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Extended-Spectrum β-Lactamase-/AmpC-Producing *Escherichia coli* and Associated Risk Factors in Shelter Dogs: A Baseline Study in North Macedonia

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Abstract

Antimicrobial resistance (AMR) is a significant public health concern in companion animals, yet systematic surveillance in North Macedonia is lacking. This study investigated the prevalence of resistance in *Escherichia coli* isolated from 112 fecal samples from dogs in six shelters in North Macedonia and evaluated the associated risk factors, providing the first baseline dataset for this population. High resistance was observed for sulfamethoxazole (68.75%), ampicillin (52.68%), and ciprofloxacin (41.07%). Multidrug resistance was present in 50% of the isolates, with 17 (15.17%) confirmed as ESBL producers. Additionally, 18 isolates (16.1%) were identified as AmpC producers, 16 of which carried the *blaCMY-2* gene. Notably, 72.2% of ESBL/AmpC isolates were resistant to ertapenem despite the absence of carbapenemase genes, a finding that warrants further investigation. Risk factors such as shared housing, longer shelter stays, and frequent empirical antimicrobial use were identified as probable contributors to the carriage of ESBL-/AmpC-producing *E. coli*. None of the shelters had antimicrobial stewardship protocols or routine diagnostic testing, revealing critical gaps in infection control and antimicrobial practices. These findings underscore the urgent need for antimicrobial stewardship and surveillance in North Macedonia's companion animal populations within the One Health framework.

Keywords: multidrug resistance; animal shelters; antimicrobial stewardship; ESBL; AmpC; beta-lactamase; *Escherichia coli*

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1. Introduction

Escherichia coli (*E. coli*) is a leading cause of infections in humans and animals and plays a crucial role in the global antimicrobial resistance (AMR) crisis [1]. In the European Union, *E. coli* is one of the most common antimicrobial-resistant bacteria in animals, including companion animals like dogs and cats [2]. The growing population of companion animals raises concerns about their role as AMR reservoirs, as close human–animal interactions

facilitate the transmission of resistant bacteria and antimicrobial resistance genes (ARGs), highlighting the need for increased monitoring [3]. Additionally, there has been a rising burden of extended-spectrum β-lactamases ESBL-/AmpC-producing *E. coli*, isolated from infection sites and the feces of healthy animals, posing a potential risk for transmission [4]. In fact, ESBL-/AmpC-producing *E. coli* is recognized as a widely distributed source of AMR in animals. Data suggest that the estimated global prevalence of ESBL-producing *E. coli* in companion dogs is approximately 6.29% [5]. Notably, this prevalence is even higher in shelter dogs, who are three times more likely to carry ESBL- or AmpC-producing *E. coli* than companion dogs [6]. Healthy shelter dogs are important reservoirs of ARGs, especially β-lactam resistance genes, which show the highest diversity among the identified ARG classes in the study of Shringi et al. [7]. The fecal shedding of these ARGs presents a significant transmission risk to both animals and humans.

The resistance mechanisms in *E. coli* are complex and primarily involve genetic adaptations that enable survival in the presence of antimicrobial agents. A significant mechanism is the production of β-lactamases, particularly ESBLs and AmpC β-lactamases, which confer resistance to a wide range of β-lactam antibiotics, including penicillins and cephalosporins [8,9]. Plasmids often carry genes that encode these enzymes, allowing transfer between bacteria and facilitating the rapid spread of ARGs within and across species [10,11].

In veterinary medicine, β-lactam agents are among the most commonly used antimicrobials for treating bacterial infections [12]. The use of critically important antimicrobials (CIAs) in small animal medicine poses an additional risk factor for the emergence and spread of AMR [1,13]. In North Macedonia, data show that β-lactam agents such as amoxicillin, amoxicillin–clavulanic acid, and fluoroquinolones are commonly prescribed for companion animals [14]. The role of antimicrobial use as a contributing factor to resistance in *E. coli* is well-documented. Studies have shown that previous antibiotic exposure significantly correlates with the emergence of resistant strains, particularly ESBL- and carbapenem-resistant *E. coli* [15]. Commensal *E. coli*, which is frequently exposed to these agents, is a key indicator for monitoring AMR within animal populations, reflecting the selective pressure imposed by antibiotic use.

Shelter dogs often have unknown medical histories and uncertain previous exposure to antimicrobials. They live in high-density environments with varying hygiene practices, which can spread antimicrobial-resistant strains [6]. Antimicrobial use in the shelters is often poorly documented and influenced by limited resources and the need to manage disease outbreaks [16]. Although the importance of antimicrobial stewardship guidelines (ASGs) in promoting responsible use of antimicrobials is well recognized, evidence of their implementation in animal shelters is still limited. This is particularly concerning within the One Health framework, as improper use of antimicrobials in high-density shelter environments can lead to the selection and proliferation of ESBL and AmpC-producing *E. coli*. Shelter policies in North Macedonia, which include returning dogs to their original locations and offering them for adoption, create a direct pathway for the dissemination of antimicrobial-resistant bacteria and resistance genes into the wider community. This highlights the importance of effective antimicrobial stewardship and infection control not just within the shelters but also in the context of public health. While data on ESBL-/AmpC-producing *E. coli* in companion animals is available from other European countries [5], Eastern and Southeastern Europe, including the Balkan region, remain underrepresented in veterinary AMR surveillance systems, creating critical blind spots in the European monitoring network [17].

