

Antimicrobial-resistant bacteria in wild game in Slovenia

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Abstract. Wildlife is usually not exposed to clinically-used antimicrobial agents but can acquire antimicrobial resistance throughout contact with humans, domesticated animals and environments. Samples of faeces from intestines (80 in total) were collected from roe deer (52), wild boars (11), chamois (10) red deer (6) and moufflon (1). After culture on ChromID extended spectrum β -lactamase (ESBL) plates to select for growth of ESBL-producing bacteria, 25 samples produced bacterial colonies for further study. Six species of bacteria were identified from the 25 samples: *Stenotrophomonas maltophilia*, *Serratia fonticola*, *Stenotrophomonas nitritireducens*, *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli*. Two ESBL enzymes were amplified from group TEM and three from group CTX-M-1. Undercooked game meat and salami can be a source of resistant bacteria when animals are not eviscerated properly.

1. Introduction

The mere occurrence of antimicrobial resistance and corresponding resistance genes in the environment is an ancient phenomenon, due to the simple fact that most of the antimicrobial substances currently in use are based on natural compounds produced by soil bacteria like streptomycetes [1]. The function of these antibiotics was presumably more related to microbial competition for ecological niches, than the role they play today in clinical settings [2,3,4].

While various bacterial species are important in terms of multiresistance and nosocomial infections in humans and veterinary medicine, we consider extended-spectrum β -lactamase (ESBL)-producing Gram-negative bacteria like *Escherichia coli* as being key indicator pathogens to trace the evolution of multiresistant bacteria in the environment and wildlife. These multiresistant bacteria have also made their way into livestock farming and companion animals [5,6,7]. Although so far it is not clear how ESBL-*E. coli* make their way into the natural environment, it seems unlikely that pathogens isolated from wildlife have acquired resistance through new parallel mutations in respective genes. Horizontal transfer of resistance genes from clinical isolates or the intake of already resistant bacteria from human waste, sewage, or domesticated animal manure might be more probable [8,3]. Antimicrobial-resistant *E. coli* isolates originating from wildlife species were reported for the first time at the beginning of the 1980s from Japanese wild birds [9,10,11]. However, the detection of ESBL-*E. coli* from wild boar was reported from Portugal and Czech Republic [12,13].

Much of the Slovenian countryside (58.3%) is covered with forest, which is why wild game presence near farms is not unusual. In 2015, according to the Republic of Slovenia Statistical Office, hunters killed 33,668 roe deer, 8,367 wild boars, 6,064 red deer and 2,302 chamois, among other wild game species. In Slovenia, game meat is treated as high quality meat and is often served as a speciality



in restaurants in the form of meat dishes and dried game meat products. For these reasons, in our study we analysed 80 samples of intestinal content of game animals for the presence of antimicrobial-resistant bacteria and resistance patterns.

2. Material and methods

2.1. Sample collection

In 2014 and 2015, a survey throughout Slovenia was performed to screen certain game animals as a potential source of enteric viruses as well as antibiotic-resistant bacteria. In total, 80 samples of game animal intestinal content were collected from five different wildlife species, comprising 52 samples from roe deer (*Capreolus capreolus*), 11 from wild boars (*Sus scrofa*), 10 from chamois (*Rupicapra rupicapra*), 6 from red deer (*Cervus elaphus*) and 1 from moufflon (*Ovis musimon*). The age of the game animals was from 5 months to 10 years and they were culled by five Slovenian hunting families between July 2014 and March 2015. Samples of intestinal content of each animal were collected by hunters after culling. The lower part of each intestine was placed in a sterile plastic bag. Samples were stored at -20°C and, as soon as possible, sent to the Veterinary Faculty where they were stored below -60°C until use.

