ISSN: 1220-3173; E-ISSN: 2457-7618

MONITORING GENOTYPIC RESISTANCE OF SOME *ESCHERICHIA COLI* STRAINS ISOLATED FROM FALLOW DEER (*DAMA DAMA*) FROM WESTERN ROMANIA

MONITORIZAREA GENOTIPICĂ A REZISTENȚEI UNOR TULPINI DE *ESCHERICHIA COLI* IZOLATE DE LA CERBUL LOPĂTAR (*DAMA DAMA*) DIN VESTUL ROMÂNIEI

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ABSTRACT | REZUMAT

The presence of antimicrobial resistance (AMR) genes in wildlife is an important indicator that resistant bacteria, of human or animal origin, are widespread in all natural environments. The aim was to identify resistance genes in Fallow deer (*Dama dama*) from Western Romania. Ten strains of *Escherichia coli* were randomly chosen for the determination of antimicrobial susceptibility using the Kirby Bauer diffusimetric method and the *Vitek2* Compact system (*Bio Mérieux, France*), and to conclude, four of the resistant strains were further studied molecularly (PCR). These strains demonstrated a high degree of resistance to beta-lactams, aminoglycosides, quinolones, tetracyclines, and furans. The initial evaluation and interpretation of the results, done using the diffusimetric method Kirby Bauer and the *Vitek-2* Compact system, revealed high resistance levels in *E. coli* samples collected from fallow deer. Results were also statistically confirmed (p <0.001). The PCR technique used for confirmation of extended-spectrum beta-lactamase resistance genes of *Escherichia coli* strains generated bands of 858 base-pairs TEM and 1016 base-pairs SHV. The PCR bands validated the *E. coli* sylvatic strains resistance genes and proved the interconnection between ecosystems, health of humans, domestic and wild animals, in the span of antimicrobial resistance.

Keywords: antibioresistance, monitoring, *Dama dama*, profiling, PCR, *E. coli*, genes, *bla*TEM, *bla*SHV

Prezența genelor de rezistență antimicrobiană (AMR) în fauna sălbatică reprezintă un indicator important pentru faptul că bacteriile rezistente, de origine umană sau animală, sunt răspândite în toate mediile naturale. Scopul acestei cercetări a fost de a identifica genele de rezistență la cerbul lopătar (*Dama dama*) din vestul României. Zece tulpini de *Escherichia coli* au fost alese aleatoriu pentru determinarea susceptibilității antimicrobiene,folosind metoda difuzimetrică Kirby-Bauer și cu ajutorul sistemului Vitek2 Compact (Bio Mérieux, Franța) și, în concluzie, patru dintre tulpinile rezistente au fost studiate în continuare din punct de vedere molecular (PCR). Aceste tulpini au demonstrat un grad ridicat de rezistență la beta-lactamine, aminoglicozide, chinolone, tetracicline și furani. Evaluarea și interpretarea inițială a rezultatelor, realizate folosind metoda difuzimetrică Kirby-Bauer, respectiv sistemul Vitek-2 Compact, au evidențiat niveluri ridicate de rezistență în probele de *E. coli* colectate de la cerbul lopătar. Rezultatele au fost, de asemenea, confirmate statistic (p <0,001). Tehnica PCR utilizată pentru confirmarea genelor de rezistență la beta-lactamaze cu spectru extins ale tulpinilor de *Escherichia coli* a generat benzi de 858 perechi de baze TEM și 1016 perechi de baze SHV. Benzile PCR au validat genele de rezistență la tulpinile silvatice de *E. coli* și au demonstrat interconexiunea dintre ecosisteme, sănătatea oamenilor, a animalelor domestice și sălbatice, în ceea ce privește rezistența antimicrobiană.