The aim of this study was to conduct initial screening of AMR in shelter dogs in North Macedonia, with a focus on commensal *E. coli* and ESBL/AmpC producers. We also

evaluated antimicrobial use practices, shelter management conditions, and associated risk factors to identify potential drivers of resistance in the country. This work provides baseline data for a previously unstudied setting, offering evidence to inform control strategies and strengthen future One Health surveillance.

2. Materials and Methods

2.1. Data and Sample Collection

The data and fecal samples were collected between March and October 2024. The study involved 119 healthy dogs housed in individual boxes across six different shelters in all eight regional areas of North Macedonia. All shelter dogs undergo quarantine upon entry, during which they are tested for *Leishmania* spp., receive a health check including blood work, and are vaccinated against rabies, dewormed, and spayed/neutered before being housed. At the time of sampling, dogs were considered apparently healthy based on observation and fecal scores, although no comprehensive clinical examinations were conducted. None of the dogs were under antimicrobial treatment at the time of sampling.

At the time of sampling, North Macedonia had 17 licensed facilities authorized to manage stray dogs, including both municipal shelters and veterinary clinics with sheltering capacity. Six facilities were included in this study (three municipal shelters and three veterinary clinics), located in Strumica, Skopje, Kumanovo, Demir Kapija, Kichevo, and Tetovo. Veterinary clinics are permitted to house dogs originating from multiple municipalities through public tenders, meaning that the animals sampled did not exclusively represent local populations but rather a broader mix of dogs from different regions.

2.2. Fecal Sampling and Bacterial Isolation

One fresh fecal sample per dog was collected aseptically from the ground using the inverted plastic bag technique, with only a portion of the upper layer taken while the part in contact with the ground was avoided. A minimum of 10 g of feces was collected per sample, and no invasive treatment was performed on the dogs. Samples were stored in sterile containers at 4 °C and transported to the Laboratory for Clinical Microbiology at the Faculty of Veterinary Medicine—Skopje within 24 h for processing.

Upon arrival at the laboratory, fecal samples were initially enriched in buffered peptone water (Oxoid, Hampshire, UK) for 30 min. Then, 10 µL of the enrichment was inoculated onto Blood agar (Oxoid, UK) and Coliform agar (Biolife, Milan, Italy) plates, which were subsequently incubated aerobically at 37 °C for 24 h. Suspected *E. coli* colonies were identified to species level by MALDI-TOF MS (Bruker, Daltonics, Germany), with bacteria scoring above 2.0.

2.3. Questionnaire and Data Collection

A questionnaire was administered to a veterinarian at each shelter to gather information regarding the shelter's capacity and policies on antimicrobial use. Furthermore, data on individual dog characteristics were collected, including sex, breed, age, original location before entering the shelter, duration of stay at the sampling shelter, history of antimicrobial therapy, and fecal score. Importantly, all participating shelters maintained treatment records, which provided documented evidence of antimicrobial therapy for each dog.

2.4. Antimicrobial Susceptibility Testing and ESBL/AmpC Phenotyping

Antimicrobial susceptibility testing (AST) was conducted on the *E. coli* isolates using the broth microdilution method with Sensititre™ plates (Thermo Scientific, Waltham, MA, USA). *E. coli* ATCC 25922 served as the quality control strain. All isolates (n = 112) were

tested with the EUVSEC3 Sensititre™ plate for broad-spectrum susceptibility screening. Isolates that showed resistance to cefotaxime and/or ceftazidime and/or meropenem on EUVSEC3 were subsequently tested with the EUVSEC2 panel to confirm the presence of ESBL/AmpC phenotypes. The antibiotics included in the susceptibility panels are listed in Table 1. The presence of ESBL phenotype was determined by evaluating the synergistic effect between cefotaxime or ceftazidime and clavulanic acid, as included in the EUVSEC2 panel. Furthermore, isolates were phenotypically categorized as AmpC producers if the cefoxitin MIC was above the corresponding ECOFF value. Moreover, multidrug resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial classes.

Table 1. EUCAST epidemiological cutoff values (ECOFFs) for antibiotics in EUVSEC3 and EUVSEC2 panels.