2.2. Bacterial isolation and identification

Suspensions (10%) of the intestinal contents were prepared in RPMI-1640 (Thermo Fisher Scientific, Carlsbad, CA, USA) and centrifuged for 10 min at 1000 g. Volumes (0.1 ml) of each supernatant were added to 0.9 ml of peptone water (Buffered Peptone Water, Biolife, Italy) and mixed on a vibromix (Tehtnica, Železniki, Slovenia). Mixtures were then incubated for enrichment at 37°C for 16-20 h. Subsequently, enriched mixtures were inoculated, using 10 µl sterile disposable sampling loops, onto selective ChromID ESBL agar plates (BioMerieux, Marcy l'Etoile, France) and incubated for 24 h at 37°C. Representative colonies from all plates showing bacterial growth were inoculated onto blood agar (Blood agar base No.2, Oxoid, Hampshire, United Kingdom, supplemented with 5% ovine blood) and incubated for 24 h at 37°C. The next day, bacterial isolates were confirmed by matrix-assisted laser desorption/ionization, time of flight (MALDI-TOF, Bruker Daltonics, Bremen, Germany) and mass spectrometry [14].

2.3. Characterization of β -lactamases

β -lactamase resistance genes, *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}, were screened by PCR [15,16]. The multiplex PCR method described by Woodford [16] did not work properly; therefore, we used the same primers in five singleplex reactions for each *bla*_{CTX-M} group (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25), respectively. The PCR products were analysed using electrophoresis on 2.0% agarose gels.

3. Results

3.1. Bacterial isolation and identification

Among 80 samples of intestinal contents, 25 (31.3%) exhibited bacterial growth on ChromID ESBL plates. Most positive samples belonged to roe deer 16 (61.5%), followed by wild boar 7 (25.9%) and red deer 2 (7.4 %). According to MALDI-TOF and mass spectrometry, bacterial cultures on ChromID ESBL agar plates belonged to six different bacterial species *Stenotrophomonas maltophilia*, *Serratia fonticola*, *Stenotrophomonas nitritireducens*, *Enterococcus faecium*, *Enterococcus faecalis* and *E. coli*.

3.2. Detection of ESBL-producing bacteria and characterisation of ESBL

Among the 25 positive bacterial isolates on ChromID ESBL plates, two (2.5%) were positive for the ESBL enzyme group, TEM. Both isolates originated from samples collected from wild boars; one

isolate was identified as *Enterococcus faecium* and the other as *Enterococcus faecalis*. Both intestinal content samples came from the same hunting family, based in the town of Škofja Loka. In Figure 1, typical positive PCR products are shown.

Bacteria producing ESBL from the ESBL enzyme group CTX-M-1 were detected in 4 out of 80 (5.0%) samples of intestinal contents. All isolates were obtained from intestinal contents of roe deer. Two isolates were identified as *Serratia fonticola*, one as *Stenotrophomonas maltophilia* and one as *Stenotrophomonas nitritireducens*. Three of these intestinal content samples were collected by the hunters from Škofja Loka while one sample came from a hunting family from Polhov Gradec (Table 1). Both hunting families hunt in adjoining territories, on the terrain of central and Gorenjska regions.

Table 1 Overview of the detected ESBL-producing isolates.

Isolate number	Animal species	Appearance of isolates on ChromID ESBL plate	MALDI –TOF identification	ESBL enzyme group	Hunting family
17/14	Roe deer	Blue colonies	<i>Serratia fonticola</i>	CTX-M-1	Škofja Loka
18/14	Roe deer	Blue colonies	<i>Serratia fonticola</i>	CTX-M-1	Škofja Loka
20/14	Roe deer	Yellow colonies	<i>Stenotrophomonas nitritireducens</i>	CTX-M-1	Škofja Loka
30/14	Wild boar	White-blue colonies	<i>Enterococcus faecium</i>	TEM	Škofja Loka
34/14	Wild boar	White-blue colonies	<i>Enterococcus faecalis</i>	TEM	Škofja Loka
67/14	Roe deer	White colonies	<i>Stenotrophomonas maltophilia</i>	CTX-M-1	Polhov Gradec

