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Original Research

Antimicrobial Resistance of Enterococci from Wild Animals in Slovakia

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Abstract

The spread of antibiotic resistant strains is not limited to the clinical environment, but the emergence of resistant bacteria and antibiotic resistance genes in the environment has now become one of the environmental pollution factors. As a model organism for the study of the spread of antibiotic resistance genes, which are a part of digestive tract, we decided to use the genus of *Enterococcus* isolated from wild living animals in Slovakia to perform screening for the presence of antibiotic resistance. In our work, two hundred and eighty- three isolates were analysed. Among isolates the *Enterococcus faecalis* (67.1%) followed by *E. hirae* (15.9%), *E. faecium* (6.4%), *E. casseliflavus* (4.2%), *E. durans* (3.5 %) and *E. mundtii* (2.8%) species dominated. The most frequently resistance to tetracycline (29.3%) and erythromycin (15.9%) was detected. In birds and mammals a similar frequency of resistant enterococci was observed. The differences in antimicrobial resistance to ampicillin and vancomycin were observed. Higher prevalence of ampicillin resistant isolates was detected in birds. On the other hand, vancomycin resistant enterococci were detected in mammals but not in birds. The presence of selected antimicrobial resistance genes was studied by PCR with tet(M) and erm(B) genes being to be the most frequently encountered. Vancomycin resistant enterococci harboured only van(C1) gene. The occurrence of antimicrobial resistance in enterococci from the digestive tract of wild living animals suggests the genetic pollution of environment which could pose a risk for human and animal health.

Keywords: *enterococci*, wild-living animals, antimicrobial resistance, resistance genes

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Introduction

Antibiotics are among the most successful and the most frequently used drugs in the treatment of diseases in humans and animals. They are naturally occurring substances produced by various microorganisms that can either directly kill other bacteria or stop their growth. Nowadays, antibiotics are losing their efficacy against bacteria and return to the pre-antibiotics era is very real. Antibiotic resistance of enterococci is point of special interest. Enterococci are the facultative anaerobic gram-positive bacteria commonly found in the human and animal gastrointestinal tract and in the environment. These bacteria are considered as indicators of faecal pollution and important nosocomial pathogens. The importance of enterococci is due to their ability to adapt and survive the wide variety of growth conditions, including temperatures from 10ºC to 45ºC, high and low salts and pH environments. Among the most distinct examples of enterococcal adaptability is the rapid acquisition of the antibiotic resistance genes. When enterococci become resistant to antibiotics, therapy may be difficult, and the diseases can be fatal. Antimicrobial resistant enterococci are very commonly found not only in the clinical environment [1] but also in farm [2], probably due to antibiotics use as feed additives in agriculture, especially in livestock farming. Waste from farms and hospitals may be contaminated either directly or by genes encoding antimicrobial resistance, which can pollute the environment [3]. The use of antibiotics as growth promoters in animal feed has been banned in the EU since 2006, however, the excessive use of antibiotics seems to persist permanently, covering also other environments and ecological niches that are not subjected to direct human action [4]. In such way, enterococci of animal origin might constitute a human hazard in themselves, but they could act as donors of antimicrobial resistance genes for other pathogenic enterococci as well [5].

The aim of the present study was to examine the distribution of enterococci in faeces of wild living animals in natural environments and to investigate the presence of antimicrobial resistance determinants in these microorganisms in Slovakia.

Material and Methods

Origin of Samples

The samples were taken either from animals living in agricultural lowland regions of Slovakia or from high altitude areas (over 1000 meters above sea level) in Tatra National Park and Low Tatras National Park. The animals lived in freedom and it is supposed that they had never received any feed or antimicrobials from humans during their lifetime. As an isolation source of enterococci from bats a sample of guano from Benus village (Banska Bystrica district, Slovakia) was used.