EUVSEC3		EUVSEC2	
Antibiotic	**ECOFF**	**Antibiotic**	**ECOFF**
Amikacin	8	Cefepime	0.125
Ampicillin	8	Cefotaxime	0.25
Azithromycin	16	Cefotaxime/clavulanic acid	0.25
Cefotaxime	0.25	Cefoxitin	16
Ceftazidime	1	Ceftazidime	1
Chloramphenicol	16	Ceftazidime/clavulanic acid	1
Ciprofloxacin	0.06	Ertapenem	0.03
Colistin	2	Imipenem	0.5
Gentamicin	2	Meropenem	0.06
Meropenem	0.125	Temocillin	16
Nalidixic acid	8		
Sulfamethoxazole	64		
Tetracycline	8		
Tigecycline	0.5		
Trimethoprim	2		

The results of the AST were interpreted following the EUCAST guidelines. Epidemiological cutoff values (ECOFFs) as defined in the Commission Implementing Decision 2020/1729 [18] were applied to classify isolates as wild-type (susceptible) or non-wild-type (resistant) populations. The corresponding ECOFFs are listed in Table 1.

2.5. Molecular Identification and Characterization of Antimicrobial Resistance Genes

Following the EUVSEC3 panel results, a total of 18 presumptive ESBL or AmpC *E. coli* isolates were tested. For DNA isolation, two to three single colonies from an overnight culture were collected using a sterile 1 µL inoculating loop and resuspended in 100 µL of DNA-/RNA-free water. Bacterial genomic DNA was isolated using the Microbial DNA kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The mechanical lysis part was performed using a TissueLyser (QIAGEN, Hilden, Germany) for 12 min at 30 Hz.

The quality of the extracted DNA was assessed using the DS-11 spectrophotometer (DeNovix, Wilmington, NC, USA). The concentration of the extracted DNA was measured using the dsDNA BR Assay Kit on the QUBIT 4 Fluorometer (Invitrogen, Carlsbad, CA, USA).

The PCR reaction mixture was prepared in 20 µL volume using the GoTaq G2 Colorless Master Mix (Promega, Medison, WI, USA) according to the following protocol: 10 µL master mix (2× concentrated), 4 µL molecular-grade water, 1 µL primer F (10 µM), 1 µL primer R (10 µM), and 2 µL DNA (0.5 ng/µL). The primers and the cycling protocol were used

according to the references presented in Supplementary Table S1 [19–40]. The SimpliAMP thermal cycler (Applied Biosystems, Carlsbad, CA, USA) was used to run the protocols according to the references presented in Supplementary Table S1.

The PCR products were separated and visualized using the Qiaxcel Advanced (QIAGEN, Hilden, Germany) capillary gel–electrophoresis system on the DNA Screening cartridge with the 15–3000 bp alignment marker and 50–2500 bp size marker. The electropherograms were analyzed using the Qiaxcel ScreenGel software v. 1.6 (QIAGEN, Hilden, Germany). The final results were concluded according to the references in Supplementary Table S1.

2.6. Statistical Analysis

To assess the impact of shelter stay duration on AMR in *E. coli* isolates, we categorized dogs based on their length of stay using a predefined threshold (short-term stay—less than 10 days, long-term stay—10 days or longer). The duration of stay was converted into a categorical variable for comparative analysis. AST results were recorded for multiple antibiotics using standard categories, susceptible (wild-type, S) and resistant (non-wild-type, R), and converted to a binary resistance variable (R = 1; S = 0). Binary resistance outcomes were computed for each antibiotic as well as for MDR and ESBL status. Two complementary statistical tests were used to compare resistance rates between the short- and long-stay groups. First, we used a Chi-square test of independence to determine whether the proportion of resistant isolates differed significantly between stay categories. Second, we applied an independent two-sample Welch's *t*-test to evaluate mean resistance rates, which accounts for unequal variance between groups.

Because multiple comparisons were performed across different antibiotics and shelter-level factors, we adjusted all *p*-values using both Bonferroni correction (to control the family-wise error rate) and the Benjamini–Hochberg False Discovery Rate (FDR) procedure (to control the proportion of false positives among significant findings). Associations were considered robust if they remained significant after Bonferroni correction, while results attained only under FDR correction were interpreted as exploratory but biologically relevant.

All analyses were performed using Python's SciPy (Version 1.16.1) and Pandas libraries (Version 2.3.2). A p-value of <0.05 was considered statistically significant.

3. Results

A total of 119 fecal samples were collected from dogs housed in animal shelters, and *E. coli* isolates were successfully identified in 112 (94.12%). Demographic analysis revealed that the samples originated from 52 female dogs (46.4%) and 60 male dogs (53.6%), representing all eight regions of North Macedonia. The majority of isolates were obtained from the Skopje region (n = 41; 36.6%), followed by the Southwestern (n = 22; 19.6%), Southeastern (n = 18; 16.1%), Polog (n = 13; 11.6%), Vardar (n = 6; 5.4%), Pelagonia (n = 5; 4.5%), Eastern (n = 4; 3.6%), and Northeastern (n = 3; 2.7%) regions. The median age of the dogs was 3 years. Most of the sampled population consisted of mixed-breed dogs (83.04%). At the same time, a smaller percentage included purebreds such as Sharplaninec, Pit Bull, Rottweiler, German Shepherd, Pointer, Hound, Kangal, Beagle, and Golden Retriever.