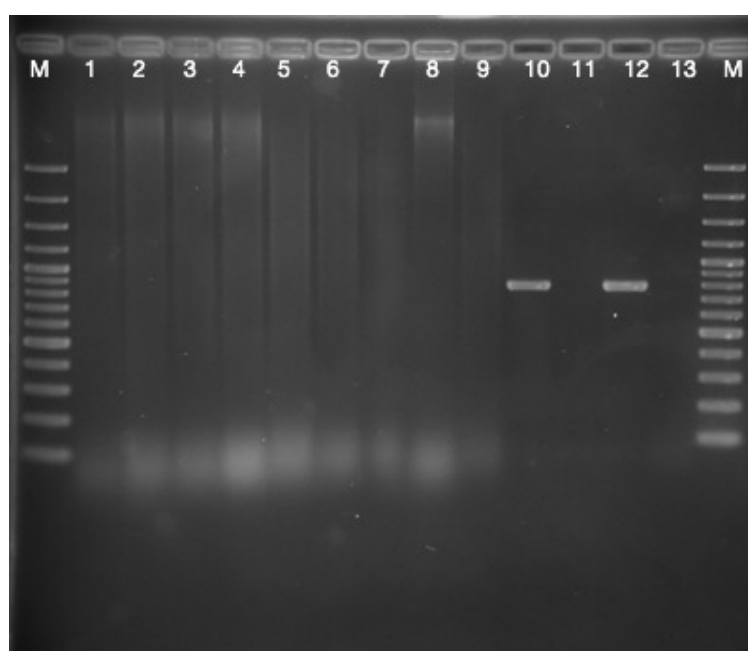


Figure 1. Agarose gel electrophoresis of amplified DNA of *bla*_{TEM} gene of *Serratia fonticola* on 2% agarose gel. (line 10 (30/14) and 12 (34/14) amplified *bla*_{TEM}); DNA marker contains bands (from bottom to the top) of 100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp, 1200bp, 1500bp, 2000bp and 3000bp.

4. Discussion

The results show that ESBL-producing bacteria are present in wild animals. However, they reveal that carriage of these multiresistant bacteria is not widespread among wild game in Slovenia. We cultivated 25 (31.3%) multiresistant bacterial isolates that grew on ChromID ESBL agar plates, and only in five isolates was a resistance gene amplified. The reasons could be that the resistance is chromosomal in origin or some new genes are involved. Most of our multiresistant isolates were common soil bacteria; however, we isolated also two strains of *S. fonticola*. Some species of the genus *Serratia* have medical significance as opportunistic pathogens [17,18]. In addition, we isolated *E. faecium*, which can be commensal in the human intestine but it can also be pathogenic, causing diseases such as neonatal meningitis or endocarditis. From the genus *Enterococcus*, we also isolated *E. faecalis*. *E. faecalis* is a commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. However, it can cause life-threatening infections in humans, especially in the nosocomial (hospital) environment, where the naturally high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity [19].

The results show that undercooked game meat and dried game meat products could be a source of multiresistant bacteria if evisceration of game is not conducted properly in the field. In addition, a further study on antimicrobial-resistant bacteria in game meat should be performed to determine the true prevalence of multiresistant bacteria with greater certainty.

5. Conclusion

As previously suggested, thorough spatial and temporal studies of antimicrobial resistance in different natural habitats are warranted [20,21] to fully understand the importance of wildlife as a source of antimicrobial drug resistance.

In our study, we only determined the ESBL enzyme group. Therefore, the next step is to perform nucleotide sequencing from these PCR products from the 25 bacterial isolates to determine the nature of their antibiotic resistance genes.

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References

- [1] D'Costa V M, et al 2011 Antibiotic resistance is ancient. *Nature* **477** 457
- [2] Martinez J L 2009a Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* **157** 2893
- [3] Martinez J L 2009b The role of natural environments in the evolution of the resistance traits in pathogenic bacteria. *Proc. Biol. Sci.* **276** 2521
- [4] Allen H K, Donato J, Wang H H, Cloud-Hansen K A, Davies J and Handelsman J 2010 Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* **8** 251
- [5] Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, Haesebrouck F and Butaye 2010 Broad-spectrum beta-lactamases among *Enterobacteriaceae* of animal origin:

