* variants (colistin)—in urban companion animals lacking agricultural exposure. Finally, our molecular analysis revealed discrepancies between genotypic carriage and phenotypic expression, suggesting the potential presence of transcriptionally inactive or "silent" genes as a working hypothesis, alongside other multifactorial resistance mechanisms. Together, these findings highlight the potential value of integrated molecular and phenotypic diagnostics to guide antimicrobial stewardship, as discussed in detail below.

The bacterial pathogens identified in this study are consistent with World Health Organization (WHO) priority classifications for AMR [23]. Specifically, *S. aureus* (28%), *Enterobacter* spp. (20%), *P. aeruginosa* (16%), and *Streptococcus* spp. (12%) represent high- and critical-priority organisms for which last-resort antibiotics are of particular concern. The detection of resistance determinants in these species therefore carries direct public health relevance within the One Health framework, and their therapeutic management should be guided by culture and sensitivity testing [24].

The genetic panel employed in this study was targeted toward specific, high-priority resistance determinants rather than providing a global resistome profile. Consequently, the absence of certain genes (e.g., bla_{IMP}) does not preclude the existence of other resistance mechanisms, and our findings should be interpreted as a focused molecular snapshot. The discrepancies observed between phenotypic resistance patterns and the PCR panel—such as imipenem resistance in the absence of bla_{IMP}—reflect the multifactorial nature of AMR in these isolates. Future studies employing broader diagnostic tools, such as whole genome sequencing (WGS), are therefore warranted.

Previous studies have reported *mcr* gene transfer predominantly in enteropathogenic *E. coli* and *Salmonella* spp. [25], with *mcr-1* detected in companion animals in China [26]

and South Korea [27]. All *mcr* variants except *mcr-6* and *mcr-7* have been identified in canine bacterial isolates [10,11]. In the present study, multiple mcr variants were detected among Gram-negative isolates (n = 9), with *mcr-3* identified in 44.4% (95% CI:18.8–73.0), *mcr-4* and *mcr-5* each in 33.3% (95% CI: 12.1–64.6), and *mcr-1* in 22.2% (95% CI: 6.3–54.7) of Gram-negative cases, while *mcr-2* was absent. When calculated across the entire cohort (n = 25), these correspond to 16%, 12%, 12%, and 8%, respectively; however, as *mcr* genes are restricted to Gram-negative bacteria, prevalence estimates are most appropriately reported within this subpopulation. As phenotypic colistin susceptibility was not assessed due to the unreliability of disk diffusion for polymyxins, the presence of these genes should be interpreted as indicative of resistance potential rather than confirmed phenotypic expression. Notably, all dogs in this cohort resided in urban housing, consumed commercial dry food, and exercised in enclosed environments, suggesting that agricultural exposure is not a prerequisite for *mcr* gene carriage and that alternative urban transmission pathways warrant further investigation [14].

A notable finding in this study was the high-level imipenem resistance observed in all *Enterobacter* spp. and 75% of *P. aeruginosa* isolates, despite the absence of the bla_{IMP} gene. This discrepancy suggests the involvement of alternative carbapenemase families not included in our PCR panel, such as bla_{KPC}, bla_{NDM}, or bla_{OXA}-48-like enzymes, which have been increasingly reported in veterinary isolates [28,29]. Additionally, resistance in *Enterobacter* spp. may be driven by overproduction of intrinsic *ampC* beta-lactamases combined with downregulation of outer membrane porins [30]. These findings are consistent with reports of carbapenem non-susceptibility in 15–23% of *P. aeruginosa* isolates from companion animals [31], and further underscore the need for WGS-based approaches to comprehensively characterize carbapenem resistance mechanisms in canine isolates. The phenotypic non-susceptibility observed in *Staphylococcus* and *Streptococcus* spp. is not interpretable per current CLSI standards, as standardized breakpoints for imipenem disk diffusion are lacking for these species in veterinary microbiology.

All isolates remained susceptible to ceftriaxone, consistent with findings from comparable studies [32]. The *penA* gene was excluded from the analysis of all pathogens, as it encodes a penicillin-binding protein specific to *Neisseria* spp. and is not applicable to the bacterial species isolated in this study.

Ciprofloxacin resistance was detected in 36% of isolates overall, with the highest rates observed in *Enterobacter* spp. (60%) and *P. aeruginosa* (50%). Although *gyrA* and *gyrB* sequences were detected across isolates, phenotypic fluoroquinolone resistance arises from specific mutations within the quinolone resistance-determining regions (QRDRs) rather than mere gene presence, consistent with findings from a comparable South Korean study that identified novel QRDR mutations in *P. aeruginosa* from canine otitis externa [33]. These findings suggest that fluoroquinolone use in canine infections, particularly those involving *P. aeruginosa*, should be guided by susceptibility testing.