Cuvinte cheie: antibiorezistență, monitorizare, *Dama dama*, profilare, PCR, *E. coli*, gene, blaTEM, blaSHV

The antibiotic resistance phenomenon described in numerous species of bacteria denotes a challenge, in both veterinary and human medicine, and is considered an issue of global importance with high zoonotic potential (29, 30). The presence of bacteria carrying antimicrobial resistance (AMR) genes in wildlife is an important indicator that resistant bacteria are widespread in all natural environments.Also, the presence of some bacteria strains carrying resistance genes to currently used antibiotics, could represent an additional challenge for humans and domestic animals,as wild animals may constitute a source of bacteria with multiple genotypic resistances to antibiotics (8, 33). However, the role of the sylvatic environment in the epidemiology of these bacteria is still insufficiently studied and rather unclear. There are several studies that analysed the phenotypic and genotypic resistance features present in various species of bacteria, from humans and food-producing animals, but bacterial resistance

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traits were observed in sylvatic environments as well (6, 8, 18). One of the most frequently identified threats is the carbapenem-resistant (CR) to *Enterobacteriaceae* (CRE) which has been studied for more than three decades, and on almost all continents, in all environments: in livestock, seafood, wildlife, and pets, posing a potential risk to public health. The primary observed result was the occurrence of CRE in samples from these animals; secondary outcomes included the CRE prevalence, genotypes, and antimicrobial susceptibilities. Wildlife acquires CRE easily, following sewage, manure, or waste contact. For this purpose, prevalence analyses using molecular and cultural microbiological methods are essential to determine the scale and diffusion of CRE (19). Though it is consistently acknowledged that bacteria found in wild animals are not subjected to direct selection pressure using antimicrobials, it has been revealed that these animals can also hold bacteria resistant to the activity of antimicrobials in their intestines. This implies that resistance to antimicrobial compounds has spread beyond human and domestic animal environments. This is why a deeper look into the epidemiology of resistance to antimicrobials in wildlife is necessary (5). Commensal bacteria appear to be important carriers of virulence and resistance genes. Current data is mostly focused on the investigation of human bacteria or those connected to human communities (18, 30). There are relatively few genomic feedbacks of commensal bacteria from hosts rarely exposed to antibiotics, such as wildlife. *Escherichia coli* has an exceptional potential to cause severe intestinal and extraintestinal diseases, such as meningitis, septicaemia, pneumonia, or urinary tract infections (5, 19, 29, 37).

This diversity, in terms of host-pathogen interactions, is due to the high plasticity of the genome of this species, which allows the bacteria to adapt to variable selective pressures exerted by different environmental factors (2).

The most identified *Enterobacteriaceae* were those resistant to the beta-lactamic group, including cephalosporins, due to the presence of extended spectrum beta-lactamases (ESBL) and to fluoroquinolones.The findings revealed that antibiotics can increase antibiotic-induced plasmid transfer (PT) in *E. coli* strains with many ESBL plasmids. Therefore, the ESBL plasmid antibiotic-induced conjugative transfer appears commonly in *E. coli,* with important implications for assessing the risks of antibiotic use (10, 24). This is why the isolation and detection of extended-spectrum beta-lactamase (ESBL) producing *E. coli* is currently one of the major concerns in the field of human and animal health, as well as an important part of the One Health approach. For this purpose, phenotypic ESBL isolates were tested for the *bla* (TEM), *bla* (CTX-M), and *bla* (SHV) genes using PCR and DNA sequencing, revealing high levels of resistance. The high incidence of resistance to these classes of antibiotics in the ESBL-producers may possibly suggest the extensive veterinary usage of these and similar molecules, demanding urgent steps to further assess the role of farm and wild animals in the diffusion of highly resistant ESBL-producing bacteria in humans (3, 15, 20, 25, 32, 35, 36).

The central objective of this research was to investigate the presence and patterns of genotypic resistance to antibiotics commonly used in the veterinary field (beta-lactams, aminoglycosides, quinolones, tetracyclines, and furans) in strains of *Escherichia coli* isolated from fallow deer (*Dama dama*) from hunting grounds in Western Romania.

MATERIALS AND METHODS

Study location

The study was carried out between 2017 and 2021, and during the open hunting seasons from October until February, the samples were taken from males (bucks) and females (deer) of fallow deer (*Dama dama*). A total of 120 animals were sampled; two were taken from each animal (i.e., 240 fresh samples, from the nasal cavity and the rectum) and directed to the microbiology laboratory of the Faculty of Veterinary Medicine of Timisoara.

The study was carried out in Socodor area, Arad County, in 7,601 hectares hunting ground (Fig. 1) (https://satellites.pro/Socodor_map#46.539757,21.447201,14).

