Microbial Drug Resistance
Mechanisms, Epidemiology, and Disease

Microbial Drug Resistance: http://mc.manuscriptcentral.com/mdr

Antibiotic susceptibility and virulence factors in E. coli from sympatric wildlife of the Apuan Alps Regional Park (Tuscany, Italy)

Journal:	*Microbial Drug Resistance*
Manuscript ID	MDR-2018-0191.R4
Manuscript Type:	Veterinary Microbiology
Date Submitted by the Author:	n/a
Complete List of Authors:	Turchi, Barbara; University of Pisa, Scienze Veterinarie Dec, Marta; Uniwersytet Przyrodniczy w Lublinie, Bertelloni, Fabrizio; University of Pisa, Scienze Veterinarie Winiarczyk , Stanislaw; Uniwersytet Przyrodniczy w Lublinie Gnat, Sebastian; Uniwersytet Przyrodniczy w Lublinie Bresciani, Flavio; University of Pisa, Scienze Veterinarie Viviani, Fabio; Parco Regionale delle Alpi Apuane Cerri, Domenico; University of Pisa, Scienze Veterinarie Fratini, Filippo; University of Pisa, Veterinary Sciences
Keyword:	E. Coli, Microbial Drug Resistance, Veterinary Microbiology, Bacteria
Manuscript Keywords (Search Terms):	wildlife, antimicrobial resistance, virulence factors, Escherichia coli
Abstract:	Today a growing number of studies are focusing on antibiotic resistance in wildlife. This is due to the potential role of wild animals as reservoirs and spreaders of pathogenic and resistant bacteria. This study focused on isolating and identifying E. coli from the faeces of wild animals living in the Apuan Alps Regional Park (Tuscany, Italy) and evaluating some of their antibiotic-resistance and pathogenicity traits. Eighty-five faecal samples from different species were studied. Seventy-one E. coli were identified by MALDI-TOF MS analysis, subjected to antibiograms and PCR for the detection of antibiotic resistance genes and pathogenicity factors. The highest resistance rates were found against cephalothin (39.4%) and ampicillin (33.8%), followed by amoxicillin-clavulanic acid (15.5%), streptomycin (12.7%) and tetracycline (5.6%). Regarding resistance genes, 39.4% of the isolates were negative for all tested genes. The remaining isolates were positive for blaCMY-2, sul2, strA-strB and aadA1, tet(B) and tet(A), encoding resistance to beta-lactams, trimethoprim-sulfamethoxazole, streptomycin and tetracycline, respectively. With regards to virulence factors, 63.4% of the isolates were negative for all genes; 21.1% carried astA alone, which is associated with different pathotypes, 9.9% carried both escV and eaeA (aEPEC); single isolates (1.4%) harboured escV (aEPEC), escV associated with astA and eaeA (aEPEC), astA with stx2 and hlyA (EHEC) or astA and stx1, stx2 and hlyA (EHEC). These results show that wildlife from non-anthropized environments can be a reservoir for antibiotic-resistant microorganisms and suggest the need for a deeper knowledge

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

	on their origin and diffusion mechanisms through different ecological niches.

SCHOLARONE™
Manuscripts

Antibiotic susceptibility and virulence factors in *E. coli* from sympatric wildlife of the Apuan Alps Regional Park (Tuscany, Italy)

Running title: Wildlife *E. coli*: drug resistance and virulence

Barbara Turchi[a*], Marta Dec[b], Fabrizio Bertelloni[a], Stanisław Winiarczyk[c], Sebastian Gnat[d], Flavio Bresciani[a], Fabio Viviani[e], Domenico Cerri[a], Filippo Fratini[a]

[a]Department of Veterinary Science, University of Pisa, Viale delle Piagge 2, Pisa, Italy

[b]Sub‑Department of Veterinary Prevention and Avian Diseases, Institute of Biological Bases of Animal Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Akademicka 12, 20‑033 Lublin, Poland

[c]Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Street Głęboka 30, 20-612 Lublin, Poland

[d]Sub-Department of Veterinary Microbiology, Institute of Biological Bases of Animal Diseases, Faculty of Veterinary Medicine, University of Life Sciences, Lublin, Akademicka 12, 20‑033, Poland

[e]Parco Regionale delle Alpi Apuane, Seravezza, Lucca, Italy

*Corresponding author: barbara.turchi@unipi.it, Viale delle Piagge 2, 56124 (Pisa), Italy, phone: +390502216959, fax: +390502216941

For Peer Review Only/Not for Distribution

Abstract

Today a growing number of studies are focusing on antibiotic resistance in wildlife. This is due to the potential role of wild animals as reservoirs and spreaders of pathogenic and resistant bacteria. This study focused on isolating and identifying *E. coli* from the faeces of wild animals living in the Apuan Alps Regional Park (Tuscany, Italy) and evaluating some of their antibiotic-resistance and pathogenicity traits. Eighty-five faecal samples from different species were studied. Seventy-one *E. coli* were identified by MALDI-TOF MS analysis, subjected to antibiograms and PCR for the detection of antibiotic resistance genes and pathogenicity factors. The highest resistance rates were found against cephalothin (39.4%) and ampicillin (33.8%), followed by amoxicillin-clavulanic acid (15.5%), streptomycin (12.7%) and tetracycline (5.6%). Regarding resistance genes, 39.4% of the isolates were negative for all tested genes. The remaining isolates were positive for bla_{CMY-2}, *sul2*, *strA*-*strB* and *aadA*1, *tet(B)* and *tet(A)*, encoding resistance to beta-lactams, trimethoprim-sulfamethoxazole, streptomycin and tetracycline, respectively. With regards to virulence factors, 63.4% of the isolates were negative for all genes; 21.1% carried *astA* alone, which is associated with different pathotypes, 9.9% carried both *escV* and *eaeA* (aEPEC); single isolates (1.4%) harboured *escV* (aEPEC), *escV* associated with *astA* and *eaeA* (aEPEC), *astA* with *stx2* and *hlyA* (EHEC) or *astA* and *stx1*, *stx2* and *hlyA* (EHEC). These results show that wildlife from non-anthropized environments can be a reservoir for antibiotic-resistant microorganisms and suggest the need for a deeper knowledge on their origin and diffusion mechanisms through different ecological niches.