Methicillin resistance, assessed by disk diffusion using cefoxitin or oxacillin disks per CLSI guidelines, was observed in 77% of *Staphylococcus* spp. isolates.

All isolates in this study remained susceptible to cefepime, in contrast to a South Korean study reporting 44.4% cefepime resistance in *P. aeruginosa* from dogs and cats [34], possibly reflecting regional differences in antimicrobial usage patterns. The *ampC* gene was detected in four of 25 isolates (16%), with distribution restricted to *Pseudomonas* and *Enterobacter* spp. (Gram-negative organisms), consistent with the known host range of plasmid-mediated ampC beta-lactamases, and suggesting its relevance as a resistance determinant in canine otitis externa within this cohort.

Since *P. aeruginosa* is intrinsically resistant to amoxicillin-clavulanic acid, the disk diffusion results for this agent are not interpretable for this species and have been excluded from

the susceptibility analysis. Accordingly, the apparent resistance reported for *P. aeruginosa* reflects intrinsic, not acquired, resistance and should not be attributed to the absence of the OXA-1 gene. Among the intrinsically susceptible species (*Staphylococcus* spp.), the *OXA-1* gene was detected in seven isolates overall; however, among the interpretable species (Staphylococcus spp.), two of these isolates were OXA-1-positive, of which one remained phenotypically susceptible. This genotype-phenotype discrepancy is consistent with previous reports indicating that *OXA-1*-mediated resistance depends not only on gene presence but also on adequate enzyme expression, beta-lactamase activity, and the broader cellular environment including efflux pump efficiency and membrane permeability [35,36]. These findings further illustrate the multifactorial nature of resistance expression in this cohort, as discussed in detail in the following section.

The discrepancies between genotypic presence and phenotypic susceptibility observed for *OXA-1*-positive/amoxicillin-clavulanate-sensitive isolates may be attributable to several molecular and cellular mechanisms:

1. Gene Expression Regulation (Silent Genes): The mere presence of a resistance gene does not guarantee its transcription.

2. Protein Functional Defects: Even if transcribed, genetic mutations can impair the final protein's function. Similarly, the *OXA-1* gene might produce an enzyme with inadequate beta-lactamase activity due to point mutations or lack of concurrent synergistic resistance mechanisms [35].

3. Efflux Pumps and Membrane Permeability: Phenotypic resistance is often multifactorial. For genes like *OXA-1*, the resistance profile is influenced by the cellular environment, including efflux pump activity and outer membrane porin permeability. If antibiotic accumulation remains below the inhibitory threshold despite gene presence, a susceptible phenotype may be observed in standard testing [36].

4. The Role of Biofilms: In canine otitis externa, the discrepancy between in vitro susceptibility and clinical resistance may also be linked to biofilm formation. Standard disk diffusion tests assess planktonic bacteria; however, in vivo, gene-carrying strains may form biofilms in the ear canal that physically restrict antibiotic penetration and alter bacterial metabolism, potentially creating a more resistant phenotype clinically than laboratory testing suggests.

Limitations and Future Perspective: The present study has several limitations that should be considered when interpreting the findings. First, the small sample size (n = 25) from a single clinical setting in Ankara, Turkey, limits the generalizability of these results to broader canine or regional populations, and the findings should be regarded as preliminary and exploratory. Second, the targeted PCR panel covered specific high-priority resistance genes and does not represent a comprehensive resistome profile; the absence of detected genes does not exclude other resistance mechanisms. Third, gene expression levels and regulatory controls were not assessed, meaning that genotype-phenotype discrepancies remain mechanistically unresolved and the 'silent gene' interpretation should be regarded as a working hypothesis requiring transcriptional validation. Fourth, the selection of a single predominant isolate per case may have introduced selection bias, as secondary pathogens with distinct resistance profiles may have been overlooked. The detection of OXA-1 in *Staphylococcus* spp. is atypical, as this gene is predominantly associated with Gram-negative Enterobacterales. While primer cross-reactivity or low-level contamination cannot be entirely excluded, the consistent phenotypic correlation in some isolates supports genuine detection. Whole genome sequencing is warranted to confirm this unexpected finding. Future studies incorporating larger, multi-center cohorts, whole genome sequencing, transcriptional assays, and metagenomic approaches are warranted to address these limitations comprehensively.

5. Conclusions

Our preliminary findings suggest the complexity of AMR in bacterial isolates from canine otitis externa. These results further emphasize the importance of comprehensive analysis of antibiograms in antibiotic selection. Importantly, a lack of agreement between the presence of resistance genes (*mcr* variants and *OXA-1*) and actual phenotype was observed. This suggests that an approach including both molecular and phenotypic testing is required. This is necessary to accurately assess resistance profiles and guide effective therapeutic strategies. This is particularly important in the context of the global threat posed by AMR and the WHO's classification of critical-priority pathogens.

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