Fig. 1. The Socodor hunting ground (orange marked line) aerial view, crossed by the Crișul Alb River

The antimicrobial susceptibility testing of the bacterial strains by the diffusimetric method

According to CLSI (2018) Guidelines and the Annex, the disc-diffusimetric method Kirby Bauer used fresh cultures (for 18-24 hours),on Müeller-Hinton (Sigma-Aldrich / Oxoid, UK) nutrient agar. To ensure the accuracy of the results, *E. coli* ATCC 25922 (*Thermo Fisher Scientific, Lake Charles, LA, USA*) strain was considered positive control (41, 42, 43). Gram-negative species were tested for antimicrobial susceptibility. In the Table 1, the identified bacterial species are presented.

The sensitivity of bacterial strains was tested to commonly used antimicrobials, i.e., beta-lactams (amoxicillin+clavulanic acid, ampicillin, ceftazidime, and cefuro-

xime), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin), and furans (nitrofurantoin and trimethoprim/sulfamethoxazole).

Table 1

Bacterial species tested by Kirby Bauertechnique sampled on Müeller-Hinton agar (Sigma-Aldrich/Oxoid, UK)

Species	Strains	Sampling zone
Escherichia coli	5	Anal
Pseudomonas oleovorans	1	Nasal
Providencia rettgeri	1	Nasal
Enterobacter aerogenes	1	Nasal
Enterobacter spp.	2	Anal

From the stock cultures of the identified genera, conserved at -50°C, in brain heart infusion broth (BHI Oxoid broth, Basingstoke, UK) with glycerol, seeds were made on Petri dishes containing Müeller-Hinton agar, then incubated at 37°C for 24 hours. Following this, the bacterial suspension was obtained and brought to 0.5 Mc Farland standard turbidity. Subsequently, the Müeller-Hinton agar Petri dishes were seeded. After calibration, plates were seeded by flooding for 15 minutes, and post-inoculum absorption (for five minutes), the 25-35 µg micro-tablets (Oxoid, Basingstoke, UK) containing antimicrobials were placed with a dispenser. Afterwards, the plates were placed in an incubator at 37°C for 24 hours, and the diameter around the micro-tablets was measured. Based on ring diameter, categories of sensitivity were established as: resistant strain (below 1 mm), moderately sensitive strain (2 to 5 mm), and sensitive (over 6 mm). For reference, naturally sensitive strains to antibiotics were used.

The antimicrobial susceptibility testing of the bacterial strains by the Vitek-2 Compact system (BioMérieux, France)

The susceptibility study to the activity of the main classes of antimicrobials was carried out both on Gram-negative (cod: AST-GN96 and AST-GN97) and Gram-positive (AST-GP79) species isolated from *Dama dama*. The antimicrobial substances used for testing bacterial species in Socodor are presented in Table 2.

Table 2

Antimicrobial substances used in Vitek-2 Compact (*BioMérieux, France*) for bacterial species testing

Antimicrobial substances used for testing Gram-negative species					
Antimicrobial	Abr.	Concentration (µg/mL)	Limits (≤ ≥)		FDA directions for use
Ampicillin	AMP	4, 8, 32	2	32	CSAGNB**
Amoxicillin/Clavulanic acid	AMC	4/2, 16/8, 32/16	2/1	32/16	CSAGNB**
Cefalexin	CN	8, 32, 64	4	64	
Cephalothin	CF	2, 8, 32	2	64	CSAGNB**
Cefquinone	CEQ	0.5, 1.5, 4	0.5	8	N/A**
Cefoperazone	CFP	4, 8, 32	4	64	N/A**
Ceftiofur	CFT	1, 2	1	8	N/A**
Imipenem	IPM	1, 2, 6, 12	0.25	16	*Enterobacter* spp., *E. coli*
Gentamicin	GM	4, 16, 32	1	16	CSAGNB**
Amikacin	AN	8, 16, 64	2	64	CSAGNB**
Neomycin	N	8, 16, 64	2	64	N/A**
Flumequine	UB	2, 4, 8	1	32	N/A**
Enrofloxacin	ENR	0.25, 1, 4	0.12	4	N/A**
Marbofloxacin	MRB	1, 2	0.5	4	N/A**
Tetracycline	TE	2, 4, 8	1	16	CSAGNB**
Nitrofurantoin	FT	16, 32, 64	16	512	CSAGNB**
Trimethoprim/Sulfamethoxazole	SXT	1/19, 4/76, 16/304	20(1/19)	320(16/304)	*Enterobacter* spp., Eco (+ETEC)**
Antimicrobial substances used for testing Gram-positive species					
Antimicrobial	Code	Concentration (µg/mL)	Limits (≤ ≥)		FDA directions for use
Amikacin	AN	16, 32, 64	2	64	N/A**
Gentamicin	GM	8, 16, 64	0.5	16	*Staphylococcus* spp.
Kanamycin	K	32, 64, 128	4	64	N/A**
Neomycin	N	16, 32, 64	2	32	N/A**
Enrofloxacin	ENR	1, 2	0.5	4	N/A**
Erythromycin	E	0.25, 0.5, 2	0.25	8	*Staphylococcus* spp.
Tilmicosin	TIL	0.5, 1, 4	0.25	4	N/A**
Tylosin	TI	2, 8, 32	1	32	N/A**
Clindamycin	CM	0.06, 0.25, 1	0.125	4	MSSA**, MSSE**
Tetracycline	TE	0.5, 1, 2	1	16	*Staphylococcus* spp.,
Florfenicol	FFC	2, 4, 16	4	32	N/A**
Trimethoprim/Sulfamethoxazole	SXT	8/152, 16/304, 32/608	10(0.5/9.5)	320(16/304)	N/A**

