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Fluoroquinolone-resistant and extended-spectrum beta-lactamase producing *Escherichia coli* isolates from free-living wild animals

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ABSTRACT

During the hunting season 2013–2014, fecal samples collected from hare, roe deer, deer and wild boars were sent to the bacteriology laboratory for the isolation of *Escherichia coli* and multidrug resistant isolates were characterized phenotypically and genotypically.

Out of 106 fecal samples, *E. coli* was isolated from 101 samples. Although the majority of isolates belonged to phylogenetic groups A and B1, 14 out of 101 isolates were affiliated to group B2. A multidrug resistance phenotype was determined in 7 isolates, all of which had distinguishable genomic macrorestriction profiles. PCR analysis and sequencing revealed a variety of resistance genes, gene cassettes and cassette arrays in these multidrug resistant isolates.

Resistance to fluoroquinolones was found in five *E. coli* isolates (two from a roe deer, one from a deer and two from a wild boar) and multiple mutations in the chromosomal topoisomerase genes were identified. In an *E.coli* isolate from a hare, the *qnrB19* gene was detected. The same isolate carried an *aadA23* gene cassette in class 1 integron. In addition, an extended- spectrum beta-lactamase $bla_{CTX\text{-}M\text{-}1}$ gene was detected in an *E. coli* isolate from a roe deer. The gene was located on a conjugative multi resistance plasmid, which was transferable to a plasmid free *E. coli* recipient.

In conclusion, a number of resistance genes and mobile genetic elements were detected in *E. coli* isolates from wildlife in Vojvodina, emphasizing the role of environmental pollution in spreading resistant bacteria.

1. Introduction

Antibiotic use in human and veterinary medicine leads to the selection of multidrug resistant (MDR) bacteria. Horizontal gene transfer associated with integrons, transposons, integrative and conjugative elements (ICEs) or plasmids enable the spread of resistance determinants and the survival of multidrug resistant bacteria in the environment and in different hosts. Of special concern is the combined resistance to important classes of antibiotics including the latest generation of penicillins and cephalosporins, carbapenems and fluoroquinolones (Levy and Marshall, 2004).

Escherichia coli (*E. coli*) are important commensal bacteria in the intestinal tract and, in addition, some *E. coli* are pathogenic and can cause diseases in both humans and animals (Kaper et al., 2004; Johnson et al., 2007). These bacteria frequently have acquired a number of resistance mechanisms including mechanisms mediating resistance to β-lactam antibiotics and quinolones (Dobiasova et al., 2013; Röderova et al. (2016)).

Wild birds present an extensive reservoir of multidrug resistant bacteria (Vittecoq et al., 2016). However, resistance to antibiotics in commensal and pathogenic isolates from free-living wild mamals is rarely investigated, so that only few reports are available in the literature (Radhouani et al., 2014).

Wild animals which have contact with waste or polluted waters become more frequently infected with the bacteria acquiring resistance to antibiotics (Radhouani et al., 2014). Other environmental factors

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such as agricultural practice, feeding, climate and migration, are also contributing to the dissemination of resistant bacteria in wildlife (Vittecoq et al., 2016). Therefore it is important to monitor health of wild animals to determine the possibility of disease transmissions, which may occur during the handling of hunted animals, food processing and preparation.

Presented in this work is the molecular characterization of multidrug resistant *E. coli* isolates from hare, roe deer, deer and wild boars hunted during 2013 and 2014 hunting seasons in the estates of the Autonomous Province of Vojvodina.

2. Material and methods

2.1. Hunting estates in the Province of Vojvodina

Seven *E. coli* isolates with multidrug resistant phenotype were found in animals hunted in four hunting estates located in the Province of Vojovodina (Supplementary Figure F1). The largest hunting estate *Vojvodina Sume* (VS) is funded by the Republic of Serbia. There are 17 open hunting grounds in VS established in the area covering 109 000 ha. Wild animals living in this nature reserve are as follows: European deer (*Cervus elaphus hippelaphus*), fallow deer (*Dama dama*), European mouflons (*Ovis orientalis musimon*), wild boar (*Sus scrofa*) and roe deer (*Capreolus capreolus*). Other hunting estates are maintained by local communities and like VS, are the members of the Hunting Association of Vojvodina. For this work the majority of samples were delivered from the following hunting grounds: Karakuša (intestinal samples from 23 wild boars and from two roe deer), Karađorđevo (intestinal samples from 15 wild boars and one sample from roe deer), Bački Petrovac (intestinal samples from 15 roe deer), Novi Sad (intestinal samples from seven roe deer, and one hare) and Kozara (three samples from deer and two samples from wild boar). One to four samples of intestines (total 37 samples) were collected from hunted animals in other hunting grounds (in total 14) in the Province of Vojvodina.