The samples were collected as soon as possible after defecation and transported to the laboratory under aseptic conditions. The average time between defecation and analysis of sample was usually less than 12 hours. Samples were taken from *faeces* of Eurasian beaver (*Castor fiber*, 8 isolates), white stork (*Ciconia ciconia*, 7 isolates), snow vole (*Chionomys nivalis*, 10 isolates), red deer (*Cervus elaphus*, 10 isolates), mallard (*Anas platyrhynchos*, 10 isolates), European pine marten (*Martes martes*, 10 isolates), red fox (*Vulpes vulpes*, 10 isolates), brown bear (*Ursus arctos*, 7 isolates), house mouse (*Mus musculus*, 10 isolates), common shrew (*Sorex araneus*, 9 isolates), Eurasian jay (*Garrulus glandarius*, 9 isolates), tawny owl (*Strix aluco*, 9 isolates), roe deer (*Capreolus capreolus*, 10 isolates), alpine marmot (*Marmota marmota*, 10 isolates), Tatra pine vole (*Microtus tatricus*, 10 isolates), Tatra chamios (*Rupicapra rupicapra tatrica*, 10 isolates), European mouflon (Ovis aries musimon, 10 isolates), striped field mouse (*Apodemus agrarius*, 10 isolates), yellow necked mouse (*Apodemus flavicollis*, 10 isolates), Ural field mouse (*Apodemus uralensis*, 10 isolates), common vole (*Microtus arvalis*, 10 isolates), rook (*Corvus frugilegus*, 8 isolates), Eurasian jackdaw (*Corvus monedula*, 10 isolates), rock dove (*Columba livia*, 10 isolates), European otter (*Lutra lutra*, 7 isolates), common pheasant (*Phasianus colchicus*, 10 isolates), black redstart (*Phoenicurus ochruros*, 10 isolates), fieldfare (*Turdus pilaris*, 9 isolates), house sparrow (*Passer domesticus*, 10 isolates) and bat guano (10 isolates). In total, 283 *Enterococcus* spp. isolates were selected for subsequent analyses.

Isolation and Identification

Faecal samples or sample of guano (of about 0.5 g) were resuspended in sterile PBS solution by intensive mixing for 20 minutes. Aliquots were spread on selective agar medium for enterococci (Bile Esculin Agar, Becton Dickinson, USA) and cultivated under aerobic conditions at 37°C for 24 h. Up to 10 morphological relevant isolates from tested individuals were randomly selected for further analysis. Selected isolates were identified by Microflex LT Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Samples for analysis were prepared by ethanol/formic acid extraction. Procedure was followed according to the manufacturer´s instruction. Results were evaluated using the software FlexControl-microflex 3.0 (Bruker Daltonics) without any user intervention.

Antimicrobial Resistance Analysis

Antimicrobial resistance to ampicillin, erythromycin, tetracycline, and vancomycin was screened by the disc diffusion method on Mueller-Hinton Agar (Oxoid, UK). Minimal breakpoint concentrations (MIC) of antibiotics

were based on Clinical and Laboratory Standards Institute guidelines (CLSI 2013) [6]. Differences in resistance between groups of isolates (birds versus mammals) were evaluated using chi-square test with a 5% significance level [7].

DNA Isolation and Detection of Antibiotic Resistance Genes

Total genomic DNA of enterococcal isolates was extracted by the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) from overnight cultures grown in Todd-Hewitt broth. Antibiotic resistance genes were detected using polymerase chain reaction (PCR). The oligonucleotide primer pairs used to amplify the genes *erm*(B), *mef*(A), *tet*(L), *tet*(M), *van*(A), *van*(C1) are listed in Table 1. As a positive control in PCR detection of resistance determinants strains according to Stovcik et al. (2008) [8] were used. PCR reactions were performed in Bio-Rad MJ Mini Personal Thermal Cycler (Bio-Rad, USA). DNA samples were tested by agarose gel (1%) electrophoresis (Bio-Rad, USA).

Results and Discussion

Five different species of enterococci were identified among 283 *Enterococcus* spp. isolates from *faeces* of wild living animals using selective cultivation media followed by MALDI-TOF analysis. This method was found to be reliable for identification of enterococci from both clinical and environmental samples as well [9].