Keywords: wildlife; antimicrobial resistance; virulence factors; *Escherichia coli*

Mary Ann Liebert, Inc., 140 Huguenot Street, New Rochelle, NY 10801

For Peer Review Only/Not for Distribution

1. Introduction

Antimicrobial resistance (AMR) is currently one of the main concerns for human and animal health.[1] A wide body of knowledge demonstrates that the improper use of antimicrobials in human and especially veterinary medicine has dramatically affected the rise of antimicrobial resistant microorganisms in the environment. [2,3]

Over the last number of years, the number of studies focusing on AMR have increased along with studies focusing on resistant bacteria from wild animals. [4,5] Indeed, a deeper knowledge on the role of different compartments in the maintenance and dissemination of resistance genes seems to be crucial to tackle the issue.

Most of the studies on AMR in wildlife have focused on microorganisms relevant for human health, such as *Escherichia coli*, [6-10] *Enterococcus* spp. [11,12] or *Salmonella* spp.. [13] According to Vittecoq *et al.*, [5] the diversity of detected resistance mechanisms and the proportion of individual hosts carrying resistant bacteria increase with the proximity to anthropized settlements.

Currently, few studies have been carried out in Italy concerning AMR in wild animals, mammals [13-15], birds[13,16,17] or both. For this reason, the aim of the present study was to investigate antimicrobial resistance and pathogenicity factors in *E. coli* isolated from wildlife ranging in Apuan Alps Regional Park, located in north-western Tuscany (Italy). The studied area extends to over 20,600 hectares and is characterized by a heterogeneous environment with hills and mountain territory. In a natural reserve, the direct impact of humans is minimum, thus a high prevalence of antimicrobial resistant bacteria could reflect the ability of these microorganisms to disseminate across environments. Here, we considered different mammal species, especially canids, to gain a deeper knowledge on the distribution of pathogenic and non-pathogenic antibiotic-resistant *E. coli* in a natural ecosystem.

2. Material and Methods

2.1 Faecal samples collection

For Peer Review Only/Not for Distribution

From April 2017 to October 2017, 85 faecal samples from wild animals were collected in the Apuan Alps Regional Park located in Tuscany, Central Italy. Forty-two samples belonged to wolves (*Canis lupus*); 13 to foxes (*Vulpes vulpes*); 6 to badgers (*Meles meles*); 3 to wild-boars (*Sus scrofa*); 3 to red deers (*Cervus elaphus*); 2 to roe deers (*Capreolus capreolus*); 9 to mouflons (*Ovis aries musimon*), 5 to wild goats (*Capra hircus*), and 2 to hares (*Lepus europeus*). Animals included in the study were not subjected to antibiotic treatment. No information on their health status was available.

2.2 E. coli isolation

For the isolation of *E. coli*, a non-selective pre-enrichment step was performed employing buffered peptone water (Biolife Italiana S.r.l.-Mascia Brunelli S.p.a., Milan, Italy) incubated at 37°C for 24 h. After incubation, a loopful from each broth culture was seeded on Tryptone Bile X-Glucuronide agar plates (Thermo Fisher Scientific, Milan, Italy) and incubated at 44°C for 48 h. From each plate, a variable number (from one to three) of colonies presumptively classified as glucuronidase positive, was selected for purification, streaking them on TBX agar plates. Once purified, isolates were grown on Brain and Heart Infusion broth (Thermo Fisher Scientific, Milan, Italy), incubated at 37 °C for 24 h. Sterile glycerol (20%) was then added to each broth culture and isolates were stored at -80 °C for further analysis.

2.3 E. coli identification via MALDI-TOF mass spectrometry (MS)

Isolated bacteria were identified by MALDI-TOF mass spectrometry (MS) using a standard extraction method as previously described. [18] Calibration was previously performed with a bacterial test standard (Bruker, Germany) containing extract of *E. coli* DH5 alpha. Measurements were performed with an UltrafleXtreme MALDI TOF mass spectrometer (Bruker, Germany) equipped with a 1000 Hz Nd-YAG laser (neodymium-doped yttrium aluminium garnet).

The mass spectra obtained from each isolate was processed with the MALDI Biotyper 3.0 software package (Bruker, Germany) and results were shown as the top 10 identification matches along with

For Peer Review Only/Not for Distribution

confidence scores ranging from 0.00 to 3.00. According to the criteria recommended by the manufacturer, a log(score) below 1.70 does not allow reliable identification; a log(score) between 1.70 and 1.99 allows identification at the genus level; a log(score) between 2.00 and 2.29 means highly probable identification at the genus level and probable identification at the species level; and a log(score) higher than 2.30 (2.30 – 3.00) indicates highly probable identification at the species level. Analysis of each sample was performed in triplicate (3 spots for each sample).

2.4 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by agar disk diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing- Version 6.0, January 2017, www.eucast.org). Isolates were revitalized on Tryptone Soy agar plates (Thermo Fisher Scientific, Milan, Italy). Bacterial suspensions with a turbidity equivalent to McFarland Standard 0.5 (approximately corresponding to 1-2*10^8 CFU/mL) were swabbed on Mueller Hinton agar plates (Thermo Fisher Scientific, Milan, Italy) with a sterile cotton swab. Antibiotic disks containing ampicillin (AM, 10 µg), amoxicillin/clavulanic acid (AMC, 20-10 µg), cefoxitin (FOX, 30 µg), cephalothin (KF, 30 µg), cefotaxime (CTX, 30 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), trimethoprim-sulfamethoxazole (SXT, 19:1; 25 µg), enrofloxacin (ENR, 10 µg), gentamicin (CN, 10 µg), streptomycin (S, 10 µg), imipenem (IPM, 10 µg), and aztreonam (ATM, 30 µg) (Kairosafe Srl, Trieste, Italy) were placed on the plates. Inhibition diameter zones, including the diameter of the 6 mm disks, were measured after incubation at 35°C for 16-20 h. Isolates were classified as resistant, intermediate and susceptible according to breakpoints provided by EUCAST or CLSI. [19,20]

2.5 DNA extraction

For Peer Review Only/Not for Distribution

E. coli isolates were cultured on Tryptic Soy Agar (TSA) at 37°C for ~20 h. Template DNA was isolated using a GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (Eurx, Poland) following the manufacturer instructions.

2.6 Detection of antibiotic resistance genes

The presence of genes encoding resistance to β-lactam antibiotics (*bla-$_{TEM}$*; *bla-$_{CMY2}$*), streptomycin (*aadA1*; *strA-strB*), tetracycline (*tetA*, *tetB* and *tetG*) and sulphonamide (*sul1*, *sul2*, *sul3*) was assessed by PCR using gene-specific primers and cycling programs according to various authors (Tab. 1). The reaction mixture (25 µl) was composed as follows: 2.5 µl of Dream Taq PCR buffer, 0.12 µl of Dream Taq DNA polymerase (5 U/ml, Thermo Scientific), 1.25 µl of 8 mM deoxynucleoside triphosphates (dNTPs, Blirt, Poland), 0.8 µl of each primer (10 pmol/µl, Genomed, Poland), 1 µl of template DNA (20 ng) and 18.5 µl of water (Sigma, Poland).