2.2. Sampling of feces and E. coli identification

In total, 106 samples of large intestines, tied at both ends, were collected from wild animals during evisceration process and delivered to the laboratory at the temperature of 4 °C. Swabs were taken from the intestine to perform the isolation and identification of *E. coli*. The pure culture of presumptive *E. coli* was subjected to identification applying BBL Crystal Enteric/Nonfermenter ID kit (Becton, Dickinson and Company, Sparks, USA) and by PCR analysis of the *gadA/B* gene encoding glutamate decarboxylase, which is highly specific for *E. coli* (McDaniels et al., 1996).

2.3. Determination of resistotype and minimal inhibitory concentration (MIC) values of antibiotics

The determination of antimicrobial susceptibility patterns was performed using the agar disk diffusion method as recommended by the Clinical and laboratory Standards Institute (2015a,b) (documents CLSI, M07-A10 and M100-S25). The following antibiotic disks were included in the resistotyping: ampicilin 10 µg (AMP), amoxicillin/clavulanic acid 20 µg + 10 µg (AMC), cefpodoxime 10 µg (CPD), cefotaxime 30 µg (CTX), ceftazidime 30 µg (CAZ), ciprofloxacin 5 µg (CIP), chloramphenicol (CHL) 30 µg, gentamicin 10 µg (GEN), nalidixic acid 30 µg (NAL), streptomycin 10 µg (STR), sulphonamides 300 µg (SA), tetracycline 30 µg (TET), trimethoprim 5 µg (TMP) and trimethoprim/sulfamethoxazole 1.25 µg + 23.75 µg (SXT), (BioRad, Marnes-la-Coquette, France). In addition, MIC values were determined by the broth microdilution method as recommended in the CLSI document M07-A10. For quality control purposes, the *E. coli* strain ATCC 25,922 was used.

2.4. DNA isolation and polymerase chain reaction (PCR)

In all experiments, DNA isolation was done by the boiling procedure (5 min at 100 °C). A master mix with hot start Taq polymerase was prepared according to the recommendation of the manufacturer (Qiagen, Hilden, Germany). The thermal cycler TECHNE (Bibby Scientific LTD, UK) was used for the amplification of PCR products. Electrophoresis was run in a 2% agarose gel, stained with ethidium bromide and visualized in a UV transilluminator (Vilber Lourmat, Cedex, France). The primers and annealing temperatures for phylogenetic typing, identification of *E. coli* and resistance gene detection are displayed in Supplementary Table S1. Sequencing of the topoisomerase genes (*gyrA*, *gyrB*, *parC* and *parE* genes) of the bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$ gene and the *qnrB* gene was done at the companies Macrogen/Holland and Eurofins. The primer walking sequencing method was used for integron analysis. The alignment analyses were done using the BioEdit and Blast search programs and also applying the software program DNAMAN version 7 (Lynnon Corporation, Quebec, Canada).

2.5. Pulsed-field-gel electrophoresis (PFGE)

The one-day (24–26 h) standardized laboratory protocol for molecular subtyping of *E. coli* O157:H7, *E. coli* Non-O157 (STEC), *Salmonella*, *Shigella sonnei* and *Shigella flexneri* by pulsed-field gel electrophoresis (PFGE), was used (http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL05_Ec-Sal- ShigPFGEprotocol.pdf). Electrophoresis was run in a CHEF DR-III (BioRad Laboratories, Hercules, CA) instrument. Fingerprints were obtained from the separation of *XbaI* (Thermo Scientific, Vilnus, Lithuania) digested genomic DNA. *Salmonella enterica* serotype Braenderup H9812 was used as a molecular size standard.