Antimicrobial susceptibility testing of the 112 *E. coli* isolates using the EUVSEC3 panel revealed significant resistance levels: sulfamethoxazole (68.75%), ampicillin (52.68%), and ciprofloxacin (41.07%), as shown in Figure 1. Complete sensitivity was observed for tigecycline, colistin, and meropenem. Of the isolates tested, 56 (50%) were determined to be multidrug-resistant, while 21 isolates (18.75%) remained susceptible to all tested antibiotics.

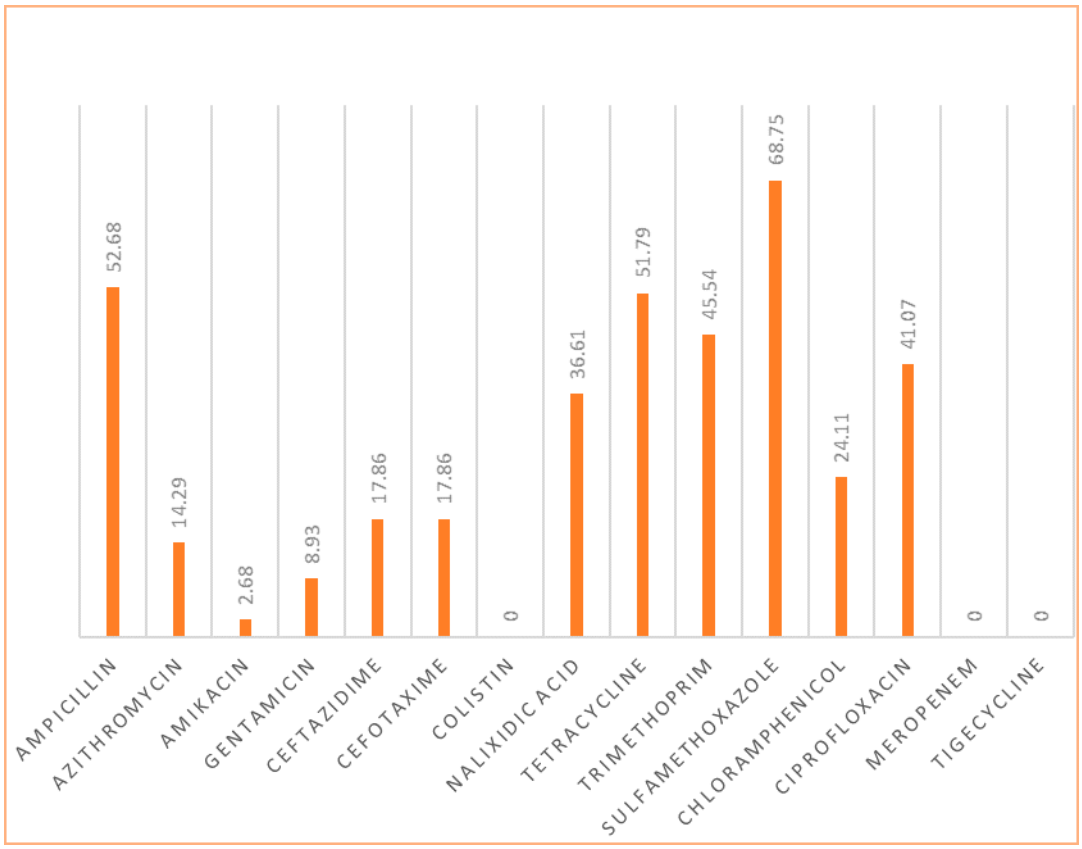

Figure 1. Antimicrobial resistance profile of *E. coli* isolates (n = 112) using the EUVSEC3 panel. Bars represent the percentage of isolates classified as non-wild type (resistant) to each antimicrobial agent tested.

Using the EUVSEC3 panel, 21 *E. coli* isolates showed resistance to both ceftazidime and cefotaxime; these were further analyzed with the EUVSEC2 panel, and 18 were classified as presumptive ESBL producers based on the synergy with clavulanic acid. The remaining three isolates were excluded, as they did not show synergy on the EUVSEC2 panel. One of the ESBL isolates met the criteria for AmpC production, with a cefoxitin MIC > 8 µg/mL. Of the ESBL-producing isolates, a high proportion demonstrated resistance to CIAs, with 17 of 18 (94.4%) also resisting cefepime and 13 (72.2%) resisting ertapenem. Detailed MIC values for all 18 ESBL/AmpC-producing isolates are presented in Supplementary Table S2 (EUVSEC3 panel) and Supplementary Table S3 (EUVSEC2 panel).

3.1. Most Frequent Resistance Patterns

Among the 112 *E. coli* isolates, the most common multidrug resistance pattern was found in 13 isolates (11.6%), which included ampicillin, ceftazidime, cefotaxime, nalidixic acid, tetracycline, trimethoprim, sulfamethoxazole, chloramphenicol, and ciprofloxacin. This pattern was particularly prevalent among isolates from Shelter 1. Further details on the remaining resistance profiles can be found in Supplementary Table S4.