Abbreviations: FDA = Food and Drug Administration; **CSAGNB = clinical significance aerobic Gram-negative bacilli; **N/A = No specific FDA indications are recommended for use. **Eco (+ETEC)=*E. coli* (including sensitive enterotoxigenic strains involved in traveler's diarrhea); NEG=Negative; POS= Positive; **MSSA =Methicillin-susceptible *S. aureus*; **MSSE = Methicillin-susceptible *S. epidermidis*

The polymerase chain reaction technique

To conclude the previously mentioned results, the polymerase chain reaction (PCR) technique was used. Of the 15 strains, ten *Escherichia coli* strains, in which significant resistance to most antimicrobial molecules was found, were chosen for PCR genotyping in order to establish the genotypic profile of antimicrobial resistance in the *Escherichia coli* strains isolated from the nasal cavity and rectum from *Dama dama*. The molecular analysis was performed in the Microbiology Laboratory of the "Louis Turcanu" Emergency Clinical Hospital for Children, Timișoara, and were based on known methodologies from the literature (3, 16, 43). The analysis of isolated *Escherichia coli* strains by PCR amplification included three successive and complementary steps:

1) *Bacterial DNA extraction* was completed from pure *Escherichia coli* cultures grown on nutrient agar (sheep blood agar and MacConkey agar) for 24 hours using the "innuPREP DNA mini" extraction kit (*Analytic, Jena, Germany*). The quality and concentration of extracted DNA were measured with a NanoDrop 1000 Spectrophotometer, all the DNA samples having values of the 260/280 and 260/230 fractions greater than 1.8.

2) *The polymerase chain reaction* was performed according to the methodology described in literature (21), aiming for the amplification of the TEM and SHV genes that confer resistance to extended-spectrum beta-lactamases. The samples were processed under sterile conditions. For amplification control, the commercially available pUC19 plasmid (Thermo Scientific). was used without any modifications, providing an amplicon of 65 bp. The optimal concentration of pUC19 was evaluated to be approximately 200 copies per PCR reaction. For each gene and the amplification control, a set of specific primer pairs was used: sense (forward, F) and antisense (reverse, R) presented in Table 3. The total volume of the PCR amplification reaction was 25.0 µL.

The PCR reaction was carried out on SureCycler thermal cycler (Agilent Technologies), and the amplification programme used for *bla*TEM and *bla*SHV genes was as follows: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of a denaturation step at 95°C for 30 seconds, primer annealing step at 54°C for 1 minute and 30 seconds, elongation step at 72°C for 1 minute, and final extension step at 72°C for 5 minutes. After the completion of the PCR programme, the gene amplification products were highlighted by electrophoretic migration in 3% agarose gel, at 100 V, 60 mA, for 60 minutes. in the presence of a DNA molecular size standard (DNA Ladder-Bioneer®100 bp) containing 11 bands from 50 bp to 1000 bp.