2.6. Mating experiments

Mating experiments were done with the *E. coli* recipient strain HK225 in Luria Bertani broth (Becton Dickinson, Le Pont de Claix, France). Transconjugants were selected with 100 mg/L of ampicilin or tetracycline and rifampicin after shaking bacteria over night at 37 °C. The macrorestriction analysis of genomic DNA by pulsed-field-gel electrophoresis was done for the transconjugants, donors and the respective recipient strain and analyzed to confirm the success of the mating experiment.

3. Results and discussion

From 106 samples of feces, *E. coli* was isolated from 101 samples. Seven isolates exhibited multidrug resistant phenotype as they were resistant to three or more antibiotics.

Most *E. coli* isolates from wildlife belong to the phylogenetic groups A and B1. In contrast, the majority of the isolates from wild boars belong to group D and, altogether, fourteen isolates were assigned to phylogenetic group B2 (Fig. 1). Among isolates with multidrug

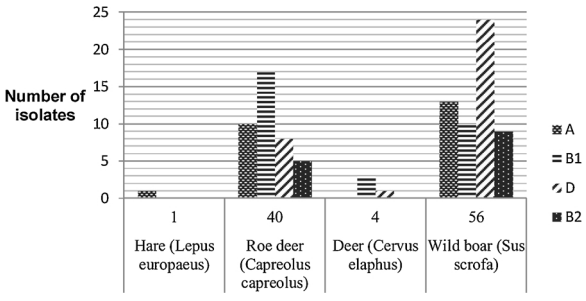

Fig. 1. Sample distribution based on phylogenetic group and origin species.

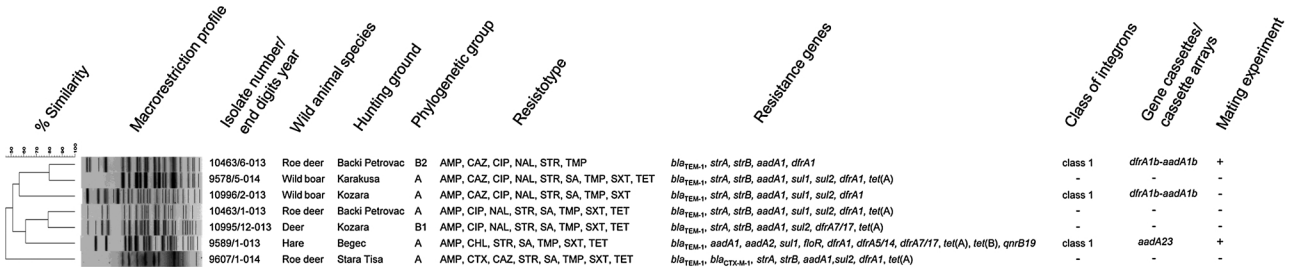

Fig. 2. Macrorestriction profiles, phylogenetic groups, resistance phenotype and genotype in *E. coli* isolates from wildlife.

resistance patterns, only one isolate from a roe deer (isolate number 10463/6) belongs to the B2 phylogenetic group. *E. coli* isolates from wild boars in Portugal exhibiting resistance to quinolones and producing an extended-spectrum β-lactamase were assigned to the B2 phylogenetic group as well (Poeta et al., 2009). Furthermore, nearly 50% of *E. coli* isolates from wild animals in Guiana are characterized as B2 group. Multi locus sequence typing revealed that isolates from wildlife were genetically more distant when compared to isolates from humans and domestic animals (Lescat et al., 2013).

Multidrug resistant isolates showed distinguishable PFGE patterns (Fig. 2).

However, it is difficult to determine the clonal relationship of *E. coli* in wildlife, even if animals share the same natural habitat. In an investigation conducted in the Czech Republic, *E. coli* isolates from gulls residing in a pond near Ostrava were compared with isolates from water samples by PFGE analysis. Although some isolates from gulls, which had the same resistance phenotype were clonally related, genetic relatedness with isolates from water samples was not observed (Dolejská et al., 2009).