2.7 Detection of virulence-associated genes

PCR was employed to detect the presence of 15 genes associated with *E. coli* virulence traits. A multiplex-PCR by Paton and Paton [21,22] was used for the detection of *stx1*, *stx2*, *hylA*, *eaeA* and *saa* genes. The cycling conditions were as follows: initial denaturation at 95°C for 5 min, 10 cycles of 95°C for 45 s, 65°C for 45 s, 72°C for 75 s, 20 cycles of 95°C for 45 s, 62°C for 45 s, 72°C for 75 s, and a final extension step at 72°C for 8 min. Detection of *ecsV*, *ent*, *bfpB*, *invE*, *astA*, *aggR*, *pic*, *elt*, *estIa* and *estIb* was performed according to Müller *et al.*. [23] Thermocycling conditions were 95°C for 5 min, 30 cycles of 95°C for 45 s, 62°C for 45 s, 72°C for 75 s and a final extension step at 72°C for 8 min. Both multiplex PCR reactions were performed in an Eppendorf Mastercycler using Dream Taq polymerase (Thermo Scientific). Table 2 shows the primers sequences and concentrations in the final volume, expected amplicon sizes and associated putative pathovars.

For Peer Review Only/Not for Distribution

PCR products (8 µl) were separated by electrophoresis (100 V) on 2% agarose gels and visualized by ethidium bromide staining. PCR products sizes were determined by comparison with a M100-M 1000 bp DNA Ladder (Blirt, Poland).

3. Results

3.1 Isolation and identification of E. coli via MALDI-TOF MS

Presumptive *E. coli* colonies were successfully obtained from 67 out of 85 faecal samples (78.8%). Of the 96 isolates selected on TBX agar, 71 (74%) were identified as *E. coli* by MALDI-TOF-MS.

For 68 (95.8%) isolates, the identification value ranged from 2,000 to 2.299, while for 3 (4.2%) it ranged from 1.943 to 1.989. For all isolates and for all the three replicates, the first and the second-best matches generated by Biotyper 3.0 indicated the same species (*E. coli*) and therefore identification at the species level was considered as reliable. Sixty-three (74.1%) *E. coli* positive faecal samples were obtained.

3.2 *Antibiotic susceptibility testing*

Thirty-two out of 71 isolates (45.1%) were susceptible to all the tested molecules. The highest resistance rates were detected for KF (39.4%) and AM (33.8%), followed by AMC (15.5%), and S (12.7%). Low percentages of resistance were observed for TE (5.6%), SXT (2.8%), CN (1.4%), CTX (1.4%), and ENR (1.4%). All the isolates were susceptible to FOX, IPM, and C. As for ATM, one intermediate isolate was obtained (isolate 13), while the others were all susceptible.

Table 3 shows the resistance phenotypes observed among the 71 isolates. The most frequently expressed phenotypes were those of exclusive resistance against KF (16.9%) or KF associated with AM (12.7%). Eight isolates (11.3%) showed multi-resistance, defined as resistance against three or more chemotherapeutic agents. In particular, isolate 66 from a wild goat and isolate 52 from a fox were resistant to 5 and 8 antibiotics, respectively.

For Peer Review Only/Not for Distribution

3.3 Detection of antibiotic resistance genes

All *E. coli* isolates were subjected to end-point PCR to detect some antibiotic resistance genes. Forty-three out of 71 isolates (60.6%) harboured at least one of the targeted genes. All the isolates were negative for bla_{TEM}. Although none of the observed resistance phenotypes suggested the possibility for extended spectrum beta-lactamases (ESBLs) production, bla_{CMY-2} gene was surprisingly detected in 54.9% (39/71) *E. coli* isolates. Among these, 53.8% (21/39) showed a resistance phenotype to different β-lactams (AM, KF, CTX, but never FOX), while 46.2% (18/39) did not show any resistance phenotype against β-lactams. A percentage of 23.9% (17/71) showed resistance against at least one of the tested β-lactams, but did not harbour bla_{CMY-2}, the remaining 21.1% (15/71) did not show any phenotypic resistance and were negative for bla_{CMY-2}.

Resistance to SXT was observed in 2/71 isolates (2.8%), both positive to *sul2*, while *sul1* and *sul3* were not detected. All the SXT susceptible isolates were negative for *sul1*, *sul2* and *sul3*.

Out of 71 *E. coli* isolates, 4 (5.6%) showed a resistance phenotype against S and tested positive for *strA-strB* or *aadA1*; conversely, 7.0% isolates (5/71) was resistant to S, but no gene coding for this resistance was identified. Furthermore, 1.4% isolates (1/71) was susceptible to S, and harboured both *aadA1* gene and *strA-strB* genes. Finally, 1.4% isolates (1/71) exhibited an intermediate phenotype against S and resulted an *aadA1* gene carrier.

With regards to TE, resistance was detected in 5.6% (4/71) isolates. No discrepancies were observed between phenotype and genotype, since *tet(B)* was detected in all resistant isolates, while *tet*(A) was found in 25% of the resistant isolates (1/4). Lastly, *tet(G)* was not detected.

Table 4 shows the resistance phenotype associated with the 43 isolates harbouring at least one antibiotic resistance gene.

3.4 Detection of virulence-associated genes

The 71 *E. coli* isolates were subjected to multiplex-PCR for the detection of gene encoding pathogenicity factors. Twenty-six out of 71 isolates (36.6%) were positive for at least one gene; 25.4%

For Peer Review Only/Not for Distribution

isolates (18/71) carried *astA*, 12.7% (9/71) *escV*, 11.3% (8/71) *eaeA*, 2.8% (2/71) *hlyA* and *stx2*, and 1.4% (1/71) carried *stx1*. *Ent, bfpB, invE, aggR, pic, elt, estIa, estIb, saa* were not detected. Table 5 shows the different gene combinations detected together with their associated pathovars. Fifteen out of 71 isolates (21.1%) carried only *astA* (EAEC), while 9.9% isolates (7/71) presented *escV* together with *eaeA* (aEPEC); single isolates (1.4%) were instead positive to *escV* (aEPEC), *escV* associated with *astA* and *eaeA* (aEPEC), *astA* associated with *stx2* and *hlyA* (EHEC) or to *astA* associated with *stx1*, *stx2* and *hlyA* (EHEC).