Table 3

The set of specific primer pairs used in this study

Specific primers: forward, F and reverse, R	
TEM-F:	5′-ATGAGTATTCAACATTTCCG-3′
TEM-R:	5′-CCAATGCTTAATCAGTGAGG-3′
SHV-F:	5′-CGCCGGGTTATTCTTATTTGTCGC-3′
SHV-R:	5′-TCTTTCCGATGCCGCCGCCAGTCA-3′
pUC19-F:	5′-CAGGATTAGCAGAGCGAGGTATG-3′
pUC19-R:	5′-CGTAGTTAGGCCACCACTTCAAG-3′

PCR amplicons were labelled with ethidium bromide and visualised under ultraviolet light. The fragments amplified with TEM primers had a size of 858 bp, and the fragments amplified with SHV primers-1016 bp. For the amplification control, the amplicon size was 65 bp (16, 32). The gel image with PCR amplification results was photographed in the traditional way, with a Polaroid system, with exposure of a few seconds, using a UVP transilluminator (BioDoc-ItTM IMAGING System). The image processing was carried out using the VilberLourmat® system (Vilber, Marne-la-Vallée, France), and the size of the amplified fragments was calculated using the USI computer programme.

Statistical analyses

The obtained values for Socodor hunting grounds were analysed by one-way ANOVA (with Bonferroni post-test) using GraphPad Prism 9.1 for Windows (GraphPad Software, San Diego, USA). Results were expressed as

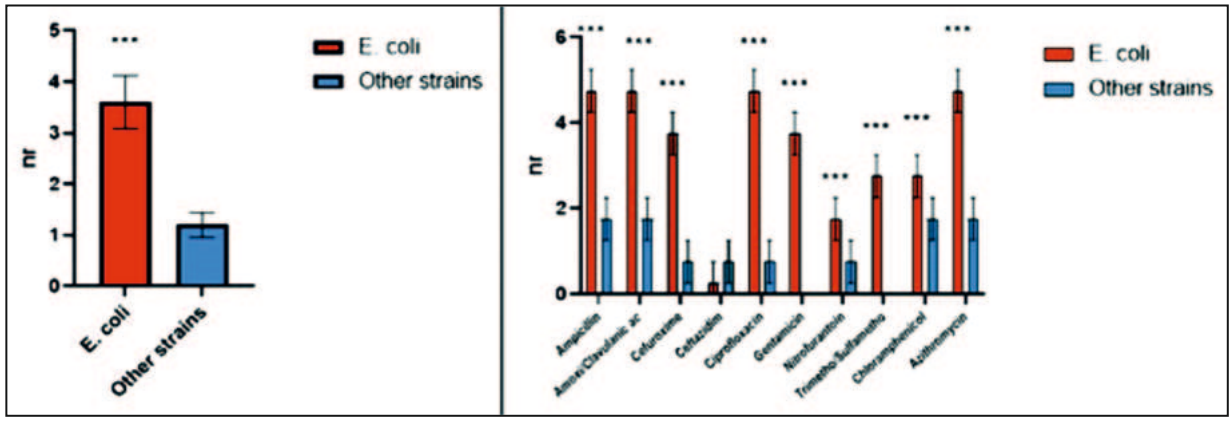

Fig. 2. Comparative overall levels of resistance between *E. coli* and other strains from Socodor hunting ground after Kirby Bauer methodology (left image, where *** = p <0.001) and the comparative antimicrobial susceptibility levels of resistance to tested antibiotics (right image, where*** = p <0.001)

mean +SEM; all values lower than p <0.05 were considered statistically significant.

RESULTS AND DISCUSSIONS

Antimicrobial susceptibility testing of the isolated strains

The initial assessment and results analysis, done by the Kirby Bauer diffusimetric method and with the *Vitek-2* Compact system (BioMérieux, France), revealed high resistance levels in *E coli* in samples collected from *Dama dama* in one of the three hunting grounds studied initially in Western Romania (respectively in the Socodor hunting ground) (36). Strains of *Enterobacteriaceae: Escherichia coli, Pseudomonas oleovorans, Providencia rettgeri, Enterobacter aerogenes,* and *Enterobacter* spp. were isolated specifically from Socodor hunting ground, providing significantly elevated resistance to most of the antibiotics tested. Significantly high resistance (between 50% and 90%) to most of the antibiotic groups tested was found and statistically confirmed ($p < 0.001$) in the strains of *E. coli* isolated from this hunting ground (Fig. 2).