Resistance to fluoroquinolones was found in five *E. coli* isolates (two from a roe deer, one from a deer and two from a wild boar) and multiple mutations in the chromosomal topoisomerase genes were identified. In isolates from roe deer (10463/1-013, 10463/6-013) and from a wild boar (9578/5-014), base pair exchanges leading to the amino acid exchanges Ser83→Leu, Asp87→Asn in GyrA and Ser80→Ile and Glu84→Ala in ParC were detected. In an isolate from a deer (10995/12-013) base pair substitutions located in the genes encoding the GyrA (Ser83→Leu, Asp87→Asn), ParC (Ser80→Ile) and ParE (Ser458→Thr) proteins were found. Multiple mutations in topoisomerase genes were also present in an isolate from a wild boar (10996/2-013) which resulted in the following amino acid exchanges: Ser83→Leu, Asp87→Asn in GyrA, Ser80→Ile and Glu84→Ala in ParC and Ser458→Thr in ParE (Table 1). Furthermore, it was identified that in the *E. coli* isolate from a hare (9589/1-013), the plasmid-mediated resistance gene *qnrB19* was responsible for the increased MIC of ciprofloxacin (MIC- 0.25 mg/L) (Supplementary Table S2).

To the best of our knowledge, this is one of a few reports on fluoroquinolones resistance in *E. coli* isolates from roe deer. For instance, in a hunting estate within a natural park situated in Southern Spain, a multidrug resistant *E. coli* from a farm deer (*Cervus elaphus*) exhibiting resistance to ciprofloxacin was reported (Alonso et al., 2016). Lonacaric et al. (2016) detected an ESBL phenotype in *E. coli* isolate no. 928 from a mouflon that was found dead in the Donau-Auen National Park, Austria, which also exhibited resistance to fluoroquinolones. Mutations found in the quinolone resistance determining region (QRDR region) in isolate 928 were similar to the mutations found in *E. coli* isolates in the course of the present study and also in a study conducted by Alonso et al (2016). A frequent occurrence of fluoroquinolone resistance was recently reported in *E. coli* isolates from bovine mastitis and pigs in the Vojovodina (Todorović et al., 2018), which suggest an overuse of fluoroquinolone antibiotics in livestock industry in Serbia.

The $bla_{\text{TEM-1}}$ gene was identified in all multidrug resistant isolates from wild animals which were resistant to ampicillin (Fig. 2). Resistance to extended-spectrum β- lactam antibiotics was determined by applying a phenotypic confirmatory test (cefotaxime and ceftazidime disk with and without clavulanic acid) to an *E. coli* isolate from a roe deer (isolate number 9607/1-014) followed by PCR analysis and sequencing and subsequently, the $bla_{\text{CTX-M-1}}$ gene was identified. The respective gene was detected on a conjugative plasmid together with the *sul2* and *tet*(A) genes. The plasmid conferred resistance to ampicillin, cefotaxime, ceftazidime, sulfonamides and tetracycline (Table 2). The enclosure of these complex genetic structures into well-established plasmids in different environment might have facilitated the spread of $bla_{\text{CTX-M}}$ (Bevan et al., 2017)

In the single isolate obtained from a hare, the dihydrofolate reductase genes, *dfrA1*, *dfrA5/14* and *dfrA7/17* were detected concomitantly. A combination of dihydrofolate reductase genes is often identified in isolates from livestock (Frech et al., 2003; Blahna et al., 2006). In the *E. coli* isolate from hare resistance to streptomycin was encoded by the *aadA1* and *aadA2* genes, while an *aadA23* (streptomycin and spectinomycin resistance) gene cassette was found to be located in class 1 integron (GenBank accession number KU956959). The *aadA23* gene cassette variant was detected for the first time in a *Salmonella enterica* subsp. *enterica* serovar Agona isolate from pig carcasses sampled in a slaughterhouse in Southern Brazil (Michael et al., 2005). After more than ten years, this cassette was found again in class 1 integron in six *Salmonella enterica* serovar Typhimurium isolates from pig carcasses and intestinal content in abattoirs from the same area (Lopes et al., 2016). Resistance to florfenicol, sulfonamides and tetracycline in *E. coli* isolate from hare was encoded by the following resistance genes: *floR*, *sul1*, *tet*(A) and *tet*(B) (Fig. 2). Most of the resistance genes including the integron 1, but not the *dfrA7/17*, *tet*(B) and *qnrB* genes were

Table 1
MIC values of CIP for *E. coli* isolated from wildlife and mutations in the topoisomerase genes.