The PCR analysis identified multiple resistance genes across the 18 analyzed *E. coli* isolates, as presented in Table 2. The genes for extended-spectrum β-lactamases, *bla*CTX-M and *bla*CTXM-1, were found in 17 isolates (94.4%), and the *bla*TEM gene was detected in 16 isolates (88.9%). PCR analysis identified *blaCMY*-1 in 18 isolates and *blaCMY*-2 in 16 isolates. The prevalence of tetracycline resistance gene *tet*A was noted in 15 isolates (83.3%), with *tet*B found in only 2 isolates. Notably, no carbapenemase genes were detected. A complete list of all genes tested in this study, including β-lactamase, tetracycline, sulfonamide, and phenicol resistance determinants, is provided in Supplementary Table S5.

Table 2. Resistance genes detected among ESBL/AmpC *E. coli* isolates (*n* = 18).

Antibiotic Class	Gene	*n*	%
β-lactams (ESBL/AmpC)	*blaTEM*	16	88.9
	blaCTX-M	17	94.4
	blaCTX-M-1	17	94.4
	blaCMY-1	18	100
	blaCMY-2	16	88.9
	blaACC	18	100
	blaFOX	18	100
Tetracyclines	*tetA*	15	83.3
	tetB	2	11.1
Sulfonamides	*sul1*	1	5.6
	sul2	16	88.9
	sul3	1	5.6
Phenicols	*cmlA*	13	72.2
	catA1	2	11.1

3.2. Correlation Analyses

No statistically significant associations were found between MDR *E. coli* and sex (χ^2 = 0.082, p = 0.7745; t-test = 0.473, p = 0.6371), age (t-test = −0.352, p = 0.7253), or breed (χ^2 = 4.284, p = 0.1174; t-test = 0.028, p = 0.9779). Grouping breeds into mixed vs. purebred also showed no significant correlation.

Fecal score was significantly associated with ESBL-producing *E. coli* (χ^2 = 13.440, raw p = 0.0093). This association did not remain significant after Bonferroni correction and should therefore be considered exploratory, although it was obtained under the FDR approach.

No significant correlation was observed between MDR or ESBL status and shelter capacity across 60%, 70%, or 80% occupancy thresholds. A non-significant trend was noted for higher ESBL prevalence at ≥70% capacity (p = 0.0843). Shelter policy type showed no significant association with resistance status (χ^2 = 0.706, p = 0.7024).

3.3. Association Between Shelter Practices and AMR

To assess the relationship between AMU policies and AMR at the shelter level, shelters were grouped based on reported antibiotic use intensity. No significant association was observed between intensive antimicrobial use and MDR prevalence (χ^2 = 0.926, p = 0.3359; t-test = −1.147, p = 0.2542). A highly significant association was found between intensive AMU and the prevalence of ESBL-producing *E. coli* (χ^2 = 15.118, p = 0.0001; t-test = −9.906, $p < 0.0001$). This relationship remained significant after both Bonferroni and FDR correction, confirming a robust association. Notably, all ESBL-producing isolates originated from shelters classified as having intensive antimicrobial use.

Shelter 1, which routinely administered combination antibiotic therapies post-surgery, exhibited the highest ESBL prevalence (66.7%) and a mean AMR burden of 6.5 resistant phenotypes per isolate. In comparison, shelters with minimal or no antibiotic use reported markedly lower ESBL rates (0–8.3%) and reduced AMR burdens. These findings indicate an association between shelter-level antibiotic practices and the emergence of ESBL-producing *E. coli*.

Shelter-specific analysis revealed a wide variance in MDR prevalence, as shown in Table 3. For example, Shelter 1 showed the highest prevalence (75% of isolates were MDR), while Shelter 4 had the lowest (13.64%).

Table 3. MDR isolates by shelter.

Shelter	Total Isolates	MDR Isolates	MDR Prevalence (%)
Shelter 1	24	18	75.00
Shelter 2	36	16	41.67
Shelter 3	13	6	38.46
Shelter 4	22	5	13.64
Shelter 5	4	2	50.00
Shelter 6	13	9	53.85

3.4. Relationship Between Duration of Stay and Antibiotic Resistance

To investigate the relationship between the length of stay at the shelter and antibiotic resistance, the dogs were divided into short-term (≤10 days) and long-term (>10 days) groups. A significantly higher prevalence of resistance to several antibiotics was found in the long-term group. Longer shelter stay was strongly associated with resistance to ceftazidime, cefotaxime, nalidixic acid, trimethoprim, and ciprofloxacin (all $p < 0.001$). These associations remained significant after both Bonferroni and FDR corrections, confirming a robust effect of stay duration. ESBL-producing *E. coli* isolates were also significantly more common in long-term-stay dogs ($\chi^2 = 7.544$, $p = 0.006$), and this association persisted after both correction methods. Associations with other antibiotics (ampicillin, sulfamethoxazole, chloramphenicol, azithromycin) were not retained after Bonferroni correction and are considered exploratory. No statistically significant correlation was found between length of stay and MDR status.