- molecular aspects, mobility and impact on public health. *FEMS Microbiol. Rev.* **34** 95
- [6] Ewers C, Grobbel M, Bethe A, Wieler L H and Guenther S 2011 Extended-spectrum beta-lactamases-producing gram-negative bacteria in companion animals: action is clearly warranted! *Berl. Munch. Tierarztl. Wochenschr.* **124** 10
 - [7] Wieler L H, Ewers C, Guenther S, Walther B and Lübke-Becker A 2011 methicillin-resistant staphylococci (MRS) and extended spectrum-beta lactamases (ESBL)-producing *Enterobacteriaceae* in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. *Int. J. Med. Microbiol.* **301** 635
 - [8] Kummerer K 2009 Antibiotics in the aquatic environment – a review – part I. *Chemosphere* **75** 417
 - [9] Sato G, Oka C, Asagi M and Ishiguro N 1978 Detection of conjugative R plasmids conferring chloramphenicol resistance in *Escherichia coli* isolated from domestic and feral pigeons and crows. *Zentralbl. Bakteriol. Orig.* **241** 407
 - [10] Kanai H, Hashimoto H and Mitsuhashi S 1981 Drug resistance and conjugative R-plasmids in *Escherichia coli* strains isolated from wild birds (Japanese tree sparrows, green pheasants and bamboo partridges). *Jpn. Poult. Sci.* **18** 234
 - [11] Tsubokura M, Matsumoto A, Otsuki K, Animas S B and Sanekata T 1995 Drug resistance and conjugative R plasmids in *Escherichia coli* strains isolated from migratory waterfowl. *J. Wildl. Dis.* **31** 352
 - [12] Poeta P, et al., 2009 Wild boars as reservoirs of extended-spectrum beta-lactamases (ESBL) producing *Escherichia coli* of different phylogenetic groups. *J. Basic Microbiol.* **49** 584
 - [13] Literak I, Dolejska M, Radimersky T, Klimes J, Friedman M, Aarestrup F M, Hasman H and Cizek A 2009 Antimicrobial-resistant fecal *Escherichia coli* in wild mammals in central Europe: multiresistant *Escherichia coli* producing extended-spectrum beta-lactamases in wild boars. *J. Appl. Microbiol.* **108** 1702
 - [14] Kohlmann R, Hoffmann A, Geis G and Getermann S 2015. MALDI-TOF mass spectrometry following short incubation on a solid medium is a valuable tool for rapid pathogen identification from positive blood cultures. *Int. J. Med. Microbiol.* **305** 469
 - [15] Dallenne C, Da Costa A, Decré D, Favier C and Arlet G 2010 Development of a set of multiplex PCR assays for the detection of genes encoding important β -lactamases in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* **65** 490
 - [16] Woodford N 2010 Rapid characterization of beta-lactamases by multiplex PCR. *Methods Mol. Biol.* (Clifton, N.J.) **642** 181
 - [17] Farmer J J, Davis B R, Hickman-Brenner F W, McWhorter A, Huntley-Carter G P, Asbury M A, Riddle C, Wathen-Grady H G, Elias C and Fanning G R 1985 Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21** 46
 - [18] Bollet C, Gainnier M, Sainty J M, Orhesser P and De Micco P 1991 *Serratia fonticola* isolated from a leg abscess. *J. Clin. Microbiol.* **29** 834
 - [19] Agudelo Higueta N I, Huycke M M 2014 Enterococcal Disease, Epidemiology, and Implications for Treatment. In: Gilmore MS, Clewell DB, Ike Y, et al., editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary; 2014. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK190429/>
 - [20] Gilliver M A, Bennett M, Begon M, Hazel S M and Hart C A 1999 Antibiotic resistance found in wild rodents. *Nature* **401** 233
 - [21] Hernandez J, Bonnedahl J, Eliasson I, Wallensten A, Comstedt P, Johanson A, Granholm S, Melhus A, Olsen B and Drobni M 2010 Globally disseminated human pathogenic *Escherichia coli* of O25b-ST131 clone, harbouring blaCTX-M15, found in glaucous-winged gull at remote Commander Islands, Russia. *Environ. Microbiol. Rep.* **2** 329