The most common species found were *Enterococcus faecalis* (67.1%) and *Enterococcus hirae* (15.9%). Less common were *Enterococcus faecium* (6.4%), *Enterococcus casseliflavus* (4.2%), and *Enterococcus durans* (3.5%). Our observations are in line with the findings of Gonçalves et al. (2013) [10], who studied enterococci originating from Iberian lynx and Stępień-Pyśniak et al. (2018) [9] who studied enterococci from

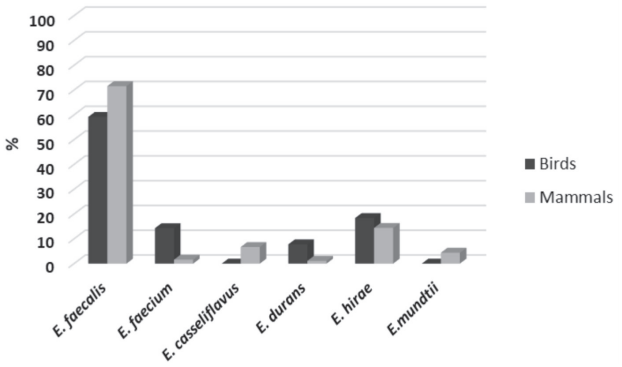

Fig. 1. Distribution of *Enterococcus* spp. in wild living mammals and birds.

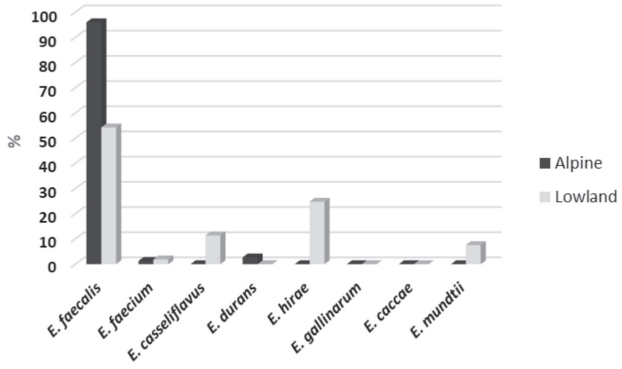

Fig. 2. Distribution of *Enterococcus* spp. among isolates from alpine and lowland area.

wild birds in Poland. Similarly, the most prevalent species of enterococci from wild rabbits were *E. faecalis* and *E. faecium* [11]. The occurrence of *Enterococcus* spp. in mammals and birds was compared (Fig. 1). Significant differences were detected in the abundance of *E. faecium*, *E. casseliflavus,* and *E. durans* species. Much higher species diversity was observed among isolates from lowland areas when *E. faecalis* was a

Table 1. Primers used for the detection of antibiotic resistance genes in enterococci.

Genes targeted	Sequence 5´-3´	Amplicon size (bp)	References
*erm*B	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	424	Soltani et al., 2000
*mef*A	CTATGACAGCCTCAATGCG ACCGATTCTATCAGCAAAG	1400	Kastner et al., 2006
*tet*M	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	657	Nonaka et al., 2007
*tet*L	CATTTGGTCTTATTGGATCG ATTACACTTGCGATTTCGG	488	Agresø et al., 2002
*van*A	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Dutka-Malen et al., 1995
*van*C-1	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	Dutka-Malen et al., 1995

dominant species detected in faecal samples from alpine animals (Fig. 2).

All identified isolates were tested for the resistance to four antibiotics – ampicillin, erythromycin, tetracycline and vancomycin. Among tested isolates, 36% exhibited resistance to one or more antibiotics. The most frequent resistance pattern observed was resistance to tetracycline (29.3%), followed by resistance to erythromycin (15.5%), to ampicillin (9.2%) and to vancomycin (5.7%). Multi-resistance (resistance to two or more antibiotics) was detected in 56 isolates. The highest frequency of antibiotic resistance was observed in *E. faecalis* species.