3.5. Shelter-Level Factors Associated with Antimicrobial Resistance

Chi-square tests revealed no significant association between the use of shared areas and MDR status ($\chi^2 = 1.103$, $p = 0.2936$). The presence of a shared area was significantly associated with ESBL production ($\chi^2 = 17.647$, $p < 0.001$). This remained significant after both Bonferroni and FDR correction, confirming a robust association.

3.6. Cleaning Frequency

Shelters were grouped according to their reported cleaning frequency (once, twice, or three times daily), as presented in Table 4. There was no significant association between cleaning frequency and MDR prevalence ($\chi^2 = 1.68$, $p = 0.431$). The ESBL prevalence differed significantly across groups ($\chi^2 = 28.01$, $p < 0.0001$). This association remained significant after both Bonferroni and FDR corrections, confirming a robust association.

Table 4. Characteristics and management practices of shelters.

Shelter	Cap. (Dogs)	Dogs (Visit Day)	Shared Area	Clean. Freq.	Hyg. Prot.	Avg. Stay (Days)	Outd. Acc.
1	48	35	Yes	2×/day	Yes	30	Yes
2	150	132	Yes	3×/day	Yes	10–15	Yes
3	35	34	No	2×/day	Yes	∞	No
4	34	25	No	1×/day	Yes	7–10	Yes
5	28	6	No	1×/day	No	3–4	No
6	19	15	No	1×/day	No	10	No

Cap. = capacity; clean. freq. = cleaning frequency; hyg. prot. = hygiene protocol; avg. stay = average stay duration; outd. acc. = outdoor access; ∞ = until adoption. Only parameters directly associated with AMR outcomes are shown; full data available in Supplementary Table S5.

The full dataset of shelter characteristics and management practices is provided in Supplementary Table S6.

3.7. Rationale for Antibiotic Selection

The rationale behind antibiotic selection was categorized into three strategies: (1) personal experience, (2) experience and literature, and (3) experience, literature, and pharmaceutical industry input. A significant association was observed between antibiotic selection strategy and ESBL prevalence ($\chi^2 = 54.15$, $p < 0.0001$). This finding remained significant after both Bonferroni and FDR correction, confirming a robust association. ESBL-producing isolates were detected only in shelter that reported using strategy 3, with a prevalence of ~67%, compared to 0% in shelters using strategies 1 and 2 (Table 5).

Table 5. Antimicrobial use practices across six shelters.

Shelter	Guidelines for AMU	Post-Op Therapy Duration	Antibiotics Used	Basis of Choice	AST Performed
1	No	3 days	ENR, CRO, SXT, AMP	Sci. lit. + Pers. exp. + Pharma info	No
2	No	3 days	AMX, PEN, STR	Sci. lit. + Pers. exp.	No
3	No	1–2 days	AMX	Sci. lit. + Pers. exp.	No
4	No	None	None	Pers. exp.	No
5	No	3 days	PEN, STR	Pers. exp.	No
6	No	>3 days	PEN, AMX	Pers. exp.	No

Abbreviations: ENR, enrofloxacin; CRO, ceftriaxone; SXT, trimethoprim/sulfamethoxazole; AMP, ampicillin; AMX, amoxicillin; PEN, penicillin; STR, streptomycin; AST, antimicrobial susceptibility testing; AMU, antimicrobial use; sci. lit., scientific literature; pers. exp., personal experience.

ESBL-producing isolates were detected only in shelters reporting strategy 3, with a prevalence of ~67%, compared to 0% in shelters using strategies 1 and 2 (Table 5).

3.8. Duration of Antibiotic Therapy

Postoperative antibiotic treatment duration was categorized into four groups: 0 days, 1–2 days, 3 days, and >3 days. A significant association was found between treatment duration and both MDR ($\chi^2 = 10.70$, $p = 0.0135$) and ESBL prevalence ($\chi^2 = 17.16$, $p = 0.0007$). The association with ESBL prevalence remained significant after both Bonferroni and FDR correction. The association with MDR was retained under FDR but not Bonferroni correction and should therefore be considered exploratory. MDR rates increased progressively with longer treatment courses, peaking at 84.6% in the >3-day group. ESBL-producing isolates were observed only in the 3-day group (29.7%).

None of the shelters had guidelines for prudent antimicrobial use, nor did they have the financial resources for bacteriology testing or AST. Shelter policies in North Macedonia included returning dogs to their original locations, offering them for adoption, and offering international adoption opportunities.

4. Discussion

This study is the first to investigate the prevalence of AMR, MDR, and ESBL/AmpC-producing *E. coli* in shelter dogs in North Macedonia. By combining phenotypic resistance profiling with molecular characterization, we identified a substantial burden of AMR, with high rates of resistance to commonly used antibiotics. The detection of ESBLs and AmpC β-lactamases further underscores the complexity of resistance mechanisms present in shelter environments.

In our study, *E. coli* isolates from shelter dogs showed a high level of resistance to sulfamethoxazole (68.75%), ampicillin (52.68%), tetracycline (51.79%), and ciprofloxacin (41.07). The significantly higher resistance rates observed in our study, compared to similar

research conducted in Italy [41], particularly for critical antibiotics such as ciprofloxacin and third-generation cephalosporins, suggest a potentially greater burden of resistance in North Macedonia. This finding calls for further investigation and offers valuable baseline data for future regional comparisons.