All strains of *E. coli* assessed were fully resistant to some of the tested compounds: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), ciprofloxacin (CIP), and azithromycin (AZM), but also fully sensitive to ceftazidime (CTZ) (36). These results were reconfirmed using the *Vitek-2* Compact (BioMérieux) methodology, where resistance to antimicrobials in the Socodor area was the greatest ($p <0.001$) (16). The system revealed high degrees of resistance for beta-lactamin group, followed by quinolones and aminoglycosides, as presented in Fig. 3.

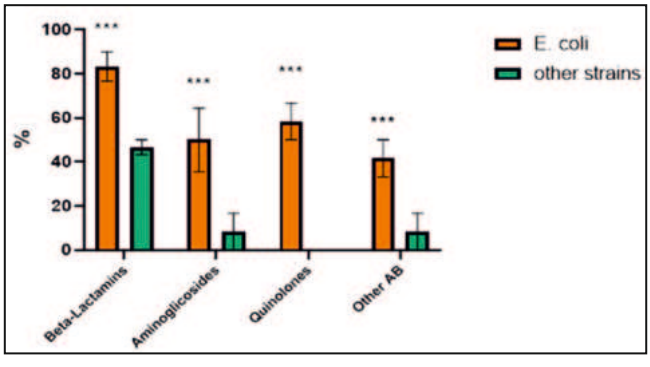

Fig. 3. The antimicrobial susceptibility of bacterial strains to the antibiotic groups established by the *Vitek2* Compact system (*BioMérieux, France*) for Socodor hunting ground (where*** = p <0.001)

Molecular analyses

Based on the analysis of the *bla*TEM and *bla*SHV genes in the studied isolates, the polymerase chain reaction (PCR) technique revealed that the sizes of the amplified fragments were approximately 858 bp (TEM) and 1016 bp (SHV), respectively (Fig. 4).

The size of these bands, with a consistent thickness and obvious brightness, confirmed that *Escherichia coli* strains isolated from fallow deer (*Dama dama*) and characterised for genotypic resistance included easily amplifiable resistance genes. Overall, the polymerase chain reaction technique, in which specific primers were used to detect resistance genes in *E. coli* strains, can be considered one with increased specificity and sensitivity for this purpose. The results confirmed that the PCR method, targeting the TEM and SHV genes, highlighted the specificity of these genes and their potential to confirm the phenomenon of antibiotic resistance for derived extended-spectrum β-lactamases (ESBLs) producing *Enterobacteriaceae*.

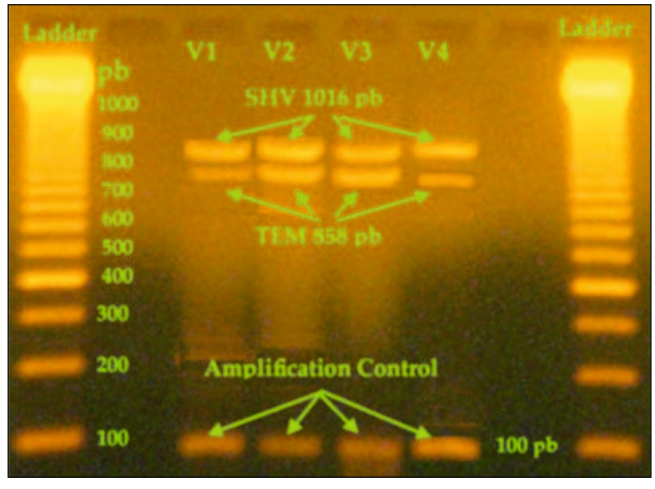

Fig. 4. The image of the gel obtained by electrophoresis for highlighting the TEM (858 bp) and SHV (1016 bp) resistance genes in sylvatic *E. coli* strains (where bp = base pair)