There are very limited data on antibiotic resistant enterococci from wild living animals in Slovakia. Intrinsic resistance to vancomycin in enterococci from chamois detected Jánošková and Kmeť (2004) [12]. On the contrary, Oravcová et al. (2016) [13] described clinically significant enterococci carrying the *van*A genes in common raven *faeces*. Studies on the resistance of enterococci from farm animals and products prepared from farm animals are more frequent [14-16] indicating that antimicrobial resistance is widespread among these bacteria. Generally, there are inconsistent data on occurrence of antibiotic resistance among bacteria from wild animals and the factors influencing the incidence of resistant enterococci are not well understood [17]. Santos et al. (2013) [18] studied the prevalence of antimicrobial-resistant bacteria in wild bird populations and found that the enterococci strains showed high percentages of resistance to tetracycline and erythromycin. Similar findings were reported by Stępień-Pyśniak et al. (2018) [9] who assessed the antimicrobial resistance of *E. faecalis* isolates from wild bird species. Except for lincomycin, the most isolates were resistant to tetracycline and erythromycin. All enterococci strains isolated from meat of wild game animals in study by Guerrero-Ramos et al. 2016 [19] showed multi-resistant phenotype. On the contrary, the study by Semedo-Lemsaddek et al. (2013) [20] showed that *E. faecalis* isolated from Eurasian otter did not carry genes of antibiotic resistance.

The analysis of resistance occurrence revealed that strains originating from birds show higher resistance levels (64 %) than strains obtained from mammals (53.3 %). For example, *E. faecalis* from mallard (30 %) and from Eurasian jay (40 %) were resistant to ampicillin, erythromycin and tetracycline. In general, wild birds are potential vector of bacteria among different environment. Bonnedahl et al. (2009) [21] suggested that wild birds could act as a reservoir of medically important pathogens and resistance genes. The reservoirs of multi-resistant enterococci are considered e.g. common buzzards (Radhouani et al. 2012) [22] and the high incidence of resistant enterococci in wild birds was documented by Klibi et al. (2015) [23]. Similar studies confirm that the use of antibiotics and chemotherapeutic compounds in intensive poultry production has led to expansion of the population of

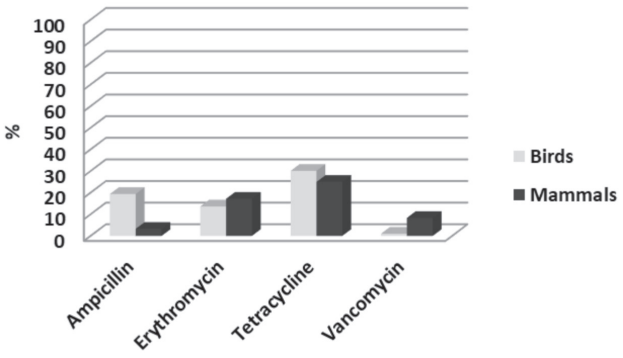

Fig. 3. The incidence of antimicrobial resistance in enterococci from birds and mammals.

multiresistant enterococci in the poultry population of Poland [24]. The dissemination of multiresistant enterococci has become an important cause of zoonotic and nosocomial infections throughout the world [25].

Statistically significant differences between birds and mammals were observed in the incidence of resistance to ampicillin (p-value<0.0001) and vancomycin (p-value 0.007) (Fig. 3) when all ampicillin resistant enterococci came from bird samples. In our study all ampicillin resistant isolates were identified as *Enterococcus faecalis*. Ampicillin resistance occurs in high level of hospital-associated *E. faecium* isolates, but it is rare in *E. faecalis* [26]. Abdel-Moein et al. (2017) [27] detected only 3.2 % ampicillin resistant *E. faecalis* isolated from dogs. Similarly, Santos et al. (2013) [18] observed low level of ampicillin resistance in *E. faecalis*, *E. faecium* and *E. durans* from wild birds. Ampicillin resistant enterococci are reported more frequently in samples from farm animals [28, 29] compared to samples from wild living animals. Zurek and Ghosh (2014) [30] assume that important link for transport of resistant genes among farm animals and wild animals represent insects. Distribution of antimicrobial resistance between different hosts species related to the type of diet was compared in meta-analysis by Vittecoq et al. (2016) [17]. The analysed data indicated