Among the ESBL/AmpC-producing isolates (n = 18), 72.2% showed reduced susceptibility to ertapenem, although no carbapenemase genes were detected. This aligns with findings reported by Johansson et al. [42] concerning imported shelter dogs, indicating that non-carbapenemase mechanisms might be contributing to this reduced susceptibility. However, it is essential to approach this observation with caution since our study design does not allow for the identification of the mechanisms involved. Potential explanations could include porin loss or efflux activity [43,44]; further molecular studies would be necessary to validate these hypotheses.

The widespread use of fluoroquinolones and third-generation cephalosporins in companion animals in North Macedonia [14], including in shelter populations, is concerning given their classification by the WHO as Highest Priority Critically Important Antimicrobials (HPCIAs) [45]. Even more alarming, our study found that these drugs are being administered empirically during elective surgical procedures, such as routine sterilizations, often in multidrug combinations (e.g., enrofloxacin and ceftriaxone in Shelter 1). According to WHO and WOAH guidance [45,46], HPCIAs should not be used prophylactically and should only be considered when no effective alternatives exist, ideally supported by AST. In our dataset, 94.4% of ESBL/AmpC-producing isolates were resistant to cefepime, a fourth-generation cephalosporin classified as an HPCIA, further underlining the risks associated with such practices. While causality cannot be established, the observed association between routine perioperative use of HPCIAs and higher MDR/ESBL prevalence suggests a critical gap in antimicrobial stewardship and infection control in shelter settings.

CTX-M-type ESBLs have emerged as the dominant β-lactamases in both commensal and pathogenic *E. coli* isolates from humans and animal sources. In the study by Biguenet et al. [47], genomic analyses showed that *E. coli* strains from companion animals displayed greater diversity than those from humans, indicating a broader range of bacterial populations. Human strains primarily showed sequence type ST131 with the *bla*CTX-M-15 gene linked to human infections. In contrast, animal strains were more likely to carry the *bla*CMY-2 and *bla*CTX-M-1 resistance genes, suggesting different resistance mechanisms in companion animals. A meta-analysis by Salgado-Caxito et al. [5] reported the presence of *bla*CTX-M-type genes in 95% of studies, highlighting their widespread occurrence in dogs and cats across all continents. In our dataset, *bla*CTX-M and *bla*CTX-M1 were detected in 15.2% of *E. coli* isolates from shelter dogs. While this proportion is lower than the rates reported in some studies .g., 79.7% in healthy dogs in Italy [48] or the combined presence of *bla*CTX-M and *bla*TEM in shelter dogs in Japan [49], it nonetheless confirms that CTX-M enzymes are circulating in shelter populations in North Macedonia. These findings are consistent with global trends and add valuable country-level evidence, supporting the view that CTX-M-type ESBLs are widely disseminated in both shelter and household dog populations.

The co-occurrence of ESBL and AmpC β-lactamases, particularly CTX-M and *bla*CMY-2, creates significant clinical challenges by limiting effective antimicrobial treatments. Although *bla*CMY-2 is frequently reported as plasmid-mediated and this raises concerns about potential spread in high-density environments such as shelters [6,48], our study design does not allow us to confirm whether dissemination in our isolates was plasmid-mediated or clonal. Our study detected both *bla*CTX-M and *bla*CMY-2 genes, which aligns with findings by Sun et al. [9], who detected the *bla*CMY-2 gene in stray dogs in China, including strains co-harboring *blaDHA-1* and *blaCTX-M-14*, highlighting the genetic

diversity and potential for co-selection of multiple resistance determinants. Additionally, in a genomic study of imported shelter dogs, Johansson et al. [42] reported that 19% of *E. coli* isolates were AmpC producers, with *blaCMY-2* being the most frequently detected gene. Notably, some isolates co-harbored *blaCMY-2* and *blaCTX-M-15.* Evidence from Romania suggests the possibility of clonal transmission of resistant *E. coli* strains between dogs and humans in shared environments [50,51]. While this risk of zoonotic transmission is already documented, our study highlights the need for further molecular epidemiological analyses within a One Health framework to determine whether dissemination in the shelter context is primarily plasmid-mediated or clonal. Such investigations should also include shelter staff, who represent a potential interface for cross-species transmission.

Despite these observations, none of the shelters in our study had antimicrobial stewardship protocols or financial resources for bacteriological testing and AST. Our previous study [14] highlighted the limited use of AST in small animal practice in North Macedonia, and the specific conditions in shelters add further complexity. As a result, veterinarians often rely on empirical antibiotic selection, which may increase the risk of inappropriate AMU and resistance development, as has also been reported in clinical staphylococci from companion dogs [52]. The detection of ESBL-/AmpC-producing *E. coli* in shelter 1, which reported relying on pharmaceutical industry input for antibiotic decisions, was associated with practices that may influence prescribing patterns. Although our data cannot establish causality, this underscores the importance of independent, evidence-based stewardship policies to guide rational antimicrobial use [53]. In contrast, a multinational survey [54] found that greater awareness and use of ASGs were associated with more judicious prescribing practices, such as targeted therapy, reduced reliance on CIAs, and improved infection control. Our findings therefore provide baseline data from North Macedonia, illustrating how gaps in stewardship and diagnostics may be associated with high and variable resistance patterns in shelter environments.