Previous research in the field revealed that the intestinal tract of wild animals could act as a reservoir of antibiotic resistance genes, especially for ampicillin (AMP), tetracycline (TE), streptomycin (STP), and Trimethoprim / Sulfamethoxazole (SXT), and it was also observed that multiresistant *E. coli* isolates were spotted in some of the tested wild animals, probably due to the fact that *E. coli* acts as a commensal bacterium, present in the normal intestinal microbiota of both farm and wild animals, normally isolated and identified in the samples collected in the study (2, 7). In a meta-study in which the aim was to investigate the presence/patterns of resistance to antibiotics in some strains of *Escherichia coli*, isolated from several species of wild animals in Germany, namely wild boar (*Sus scrofa*), deer (*Capreolus capreolus*), mallards (family *Anatidae*, subfamily *Anatinae*), and geese (family *Anatidae*, subfamily *Anserinae*), the authors found that the most prevalent ESBL genes were *bla*CTX-M-1 (56%), followed by *bla*CTX-M-15 (20%) and *bla*CTX−M−14-like (12%) (28). In this respect, most researchers in the field believe that the most common genes that confer genotypic resistance to extended-spectrum beta-lactamases, in animals are: *bla*CTX-M1, *bla*CTX-M14, followed by *bla* TEM-52 and, respectively, *bla*SHV-12, while the gene mainly associated with resistance to AmpC-type β-lactamases is *bla*CMY-2. Moreover, in enteric bacteria isolated from domestic animals in Europe, *bla*CTX-M1 is one of the

most widespread ESBL genes, frequently identified in *Enterobacteriaceae*, including game meat (7, 16, 28).

For example, in a study profiling antimicrobial resistance and prevalence of extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamases, and colistin resistance (*mcr*) genes in *E. coli* from swine between 1999 and 2018, the authors found 100% resistance for amoxicillin and tetracycline, and a progressive growth in ceftiofur resistance over almost 20% (24). The most common antimicrobial resistance genes found were the ESBL bla CTX-M (13.5%), AmpC-type β-lactamase (AmpC) bla CMY-2 (3%), and colistin resistance genes *mcr*-4 (13%), *mcr*-1 (7%) and to a lesser extent *mcr*-5 (3%). These *mcr* genes were identified in strains initially isolated in 2000 (1).

In a prominent study, the latent influence of food-producing animals on public health risks (by ESBL and/or AmpC-producing bacteria) was associated with specific plasmid-mediated ESBL and/or AmpC genes. The most common genes correlated with resistance in animals were *bla*CTX-M-1 and *bla*CTX-M-14, followed by *bla*TEM-52 and *bla*SHV-12. Among the genes encoding AmpC-type β-lactamases, *bla*CMY-2 was the most common (44). In accordance with the present results and important reviews that aimed to abridge the current knowledge on ESBL-*E. coli* in wildlife, the necessity for more large-scale investigations, in particular sentinel studies, to monitor the impact of multiresistant bacteria on wildlife was imposed (12, 13). In a study in the Emilia Romagna region, Italy, the purpose was to assess in parallel the prevalence of ESβL, AmpC, and colistin-resistant *E. coli* isolated from pork and wild boar meat. The ESβL and AmpC *E. coli* prevalence were genotypically established and were found to be higher in pork meat compared to wild boar meat. Four *E. coli* samples from wild boar meat were resistant to colistin but did not carry the *mcr*-1 gene. *E. coli* isolated from wild boar meat appeared to show a particular antimicrobial resistance mechanism for the cephalosporin group and colistin. Though the prevalence of resistant isolates in wild boar is not as alarming as in domestic pigs, the potential health risk to consumers remains, and requires further investigation (31). It is known that the bacterial membrane plays an important role in the survival of bacteria and the efficacy of antimicrobials. Important differences can be found between susceptible vs. resistant *E. coli.* In a preliminary study, making correlations between antibiotic resistance and bacterial lipid composition in *E. coli*, based on the function and arrangement of the bilipid coating of the bacterial cell, intimately associated with the path of antimicrobials through membranes, the methyl-(Z)-11-tetradecenoate acid was observed to have a clear relationship with the susceptibility in *E. coli* populations.

Compared to the presented results, resistance genes in *E. coli* strains isolated from animals in a sylvatic environment are more numerous and diverse. For example, in a study carried out in Portugal, a strain of *Escherichia coli* resistant to the main classes of antibiotics was isolated from red deer with the genes *bla*TEM-1D, *qnr*B36, *qnr* B19, *qnr*B82, and *gyr*A, and in Poland, variants of the *mcr*-1 to *mcr*-9 genes have been identified (27, 39).