4. Discussion

As reviewed by Vittecoq *et al.*, [5] several authors reported that depending on the diet, the incidence of resistant strains could vary, with carnivores more prone to be colonized by resistant bacteria than herbivores. The present study took into consideration antibiotic resistance and virulence factors in *E. coli* isolates from faecal samples from different animal species in Apuan Alps Regional Park (Tuscany; Italy). Most of the collected samples (64.7%) and isolates (56.3%) belonged to *Canidae* (wolves, and foxes), while a lower number of samples belonged to other animal species. Since canids as predators occupy the top-position on the trophic pyramid, they carry and accumulate microorganisms from all the lower levels. Thus, the rate of antibiotic-resistance detected from these animals could be considered representative of the entire ecosystem.

Globally, high percentages of resistance against KF (39.4%) and AM (33.8%) were obtained, followed by AMC (15.5%), and S (12.7%). Resistance against TE, SXT, CN, CTX, and ENR was much lower (5.6%, 2.8%, 1.4%, 1.4%, and 1.4%, respectively). The same situation can be observed when considering the sole family of *Canidae*, with the highest rates of resistance against KF and AM (47.5% and 40%, respectively), followed by AMC (20%), S (15%), TE (7.5%), SXT (5%), CTX, ENR and CN (all with 2.5%).

Studies by different authors reported quite different results and highlighted overall high rates of resistance against TE. Most of the European studies focusing on antimicrobial resistance in wild

For Peer Review Only Not for Distribution

1
2
3 207 animals from natural parks were performed in Portugal. However, the comparison with other studies
4
5
6 208 seems to be difficult, due to the variability of species included in sampling plans.
7
8 209 In a study similar to ours, Gonçalves *et al.* [24] studied a Portuguese population of Iberian wolves and
9
10 210 isolated *E. coli* from 195 faecal samples. In contrast to our observations, they detected the highest
11
12
13 211 levels of resistance against TE (30%), AM (25%), S (25%) SXT (12%) and C (5%), while resistance
14
15 212 to AMC, FOX, and CN was lower than 1%. Considering studies looking at top predators from a
16
17 213 natural reserve, Gonçalves *et al.* [25] studied the antimicrobial resistance in *E. coli* from 30 faecal
18
19
20 214 samples of Iberian lynx (*Lynx pardinus*), which is an endangered species living in Southern Spain.
21
22 215 Among 18 recovered *E. coli* isolates, they detected high levels of resistance against TE (33%), S and
23
24 216 nalidixic acid (28%), and SXT (22%). Taken together, these results suggest that in the Iberian natural
25
26
27 217 parks there is a different distribution of resistance determinants compared to what was observed in
28
29 218 Apuan Alps Regional Park.
30
31 219 Considering the Iberian Peninsula, Costa *et al.* [6] in accordance with Gonçalves *et al.* [24 and25], analysing
32
33
34 220 112 *E. coli* from 72 faecal samples from a wide variety of different wild animals, also confirmed the
35
36 221 highest resistance rate against TE (34.8%), which was followed by AM (22.3%), S (22.3%) and SXT
37
38 222 (18.8%). Lower percentages of resistance were observed against AMC (7.1%), C (6.3%), CN (6.3%),
39
40
41 223 CTX (1.8%) and ATM (1.8%). No resistance was detected against FOX and IPM. Remaining in
42
43 224 Portugal, Dias *et al.* [26] analysing 152 *E. coli* from 67 faecal samples from wild ungulates, which
44
45 225 represent the main prey for wolves, detected the highest resistance against AMC (16.45%); followed
46
47
48 226 by AM (9.87%), TE (8.55%), S (4.61%) and FOX (0.66%). All isolates were susceptible to ATM,
49
50 227 CTX, C, and IPM. The reported AMC resistance rate was higher than that of AM. This is an unusual
51
52 228 result, since resistance to AMC is usually equal to or lower than AM resistance. We also observed
53
54
55 229 few *E. coli* isolates resistant to AMC and susceptible to AM. As suggested by Dias *et al.*, [26] this
56
57 230 uncommon phenotype is not related to a particular resistance mechanism but is probably due to the
58
59 231 use of EUCAST AMC cut-off value for interpretation of inhibition diameter zone (R<19 mm), instead
60
232 of that proposed by CLSI, which is lower (R<14 mm).

For Peer Review Only/Not for Distribution

In Italy, few studies on AMR in *E. coli* from wildlife are available, and none of them focus on wolf populations. In particular, Foti *et al.* [16] and more recently Botti *et al.* [13] and Foti *et al.* [17] studied antimicrobial resistance in several *Enterobacteriaceae* isolated from wildlife. In both their works Foti *et al.* [16,17] focused on antimicrobial resistance from wild birds passing through the territory of Ustica island, Sicily (Italy) and Metaponto, Basilicata (Italy), respectively. In their first work, the authors isolated 183 strains belonging to 28 different species of the *Enterobacteriaceae* family, among which *E. coli* (53 strains) was identified. Despite the different animal species considered, similar to what we obtained, they reported the highest percentages of resistance against AM (42.6%), AMC (42.6%) and S (43.7%) and lower resistance rates against TE (6.6%), C (4.4%), FOX (1.2%) and SXT (0.6%). In their more recent work, Foti *et al.* [17] analysing 121 cloacal swabs from migratory birds, isolated 122 strains belonging to 18 distinct species, none of them belonging to *E. coli*. They observed the highest resistance rates against amoxicillin (64.8%); AM (63.1%); rifampicin (61.5%); AMC (54.1%), while lower rates were detected for IPM (25.4%) and meropenem (6.6%). Only, Botti *et al.* [13] looked at the presence of antibiotic resistant microorganisms from a wide collection of wild animals' samples (2,713) built during the period 2002-2010 in North-Western Italy. The sampling plan included a substantial proportion of canids, all belonging to *Vulpes vulpes* (1,222), but also birds (1,101), mustelids (221), rodents (100) and ungulates (69). However, they focused on *Salmonella* and observed the highest resistance values against tetracycline class.

With respect to antibiotic resistance genes, none of our isolates demonstrated a typical ESBL-phenotype, which is characterized by resistance to penicillins; first, second, and third-generation cephalosporins and aztreonam. Thus, the high percentage of bla_{CMY2} positive isolates (54.9% considering all isolates and 52.5% for canids) was unexpected. Indeed, bla_{CMY2} encodes an AmpC type beta-lactamase (cephamycinase) and is the most widespread plasmid-borne β-lactamase detected from *E. coli* and *Salmonella* spp. of animal origin. [27] Our result was surprising since the presence of bla_{CMY2} is associated with a broader spectrum of antimicrobial activity than those observed among the studied isolates. Indeed, isolates harbouring bla_{CMY2} were in almost all cases, AM-resistant and FOX-

For Peer Review Only/Not for Distribution

susceptible or even susceptible to all β-lactams tested. Hinthong *et al.* [28] detected isolates displaying the same characteristic among non-pathogenic *E. coli* from a water supply in Thailand. Davis *et al.* [29] demonstrated that in the absence of a selective pressure, which can be observed in natural settings where antibiotic presence is limited, microorganisms are more prone to accumulate mutations leading to dysfunctional pseudogenes (also antibiotic resistance pseudogene), which lost their ability to be expressed. In our specific case, to confirm this hypothesis, sequencing analysis of amplification products would be needed.