Shelter management practices such as returning dogs to their original locations or offering them for adoption may also facilitate the dissemination of resistant bacteria beyond the shelter environment [55]. This concern extends to international dog movement, which has been identified as a major pathway for the spread of AMR in Northern Europe [56]. Recent reports, including recommendations from the Finnish Food Authority, emphasize the importance of screening shelter dogs for resistant bacteria before adoption or cross-border transfer [42,56]. To address such risks, the development of independent national or regional guidelines, enhancement of Antimicrobial Stewardship Training capacity, and the establishment of screening protocols in the shelters before adoption or transfer should be considered.

Environmental and housing conditions also appeared to influence resistance patterns. In our study, shared living areas were significantly associated with ESBL-producing *E.coli*, suggesting that close contact and communal environments could contribute to the spread of resistant bacteria and ARGs. This observation is consistent with findings by Weese et al. [57], who reported rapid transmission of ESBL-producing *Enterobacterales* in shelter cats upon admission. In our study, longer duration of stay was significantly associated with ESBL carriage, indicating that prolonged exposure to shared environments may facilitate the persistence of resistant strains. Another noteworthy result is that the fecal score was significantly associated with the presence of ESBL-producing *E. coli* ($\chi^2 = 13.440$, $p = 0.0093$). While the mechanism underlying this relationship remains unclear, one possible explanation is that colonization by ESBL-producing *E. coli* may be linked to altered gut health [58]. However, further studies would be required to confirm whether this reflects gut dysbiosis or other confounding factors.

This study has several limitations that should be considered when interpreting the findings. First, the number of dogs sampled in some shelters was relatively small since sampling was performed on a single day in each facility and the number of available dogs reflected shelter capacity at that moment. This uneven distribution reduces statistical power, increases the risk of type I error in some associations, and may have introduced sampling bias between shelters. Second, fecal samples were collected from the ground. Although aseptic techniques were applied, the possibility of environmental contamination cannot be excluded, which may bias the results toward more environmentally resilient *E. coli* strains. Third, although molecular detection of ESBL and AmpC genes offered valuable insights, we did not perform multilocus sequence typing (MLST), plasmid typing, or whole-genome sequencing, which would be required in determining clonal relatedness and transmission pathways. Fourth, while treatment records supported antimicrobial use data, they were still partly dependent on veterinarian reporting and may be subject to recall bias. Finally, as the study design was cross-sectional, it does not allow for conclusions regarding causality, persistence, or temporal dynamics of colonization.

This study provides the first baseline epidemiological dataset on AMR in shelter dogs in North Macedonia. While molecular typing was not performed, the epidemiological insights presented here establish a foundation for future research, which should integrate molecular tools to better characterize clonal relatedness and transmission dynamics. At the same time, these findings emphasize the importance of a One Health perspective, as resistant bacteria in shelter animals may contribute to broader dissemination. Looking ahead, future efforts should not only combine epidemiological and molecular approaches but also consider the development of novel antimicrobial strategies, such as antibiotics targeting bacterial metallophores [59] or employing Trojan horse approaches to enhance intracellular delivery [60], as potential long-term solutions to AMR.

5. Conclusions

In conclusion, this study presents the first comprehensive dataset on AMR in commensal *E. coli* from shelter dogs in North Macedonia, highlighting high rates of resistance to CIAs. Associations with antimicrobial use practices and shelter conditions suggest potential drivers of resistance. The prevalence of ESBL/AmpC genes and empirical antimicrobial use underscore the potential for shelter dogs to serve as reservoirs and amplifiers of antibiotic resistance in North Macedonia. These results emphasize the importance of integrating companion animals, including shelter populations, into AMR surveillance and support the need for developing antimicrobial stewardship and infection control measures within a One Health framework.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres16090206/s1, Table S1: List of primers for detection of antimicrobial resistance genes; Table S2: Antimicrobial susceptibility profiles of 18 ESBL/AmpC-producing Escherichia coli isolates identified by the EUVSEC3 panel; Table S3: MIC values of 18 ESBL/AmpC-producing Escherichia coli isolates tested with the EUVSEC2 panel; Table S4: Resistance patterns from 112 isolates with EUVSEC3 panel; Table S5: PCR Analysis of Resistance Genes in 18 ESBL and AmpC E. coli isolates; Table S6: Characteristics and Management Practices of Shelters.

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Institutional Review Board Statement: In this study, non-invasive fecal samples were collected from dogs at animal shelters without touching or treating the animals. The process ensured no harm, stress, or discomfort to the dogs. All shelter staff consented to the collection of samples and data related to shelter practices. 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, 31 March 2023).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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