Isolates and origins	MIC-CIP mg/L	*gyrA*	*gyrB*	*parC*	*parE*	PMQR genes
9589/1-013, Hare	0.25	wt	wt	wt	w	*qnrB19*
10463/1-013 Roe deer	> 128	Ser83Leu Asp87Asn	wt	Ser80Ile Glu84Ala	wt	–
10463/6-013 Roe deer	16	Ser83Leu Asp87Asn	wt	Ser80Ile Glu84Ala	wt	–
9607/1-014 Roe deer	0.062	wt	wt	wt	wt	–
10995/12-013 Deer	> 128	Ser83Leu Asp87Asn	wt	Ser80Ile	Ser458Thr	–
9578/5-014 Wild boar	> 128	Ser83Leu Asp87Asn	wt	Ser80Ile Glu84Ala	wt	–
10996/2-013 Wild boar	16	Ser83Leu Asp87Asn	wt	Ser80Ile Glu84Ala	Ser458Thr	–

CIP = ciprofloxacin; wt = wild type; PMQR = plasmid-mediated quinolone resistance; No PMQR genes found (-).

Table 2
Resistance geno- and phenotypes of *E. coli* transconjugants from wildlife.

Isolates and origins	Resistance phenotype in donor	Resistance genes in donor	Resistance phenotype in transconjugant	Resistance genes in transconjugant
9589/1-013, Hare	AMP, CHL, STR, SA, TMP, SXT, TET	bla_{TEM-1}, *aadA1*,*aadA2*, *sul1*, *floR*, *dfrA1*,*dfrA5/14*, *dfrA7/17*, *tet*(A), *tet(B)*, *qnrB19*	AMP, CHL, STR, SA, TMP, SXT, TET	bla_{TEM-1}, *floR*, *sul1*, *dfrA1*,*dfrA5/14* *aadA1*,*aadA2*, *tet*(A), integron 1
9607/1-014, Roe deer	AMP, CTX, CAZ, STR, SA, TMP, SXT, TET	bla_{TEM-1}, $bla_{CTX-M-1}$, *strA*, *strB*, *aadA1*, *sul2*, *dfrA1*, *tet(A)*	AMP, CTX, CAZ, SA, STR, TET	$bla_{CTX-M-1}$, *sul2*, *tet*(A)

AMP = ampicillin; CHL = chloramphenicol; CTX = cefotaxime;CAZ = ceftazidime; STR = streptomycin; SA = sulfonamides; TMP = trimethoprim ;SXT = sulfamethoxazole/trimethoprim; TET = tetracycline.

detected on conjugative plasmids (Table 2).

Two isolates from roe deer (10463/1-013 and 10463/6-013) from the hunting estate Backi Petrovac, carried *strA/strB*, *aadA1*, and *dfrA1* genes. In addition, *sul1* and *sul2* genes conferring resistance to sulfonamides and a *tet*(A) gene, which mediates resistance to tetracycline, were identified in isolate 10463/1-013. Furthermore, isolate 10463/6-013 harbors a gene cassette array *dfrA1b-aadA1b* located in a class 1 integron (GenBank accession number MG712782). Both isolates from wild boars (9578/5-014 and 10996/2-013) carried *strA/strB, aadA1*, *sul1*, *sul2* and *dfrA1* genes and isolate 9578/5-014 additionally carried a *tet*(A) gene. In the wild boar isolate 10996/2-013, class 1 integron 1 was detected and sequencing revealed that an *dfrA1b-aadA1b* gene cassette array was integrated within this integron1 (GenBank accession number MG712783). An isolate from a deer (10995/12-013) carried similar resistance genes, but resistance to trimethoprim was encoded by a *dfrA7/17* gene (Fig. 2).

4. Conclusion

High level resistance to fluoroquinolones was detected in five *E. coli* isolates from wildlife in the Province of Vojovodina. In addition, many other resistance genes were identified on mobile genetic elements (conjugative plasmids and integrons) in two and three isolates, respectively. In conclusion, it has been shown that significant resistance mechanisms and genes mediating resistance to clinically important antimicrobial agents are present in an environment with only low or without selective pressure. As reasons for the occurrence of resistance in *E. coli* from free-living wild animals, natural conditions and human activities responsible for environmental pollution can be assumed.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.08.011.

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