Regarding S antibiotic resistance genes, we detected some discrepancies between phenotypes and genotypes, since 5 resistant isolates did not harbour any of the investigated genes, one intermediate isolate harboured *aadA1*, and one susceptible isolate harboured both *aadA1* and *strA-strB*. The isolates not possessing any of these two genes but displaying a resistant-phenotype may harbour other genes mediating S resistance, or resistance could be due to chromosomal mutations altering the ribosomal binding site of S. Sunde and Norström [30] observed that *aadA* genes are involved in low levels of S-resistance, while *strA-strB* genes probably confer high-level resistance to S, this could explain the intermediate phenotype. Lastly, Davis *et al.* [29] observed in their study many isolates with inactive S-resistance gene, mostly *strA-strB* and *aadA2* consistent with our observation of susceptible phenotype.

The SXT and TE resistant isolates which were in a lower percentage, all presented with a consistent genotype. In particular, *sul2* was detected in the two SXT-resistant isolates from a fox, *tet(B)* in all TE resistant isolates, while *tet(A)* was detected in one out of 4 (25%) TE resistant isolates. Isolates carrying *tet* genes were all but one of canids origin. The presence of these genes among isolates from wild animals, especially predators, was also reported by Gonçalves *et al.* [31,24], and also by Costa *et al.*[6] who considered different animal species.

Understanding the distribution of zoonotic pathogens in specific ecological niches is fundamental to evaluate the risk derived from the exposure to that specific environment. Thus, all the isolates were screened for the presence of some of the main virulence-associate genes. Most of the isolates (63.4%)

For Peer Review Only/Not for Distribution

For Peer Review Only/Not for Distribution

did not present any of the tested genes, suggesting a prevalent circulation of non-pathogenic isolates. This percentage increases, if only *Canidae* samples are considered (72.5%). Moreover, 21.1% total isolates and 1.5% canid isolates presented only *astA* encoding for EAST1, a heat-stable enterotoxin. This gene was at first associated with EAEC, but was later detected in other pathotypes. The role of *astA* as marker for pathogenic *E. coli* is still unclear, as it was reported that *astA* contributes to pathogenicity only when in combination with other virulence-associated genes. [32] Eight isolates (4 of them from canids) presented an EPEC putative pathotype, since they showed the presence of *eaeA* and lacked *stx1*, *stx2*. In particular, these isolates could be ascribed to the atypical EPEC (aEPEC) group, due to the absence of *bfp* encoding the bundle encoding pilus (BFP) characteristic of typical EPEC (tEPEC). An additional isolate from a wolf presented with *escV* alone and could be attributed to the aEPEC group as well. This is in accordance with several authors reporting that while humans seem to be the only reservoir of tEPEC, aEPEC can be isolated from humans as well as from a wide variety of animals. [33,34] Some aEPEC strains from animals have been associated to human diseases, suggesting that animals could represent important reservoirs of zoonotic aEPEC. [35] Moreover, two putative EHEC isolates were detected from a wild boar and roe deer.

5. Conclusion

The present work highlights the prevalent presence of non-pathogenic *E. coli* with antibiotic-resistant traits in faecal samples from wild animals living in a natural park with a minimum exposure to a selective pressure. The highest percentages of resistance were observed against a first-generation cephalosporin (cephalothin) and ampicillin, while ESBL-producers were not detected. Most of the analysed samples belonged to canids, such as wolves and foxes, top predator carnivorous species, able to acquire antibiotic resistant genes from diverse environments and disperse them across large distances. Indeed, due to the reduction of natural prey, farm animals become an important nutritional source, pushing predators towards anthropized settlements. In addition, considering their wide home range, such species may play a key role concerning AMR dynamics in natural ecosystems. On the

other hand, species belonging to wild ungulates, especially wild boars, are frequently present near houses and farms, meaning that they could represent an important epidemiological link between domestic animals, humans and wildlife.

Acknowledgments

The authors would like to thank Dr. Anna Paola Biagi for the English revision.

Author Disclosure Statement

No competing financial interests exist.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. World Health Organization, 2014 Antimicrobial resistance global report on surveillance: 2014 summary. http://www.who.int/drugresistance/documents/surveillancereport/en

2. Singer, R.S., Finch, R., Wegener, H.C., Bywater, R., Walters, J., Lipsitch, M. 2003. Antibiotic resistance--the interplay between antibiotic use in animals and human beings. Lancet Infect. Dis. 3(1):47-51.

3. Tasho, R.P., Cho, J.Y. 2017. Entry Routes of Veterinary Antibiotics in the Environment. In: Hashmi M., Strezov V., Varma A. (eds) Antibiotics and Antibiotics Resistance Genes in Soils. Soil Biology, vol 51. Springer, Cham.

4. Radhouani, H., Silva, N., Poeta, P., Torres, C., Correia, S., Igrejas, G. 2014. Potential impact of antimicrobial resistance in wildlife, environment and human health. Front. Microbiol. 5(FEB):23.