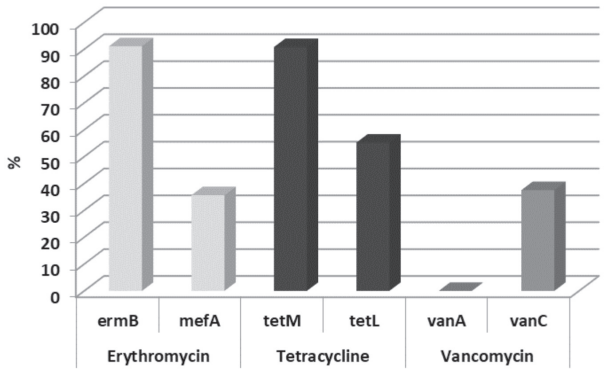

Fig. 4. Occurrence of antibiotic resistance genes in wild living animals.

that carnivores and omnivores are probably most at risk in antibiotic resistance transfer. Our results supported this hypothesis as the highest level of antimicrobial resistance was detected in omnivores compared to other nutrition type animals.

The presence of antibiotic resistance genes was tested by PCR. The *tet*M gene was found as predominant (90.8%) in tetracycline resistant isolates, while the *tet*L gene was confirmed in 55.2% isolates (Fig. 4). The combination of *tet*M and *tet*L was detected in 52.9% isolates. Enterococci from broiler chicken harboured both *tet*M and *tet*L gene at similar high frequency (68.1%) (Diarra et al. 2010) [31] and Rahman et al. (2015) [32] found tetracycline resistant bacteria (harbouring *tet*M gene) in faecal microbial community of Adélie penguin in Antarctica.

The *erm*B gene was the most prevalent resistance determinant found in erythromycin-resistant isolates. The *mef*A gene, coding for a macrolide efflux, was found in 35.6% isolates. The co-occurrence of the *erm*B and *mef*A genes was detected in 33.3% isolates. For comparison all clinical enterococcal isolates with high level of resistance to erythromycin carried the *erm*B gene but no isolate carried *mef*A gene [33]. Similar results were documented in sheep [5] where *mef*A gene was present in 3 of 14 (21.4%) isolates from birds and in 13 of 30 (43.3%) isolates from mammals.

Using PCR no *van*A positive vancomycin resistant isolates were found in tested collection of isolates. Oravcová et al. (2014) [34] detected *van*A gene in 2.5% of enterococci isolates from wild American crows and Mallon et al. (2002) [35] observed *van*A genotype in *E. faecium* isolated from woodmice (4.6 %) and badgers (1.2 %). However, 37.5% isolates, identified as *E. faecalis* and *E. hirae,* were positive for *van*C1 gene. All these isolates were correctly identified by MALDI TOF MS with score values of >2.30 what indicates highly probable species identification. While VanC1 phenotype is common in *E. faecalis*, it was found in *E. hirae* as well [36]. On the other hand, Drobni et al. (2009) [37] collected faecal samples from birds at the sites with no or low human population and detected vancomycin resistant enterococci isolates.

All isolates from high altitude areas were taken from mammals from the territories of two Slovak national parks. Surprisingly, much higher frequency of tetracycline and erythromycin resistance was observed between enterococci isolated from mammals in Tatra National Park (TANAP) compared to Low Tatras National Park (NAPANT) despite both national parks cover alpine parts of mountains around river Vah with similar climatic conditions. One of the possible explanations for the differences in antimicrobial resistance occurrence are differences in anthropogenic influences (tourism). Recent analyses of bacterial communities from two localities in Tatra National Park suggested different bacterial diversity, which corresponds probably to lower intensity of visitors and less anthropogenic impact [38].

A study of the occurrence of antimicrobial resistance in bacteria is needed not only in hospitals but also in farm environment and nature to understand the role of wild living animals in spreading resistant bacteria and antibiotic resistance genes in the environment.

Conclusions

We studied the occurrence of antimicrobial resistance in enterococci in wild animals in Slovakia. The frequency of resistance of enterococci from the digestive tract of wild living animals suggested the genetic pollution of environment and represents a risk for human and animal health. Is therefore necessary to observe and study the spread of antimicrobial resistance in enterococci in the environment.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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