In Finland, in a study conducted on faecal samples collected from reindeer in the north of the country, strains of *E. coli* were isolated and identified. Although antibiotic resistance was quite low, only in 5% of the 95 *Escherichia coli* strains isolated, genes for *Shiga* toxins (*stx*1 and/or *stx*2) were detected, with a remarkable prevalence of 32.6% (22). Holtmann et al. (2021) conducted a larger study in animals from the sylvatic environment in which they tracked the presence of ESBL-/AmpC *E. coli* in wild boar. Following the determinations carried out on a total of 375 faecal samples and 439 nasal samples, they isolated and identified *Escherichia coli* in 22 faecal samples (5.9%). Among the 22 strains, 19 were confirmed as ESBL producers and carried genes belonging to *bla*CTX-M group 1 or *bla*SHV-12. The other three strains carried the AmpC-β-lactamase gene *bla*CMY-2. *E. coli* ESBL-/AmpC occurrence in wild boar was significantly and positively associated with human population density (14). Through this study, both the authors mentioned above and other researchers demonstrate that in the sylvatic environment, wild boars can also serve as sources for *Escherichia coli* producing ESBL/AmpC and multiple resistances to antimicrobials (13, 34, 38). The presence of resistance genes was also detected in bacteria isolated from wild birds. Thus, Athanasakopoulou et al., following a study carried out in Greece, isolated and identified, from eight different species of wild birds, 12 strains of *Escherichia coli* with multiple resistances to most classes of antibiotics. In 11 of the 12 isolated strains, they highlighted the presence of the *bla*CTX-M-1 group of genes, alone or in combination with *bla*TEM, and one *bla*TEM alone (4).

The importance of the latent impact of the continuous proliferation of resistant *Enterobacteriaceae* increasingly observed in natural ecosystems alerts to the fact that wildlife has a proven capability to host organisms, including resistant bacterial strains, predominantly *E. coli*.

These different and abundant results prove the assumption that the health of humans, domestic and wild animals, as well as ecosystems, are interconnected, underscoring the importance of the *One Health* approach for enhanced monitoring and control of real threats to public health (17, 21, 23, 26). The plentiful studies carried out in diverse areas of the world confirm the fact that multiple resistances to antimicrobials, within *Enterobacteriaceae* serotypes can display significant disparities, from one geographical area to another and from one isolate to another. The occurrence of resistance is also dependent on the way antimicrobial compounds are used, both in veterinary and human medicine (40). The identification of antimicrobial resistance genes in *Escherichia coli* in the present study opens further opportunities for monitoring many other genes in domestic and wild animal herds in Europe. In accordance with other authors, the AMR surveillance can be successfully performed using the local wild deer as a sentinel species. In an interesting study on Scotland's mainland wild life, *Shiga* toxin-negative wild deer faecal samples were ascertained and screened for the presence of AMR *E. coli* and examined for latent risk factors related to AMR incidence. The resistance features

were targeted, resulting in 99% of the samples gathered containing *E. coli*, with a 21.8% occurrence of resistant *E. coli* in faecal samples for tetracycline (TE), 6.5% for cefpodoxime (CPD), 0.3% for ciprofloxacin (CIP), and no documented resistance to meropenem (MEM). Potential risk factors for the first occurring resistant *E. coli* strains (TE and CPD) were further investigated. The authors observed that the associated risk factors contrasted throughout the resistance phenotype and deer genus, with the vicinity or density of horses, being an important indicator of significantly decreased/increased risk. The conclusion of this study was that the resistance to critically important antibiotics was observed to be modest in the reviewed population, insinuating no immediate cause for concern regarding human health (11).

CONCLUSIONS

The PCR confirmed the extended-spectrum beta-lactamase resistance genes in four, out of ten initially studied, *Escherichia coli* strains gathered from fallow deer (*Dama dama*) generating 858 bp TEM and 1016 bp SHV bands. This is the first time ESBL genes were ascertained in silvatic environments in Western Romania. The identification of *Escherichia coli* resistance genes to antimicrobials in the present study, in wild species, opens additional opportunities for monitoring further resistance genes. This supports the hypothesis that the health of humans, domestic and wild animals, and ecosystems are interconnected, reinforcing the importance of the One Health approach for better monitoring and control of threats to public health.

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