Mary Ann Liebert, Inc., 140 Huguenot Street, New Rochelle, NY 10801

For Peer Review Only/Not for Distribution

1
2
3 331 5. Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., Arnal, A., Aberkane,
4
5
6 332 S., Jean-Pierre, H., Gauthier-Clerc, M., Thomas, F., Renaud, F. 2016. Antimicrobial resistance in
7
8 333 wildlife. J. Appl. Ecol. 53(2):519-529.
9
10
11 334 6. Costa, D., Poeta, P., Saenz, Y., Vinué, L., Cohelo, A.C., Matos, M., Rojo-Bezares, B., Rodrigues,
12
13 335 J., Torres, C. 2008. Mechanisms of antibiotic resistance in *Escherichia coli* isolates recovered from
14
15
16 336 wild animals. Microb. Drug Resist. 14:71–7.
17
18
19 337 7. Poeta, P., Radhouani, H., Igrejas, G., Gonçalves, A., Carvalho, C., Rodrigues, J., Vinué, L., Somalo,
20
21 338 S., Torres, C. 2008. Seagulls of the Berlengas natural reserve of Portugal as carriers of fecal
22
23 339 *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. Appl. Environ.
24
25
26 340 Microbiol. 74(23):7439-7441.
27
28
29 341 8. Poeta, P., Radhouani, H., Pinto, L., Martinho, A., Rego, V., Rodrigues, R., Gonçalves, A.,
30
31 342 Rodrigues, J., Estepa, V., Torres, C., Igrejas, G. 2009. Wild boars as reservoirs of
32
33 343 extended‐spectrum beta‐lactamase (ESBL) producing *Escherichia coli* of different phylogenetic
34
35
36 344 groups. J. Basic Microbiol. 49(6):584-588.
37
38
39 345 9. Radhouani, H., Pinto, L., Coelho, C., Gonçalves, A., Sargo, R., Torres, C., Igejas, G., Poeta, P.
40
41 346 2010. Detection of *Escherichia coli* harbouring extended-spectrum β-lactamases of the CTX-M
42
43
44 347 classes in faecal samples of common buzzards (*Buteo buteo*). J. Antimicrob. Chemother. 65(1):171-
45
46 348 173.
47
48
49 349 10. Radhouani, H., Igrejas, G., Gonçalves, A., Estepa, V., Sargo, R., Torres, C., Poeta, P. 2013.
50
51 350 Molecular characterization of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates
52
53
54 351 from red foxes in Portugal. Arch. Microbiol. 195(2):141-144.
55
56
57 352 11. Klibi, N., Amor, I. B., Rahmouni, M., Dziri, R., Douja, G., Said, L. B., Lozano, C., Boudabous,
58
59 353 A., Slama, K.B., Mansouri, R., Torres, C. 2015. Diversity of species and antibiotic resistance among
60

For Peer Review Only/Not for Distribution

354 fecal enterococci from wild birds in Tunisia. Detection of vanA-containing *Enterococcus faecium*
355 isolates. Eur. J. Wildlife Res. 61(2):319-323.

356 12. Lozano, C., Gonzalez-Barrio, D., Camacho, M. C., Lima-Barbero, J. F., de la Puente, J., Höfle,
357 U., Torres, C. 2016. Characterization of fecal vancomycin-resistant enterococci with acquired and
358 intrinsic resistance mechanisms in wild animals, Spain. Microb. Ecol. 72(4):813-820.

359 13. Botti, V., Navillod, F.V., Domenis, L., Orusa, R., Pepe, E., Robetto, S., & Guidetti, C. 2013.
360 *Salmonella* spp. and antibiotic-resistant strains in wild mammals and birds in north-western Italy from
361 2002 to 2010. Vet. Ital. 49(2):195-202.

362 14. Caprioli, A., Donelli, G., Falbo, V., Passi, C., Pagano, A., Mantovani, A. 1991. Antimicrobial
363 resistance and production of toxins in *Escherichia coli* strains from wild ruminants and the alpine
364 marmot. J. Wildl. Dis. 27(2):324-327.

365 15. Zottola, T., Montagnaro, S., Magnapera, C., Sasso, S., De Martino, L., Bragagnolo, A., D'Amici,
366 L., Condoleo, R., Pisanelli, G., Iovane, G., Pagnini, U. 2013. Prevalence and antimicrobial
367 susceptibility of *Salmonella* in European wild boar (*Sus scrofa*); Latium Region–Italy. Comp.
368 Immunol. Microbiol. Infect. Dis. 36(2):161-168.

369 16. Foti, M., Rinaldo, D., Guercio, A., Giacopello, C., Aleo, A., De Leo, F., Fischella, V., Mammina,
370 C. 2011. Pathogenic microorganisms carried by migratory birds passing through the territory of the
371 island of Ustica, Sicily (Italy). Avian Pathol. 40(4):405-409.

372 17. Foti, M., Mascetti, A., Fisichella, V., Fulco, E., Orlandella, B.M., Piccolo, F.L. 2017. Antibiotic
373 resistance assessment in bacteria isolated in migratory Passeriformes transiting through the
374 Metaponto territory (Basilicata, Italy). Avian Res. 8(1):26.

For Peer Review Only Not for Distribution

375 18. Dudzic, A., Urban-Chmiel, R., Stępień-Pyśniak, D., Dec, M., Puchalski, A., Wernicki, A. 2016.
376 Isolation, identification and antibiotic resistance of *Campylobacter* strains isolated from domestic and
377 free-living pigeons. Br. Poult. Sci. 57:172-8.

378 19. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for
379 interpretation of MICs and zone diameters. Version 8.1, 2018. http://www.eucast.org.

380 20. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth
381 Informational Supplement. 2015. CLSI document M100-S25. Wayne, PA: Clinical and Laboratory
382 Standards Institute.

383 21. Paton, A.W., Paton, J.C. 1998. Detection and Characterization of Shiga toxigenic *Escherichia*
384 *coli* by using multiplex PCR assays for *stx 1*, *stx 2*, *eaeA*, enterohemorrhagic *E. coli* hlyA, rfb O111,
385 and rfb O157. J. Clin. Microbiol. 36(2):598-602.

386 22. Paton, A. W., Paton, J. C. 2002. Direct detection and characterization of Shiga toxigenic
387 *Escherichia coli* by multiplex PCR for *stx1*, *stx2*, *eae*, *ehxA*, and *saa*. J. Clin. Microbiol. 40(1):271-
388 274.

389 23. Müller, D., Greune, L., Heusipp, G., Karch, H., Fruth, A., Tschäpe, H., Schmidt, M.A. 2007.
390 Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing
391 intermediate virulence factor profiles by using a novel single-step multiplex PCR. Appl. Environ.
392 Microbiol. 73(10):3380-90.

393 24. Gonçalves, A., Igrejas, G., Radhouani, H., Correia, S., Pacheco, R., Santos, T., Monteiro, R.,
394 Guerra, A., Petrucci-Fonseca, F., Brito, F., Torres, C., Poeta, P. 2013. Antimicrobial resistance in
395 faecal enterococci and *Escherichia coli* isolates recovered from Iberian wolf. Lett. Appl. Microbiol.
396 56(4):268-274.

For Peer Review Only Not for Distribution

25. Gonçalves, A., Igrejas, G., Radhouani, H., Santos, T., Monteiro, R., Pacheco, R., Alcaide, E., Zorrilla, I., Serra, R., Torres, C., Poeta, P. 2013. Detection of antibiotic resistant enterococci and *Escherichia coli* in free range Iberian Lynx (*Lynx pardinus*). Sci. Total Environ. 456:115-119.

26. Dias, D., Torres, R. T., Kronvall, G., Fonseca, C., Mendo, S., Caetano, T. 2015. Assessment of antibiotic resistance of *Escherichia coli* isolates and screening of *Salmonella* spp. in wild ungulates from Portugal. Res. Microbiol. 166(7):584-593.

27. Li, X. Z., Mehrotra, M., Ghimire, S., Adewoye, L. 2007. β-Lactam resistance and β-lactamases in bacteria of animal origin. Vet. Microbiol. 121(3-4):197-214.

28. Hinthong, W., Pumipuntu, N., Santajit, S., Kulpeanprasit, S., Buranasinsup, S., Sookrung, N., Chaicumpa, W., Aiumurai, P., Indrawattana, N. 2017. Detection and drug resistance profile of *Escherichia coli* from subclinical mastitis cows and water supply in dairy farms in Saraburi Province, Thailand. PeerJ, 6:e3431.

29. Davis, M. A., Besser, T. E., Orfe, L. H., Baker, K. N., Lanier, A. S., Broschat, S. L., New, D., Call, D. R. 2011. Genotypic-phenotypic discrepancies between antibiotic resistance characteristics of *Escherichia coli* from calves in high and low antibiotic use management settings. Appl. Environ. Microbiol. 77:3293-3299.

30. Sunde, M., Norström, M. 2005. The genetic background for streptomycin resistance in *Escherichia coli* influences the distribution of MICs. J. Antimicrob. Chemother. 56(1):87-90.

31. Gonçalves, A., Igrejas, G., Radhouani, H., Estepa, V., Pacheco, R., Monteiro, R., Brito, F., Guerra, A., Petrucci-Fonseca, F., Torres, C., Poeta, P. 2012. Iberian wolf as a reservoir of extended-spectrum β-lactamase-producing *Escherichia coli* of the TEM, SHV, and CTX-M groups. Microb. Drug Resist. 18(2):215-219.

For Peer Review Only/Not for Distribution

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

419 32. Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D. M., Fairbrother, J. M. 2003. Isolation
420 and association of Escherichia coli AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in
421 piglets and antibiotic sensitivity of isolates. J. Vet. Diagn. Invest. 15(3):242-252.

422 33. Blanco, M., Schumacher, S., Tasara, T., Zweifel, C., Blanco, J. E., Dahbi, G., Blanco, J., Stephan,
423 R. 2005. Serotypes, intimin variants and other virulence factors of eae positive *Escherichia coli*
424 strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-
425 η2). BMC Microbiol. 5(1):23.

426 34. Chandran, A., Mazumder, A. 2013. Prevalence of diarrhea-associated virulence genes and genetic
427 diversity in *Escherichia coli* isolates from fecal material of various animal hosts. Appl. Environ.
428 Microbiol. 79(23):7371-7380.

429 35. Hernandes, R. T., Elias, W. P., Vieira, M. A., Gomes, T. A. 2009. An overview of atypical
430 enteropathogenic *Escherichia coli*. FEMS Microbiol. Lett. 297(2):137-149.

For Peer Review Only/Not for Distribution

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Tab 1 Targeted resistance gene, primers sequences, annealing temperature, expected amplicon size and references

Gene	Primers (5'-3')	Annealing (°C)	Amplicon size (bp)	Reference
bla-TEM	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	50	857	Maynard et al. (2003)
bla-CMY2	GACAGCCTCTTTCTCCACA TGGACACGAAGGCTACGTA	54	1000	Kozac et al. (2009)
strA-strB	ATGGTGGACCCT AAAACTCT CGTCTAGGATCGAGACAAAG	62	891	Tamang et al. (2007)
aadA1	GTGGATGGCGGCCTGAAGCC AATGCCCAGTCGGCAGCG	65	525	Kozac et al. (2009)
tetA	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	64	210	Dahshan et al. (2010)
tetB	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	64	659	Dahshan et al. (2010)
tetG	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	59	468	Dahshan et al. (2010)
sul1	TGGTGACGGTGTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG	63	789	Costa et al. (2008)
sul2	CGGCATCGTCAACATAACC GTGTGCGGATGAAAGTCAG	50	722	Costa et al. (2008)
sul3	GAGCAAGATTTTTGGAATCG CATCTGCAGCTAACCTAGGGTTTGG A	51	792	Costa et al. (2008)

For Peer Review Only/Not for Distribution

Tab 2 Targeted virulence factors genes, primers sequences, primers concentrations, expected amplicon sizes, associated pathovars and references

Gene	Primers (5'-3')	Concentration (µM)	Amplicon size (bp)	Associated pathovars	Reference
escV	ATTCTGGCTCTCTTCTTCTTTATGGCTG CGTCCCCTTTTACAAACTTCATCGC	0.4	544		Müller et al. (2007)
ent	TGGGCTAAAAGAAGACACACTG CAAGCATCCTGATTATCTCACC	0.4	629	EHEC, EPEC	
eaeA	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	0.3	384		Paton and Paton (1998)
bfpB	GACACCTCATTGCTGAAGTCG CCAGAACACCTCCGTTATGC	0.1	910	Typical EPEC	Müller et al. (2007)
stx1	ATA AAT CGC CAT TCG TTG ACT AC AGA ACG CCC ACT GAG ATC ATC		180		
stx2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	0.3	255	EHEC	Paton and Paton (1998)
hlyA	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT		534		
saa	CGTGATGAACAGGCTATTGC		119		

For Peer Review Only/Not for Distribution

	ATGGACATGCCTGTGGCAAC				
invE	CGATAGATGGCGAGAAATTATATCCCG CGATCAAGAATCCCTAACAGAAGAATCAC	0.2	766	EIEC	
astA	TGCCATCAACACAGTATATCCG ACGGCTTTGTAGTCCTTCCAT	0.4	102		
aggR	ACGCAGAGTTGCCTGATAAAG AATACAGAATCGTCAGCATCAGC	0.2	400	EAEC	
pic	AGCCGTTTCCGCAGAAGCC AAATGTCAGTGAACCGACGATTGG	0.2	1,111		Müller et al. (2007)
elt	GAACAGGAGGTTTCTGCGTTAGGTG CTTTCAATGGCTTTTTTTTGGGAGTC	0.1	655		
estIa	CCTCTTTTAGYCAGACARCTGAATCASTTG CAGGCAGGATTACAACAAAGTTCACAG	0.4	157	ETEC	
estIb	TGTCTTTTTCACCTTTCGCTC CGGTACAAGCAGGATTACAACAC	0.2	171		

2

For Peer Review Only/Not for Distribution

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Tab 3 Resistance phenotypes (number and percentage of detection) observed among the 71 *E. coli* isolates from wildlife

Resistance phenotypes	**Number of isolates**	**Percentage of isolates (%)**
Susceptible to all tested molecules	32	45.1
KF	12	16.9
AM-KF	9	12.7
AM-AMC	4	5.6
AM-S	2	2.8
AMC	2	2.8
AM-AMC-KF-S	1	1.4
AM-AMC-KF	1	1.4
AM	1	1.4
S	1	1.4
AM-AMC-TE	1	1.4
AM-KF-CTX-S	1	1.4
AM-KF-SXT-S	1	1.4
AM-KF-TE-S	1	1.4
AM-AMC-KF-TE-S	1	1.4
AM-AMC-KF-TE-SXT-ENR-CN-S	1	1.4
Resistant to all tested molecules	0	0

AM: amoxicillin; AMC: amoxicillin+clavulanic acid; KF: cephalothin; CTX: cefotaxime; TE: tetracycline; SXT: trimethoprim-sulfamethoxazole; ENR: enrofloxacin; CN: gentamicin; S: streptomycin.

For Peer Review Only/Not for Distribution

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Tab 4 Resistance phenotypes, resistance genotypes, virulence genes and putative associated pathovars in 52 isolates harbouring at least one resistance or virulence gene.

Isolate ID	Animal species	*Resistance phenotype*	*Resistance genotype*	*Virulence associated genes*	*Putative pathovars*
A1	wolf	AM-AMC-KF	*bla*$_{CMY-2}$	-	-
A3	wolf	AM-KF	*bla*$_{CMY-2}$	*astA*	VARIOUS
A5a	wolf	AMC	*bla*$_{CMY-2}$	*astA*	VARIOUS
A5b	wolf	KF	*bla*$_{CMY-2}$	*astA*	VARIOUS
A6b1	wolf	AM-KF	*bla*$_{CMY-2}$	-	
A6b2	wolf	KF	*bla*$_{CMY-2}$	-	
A9b	wolf	AM-S	*bla*$_{CMY-2}$	*astA*	VARIOUS
A10b	wolf	AM-KF	*bla*$_{CMY-2}$	-	
1a	wolf	AM-KF-CTX-S	*bla*$_{CMY-2}$	-	
3	badger	AM-AMC-KF-S	*bla*$_{CMY-2}$	-	
4	mouflon	-	*bla*$_{CMY-2}$	-	
5a	badger	-	*bla*$_{CMY-2}$	-	
5b	badger	-	*bla*$_{CMY-2}$	*escV-eaeA*	aEPEC
6a	mouflon	KF	*bla*$_{CMY-2}$	*escV-eaeA*	aEPEC
10a	fox	S	*bla*$_{CMY-2}$	-	
11	badger	-	*bla*$_{CMY-2}$	-	
12a	wolf	AM-AMC	*bla*$_{CMY-2}$	-	
12b	wolf	AM-KF	*bla*$_{CMY-2}$	-	
12c	wolf	KF	*bla*$_{CMY-2}$	-	
13	wolf	-	*bla*$_{CMY-2}$	-	
14	wolf	AM-AMC	*bla*$_{CMY-2}$	-	
19	wild boar	AM-AMC	*bla*$_{CMY-2}$	*astA-escV-eaeA*	aEPEC
20a	badger	-	*bla*$_{CMY-2}$	-	
20b	badger	-	*bla*$_{CMY-2}$	-	
21a	badger	-	*bla*$_{CMY-2}$	*astA*	VARIOUS
22a	wild boar	-	*bla*$_{CMY-2}$	*astA-stx1-stx2-hlyA*	EHEC
24a	mouflon	-	*bla*$_{CMY-2}$	-	
25a	mouflon	-	*bla*$_{CMY-2}$	-	

For Peer Review Only/Not for Distribution

25c	mouflon	AM-KF	*bla*$_{CMY-2}$	*astA*	VARIOUS
33a	wolf	-	*bla*$_{CMY-2}$	-	
34a	fox	AM-AMC-TE	*bla*$_{CMY-2}$*-aadA1-tet(B)*	-	
35a	wild boar	AM-S	*bla*$_{CMY-2}$	*astA*	VARIOUS
36a	wolf	-	-	*escV-eaeA*	aEPEC
37a	wild goat	-	*bla*$_{CMY-2}$	-	
40	mouflon	-	*bla*$_{CMY-2}$	*escV-eaeA*	aEPEC
43	wolf	KF	*bla*$_{CMY-2}$	*escV*	aEPEC
46	wild goat	-	-	*astA*	VARIOUS
47	wild goat	-	*bla*$_{CMY-2}$	*astA*	VARIOUS
49	fox	-	*bla*$_{CMY-2}$*-strA/strB-aadA1*	*astA*	VARIOUS
50	fox	AM-KF-SXT-S	*strA/strB-sul2*	*astA*	VARIOUS
52	fox	AM-AMC-KF-TE-SXT-ENR-CN-S	*strA/strB-tet(A)-tet(B)-sul2*	-	
54	wolf	AM-KF-TE-S	*strA/strB-tet(B)*	-	
58	hare	-	-	*astA*	VARIOUS
59	wolf	-	*bla*$_{CMY-2}$	*escV-eaeA*	aEPEC
61	wolf	AMC	*bla*$_{CMY-2}$	-	
63	fox	-	-	*escV-eaeA*	aEPEC
64	wolf	-	-	*escV-eaeA*	aEPEC
66	wild goat	AM-AMC-KF-TE-S	*strA/strB-tet(B)*	-	
68	red deer	-	-	*astA*	VARIOUS
72	hare	-	-	*astA*	VARIOUS
73	roe deer	-	-	*astA-stx2-hlyA*	EHEC
74	red deer	-	-	*astA*	VARIOUS

AM: amoxicillin; AMC: amoxicillin+clavulanic acid; KF: cephalothin; CTX: cefotaxime; TE: tetracycline; SXT: trimethoprim-sulfamethoxazole; ENR: enrofloxacin; CN: gentamicin; S: streptomycin

For Peer Review Only/Not for Distribution

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Tab 5 Virulence gene combinations (number and percentage of isolates) and associated putative pathovars detected among 71 *E. coli* isolates from wildlife.

Virulence gene combination	Number of isolates	Percentage of isolates (%)	Putative pathovars
No genes detected	45	63.4	-
astA	15	21.1	VARIOUS
escV - eaeA	7	9.9	aEPEC
escV	1	1.4	aEPEC
astA - escV - eaeA	1	1.4	aEPEC
astA - stx2 - hlyA	1	1.4	EHEC
astA - stx1 - stx2 - hlyA	1	1.4	EHEC

For Peer Review Only/Not for Distribution