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A COMPARISON OF ANTIBIOTIC RESISTANCE AND MULTIPLE ANTIBIOTIC RESISTANCE INDEX IN WILD BOARS FROM COVASNA AND CLUJ COUNTIES

Emőke PALL[1], Marina SPÎNU[1], Carmen Dana ȘANDRU[1], Gheorghiță DUCA[2], Monica Ioana SUĂTEAN[1], Andrea-Angela SZAFTA[1], Olah Diana[1], Aurel VASIU[1]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca,
3-5 Calea Mănăştur, 400372, Cluj-Napoca, Romania
[2]Institute of Research and Development for Montanology, 53 XIII Street, 557085,
Cristian, Sibiu, Romania.

Corresponding author email: emoke.pall@usamvcluj.ro

Abstract

Due to the global decline of bacterial antimicrobial sensitivity, international organizations such as Word Health Organization, Food and Agriculture Organization, and World Organization for Animal Health consider it necessary to implement an integrative approach to the fight against antibiotic resistance in the light of the One Health concept. The purpose of the study was to investigate the degree of interchangeability of the antibiotic resistance of the isolated microbiome from wild boars and their living environment. The samples (n=24) were collected from two hunting funds in Covasna and Cluj counties. The identification of microorganisms was performed using the Vitek®2 System and, subsequently, their susceptibility was evaluated using the agar diffusion method. The isolated strains *were identified as Pseudomonas spp., Pseudomonas aeruginosa, Pseudomonas luteola, Brevibacterium spp., Aerococcus viridans, Aeromonas sobria, Campylobacter coli, Campylobacter lari, Staphylococcus aureus, and Streptococcus suis. The highest multiple antibiotic resistance index (MAR 0.666) was identified in strains isolated from Cluj County, while the lowest (MAR 0.111) was obtained in strains isolated from Covasna County. The results of this study further acknowledge the bacterial resistance in wildlife.*

***Key words**: multidrug resistance, wild boar, bacterial microbiome, One Health.*

INTRODUCTION

Nowadays antibiotic resistance is a complex global and extraordinarily complicated problem with a major impact on human, animal, and environmental health (Malik & Bhattacharyya 2019; Boyd et al., 2021). The identification of the antimicrobial potential of some substances and their routine administration for the prophylaxis or treatment of some infections have revolutionized modern medicine and often changed the therapeutic paradigm. Thus, substances with antimicrobial potential have become very important in the development of complex medical approaches (Munita & Arias, 2019). Antibiotics are natural substances, produced by microorganisms or artificially synthesized, with selective toxicity to bacteria, so they can destroy the microorganism without affecting the host (Rossiter et al., 2017; Seal et al., 2018). However, excessive and irrational use of these substances has led to the development of antibiotic resistance (Ventola, 2015).

Antibiotic resistance was first observed in 1940, in an *Echerichia coli* strain, resistant to penicillin, later identifying that the penicillinases were responsible for this phenomenon. In 1942 the same phenomenon was observed in four clinical isolates of *S. aureus* (Rammelkamp & Stolzer, 1961; Boyd et al., 2021). The use of antibiotics in human and veterinary medicine as well as in agriculture to prevent disease or as a growth promoter in mammals and birds, together with their use in farmed fish has led to contamination of water and soil with antibiotic-resistant microorganisms. The pattern of antibiotic resistance varies widely between regions and countries and is directly correlated with their degree of use (Sahoo et al., 2012; Manyi-Loh et al., 2018). The phenomenon of antibiotic resistance has long been known, but the role of wildlife as natural reservoirs in this process is not fully elucidated. However, it is considered that both birds, wild rodents, and mammals in the vicinity of human settlements are potentially coming into contact with household waste and improperly stored faeces may be contaminated from these sources with resistant microorganisms. Due to the large territory, they cross, their role in the distribution of resistant microorganisms must also be considered (Radhouani et al., 2014).

Human density and proximity have a direct influence on the wild environment and vice versa. Thus, the identification of bacteria and the verification of antibiotic resistance in wild animals offers the possibility of monitoring microorganisms with pathogenic potential. Due to the rapid and continuous adaptation of bacteria, the importance of knowing the types of antibiotics, which have low efficacy, is of utmost importance.

The study aimed to investigate the interchangeability degree of the microbiome antibiotic resistance isolated from wild boars as well as their habitat with potential resistant flora to antimicrobials used in farm animal therapy or in the treatment of bacterial diseases in humans and also defining the antibiotic resistance profile (MAR index) of wild boar bacteriome.

MATERIALS AND METHODS

The samples (n=24) were collected from two hunting funds in Covasna and Cluj counties. The identification of bacterial strains was performed by standard microbiological methods adopted from the Clinical and Laboratory Standards Institute (CLSI) guideline. For initial microbiological analyses, the samples were inoculated in nutrient broth and MacConkey agar in aerobic conditions at 37°C for 24 hours. The cultures were repeatedly passed through nutrient agar to obtain isolated colonies. Macroscopic aspects such as colour, size, transparency of the colonies were the basis for their differentiation, followed by repeated microscopic examinations using Gram staining. The identification of microorganisms was performed using the Vitek®2 System. The antimicrobial sensitivity patterns of the isolated strains were evaluated using the standard Kirby-Bauer disk diffusion method according to the CLSI guidelines. The strains were tested towards 9 antimicrobials: amoxicillin/clavulanic acid (AMC, 20/10 µg; Oxoid, UK), doxycycline (DO, 30 µg; Oxoid, UK), enrofloxacin (ENR, 5 µg; KRKA, Slovenia), florfenicol (FFC, 30 µg; Oxoid, UK), penicillin (P, 10UI; Oxoid, UK), oxytetracycline (OT, 30 µg; Oxoid, UK), cephalexin (CEP, 30 µg; Oxoid, UK), neomycin (N, 30 µg; Oxoid, UK), and tylosin (TY, 30 µg; Oxoid, UK). Based on the growth inhibition zone diameters (mm), the bacterial strains were recorded as resistant (R), intermediate (I), and susceptible (S). For further analysis, intermediate and resistant pattern isolates were grouped as resistant. The multiple antibiotic resistance index was recorded according to the procedure described by Krumperman (Krumperman, 1983), so for the calculation of the MAR index, the total number of antibiotics to which the isolate was resistant / the total number of antibiotics tested was considered. According to Krumperman, values lower than 0.2 are considered low risk, while values higher than 0.2 indicate a high risk (Krumperman 1983). For each antibiotic, the MAR index was calculated, interpreted as the number of isolates resistant to the selected antibiotics, divided by the sum of the number of antibiotics used, multiplied by the number of isolates (Tambekar 2006; Pall et al., 2021). Classification of multidrug resistance (MDR) was carried out according to Magiorakos et al. (2012). MDR was considered as the resistance to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). The results of the study were analysed with GraphPad Prism 5.00 software (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel. The data were presented as average values, and Excel was used for the graphical construction.

RESULTS AND DISCUSSIONS

Out of 24 (n=24) pharyngeal exudate samples collected from wild boars from Covasna and Cluj counties, 20 different single bacterial colonies were obtained. The isolated strains were identified as *Pseudomonas* spp., *Pseudomonas aeruginosa, Pseudomonas luteola, Brevibacterium* spp., *Aerococcus* *viridans, Aeromonas sobria, Campylobacter coli, Campylobacter lari, Staphylococcus aureus, Streptococcus suis.* Most isolated bacteria were *Pseudomonas aeruginosa* (20%), *Pseudomonas luteola*, (15%), *S. aureus* (15%), and *Streptococcus suis* (15%) (Fig. 1).

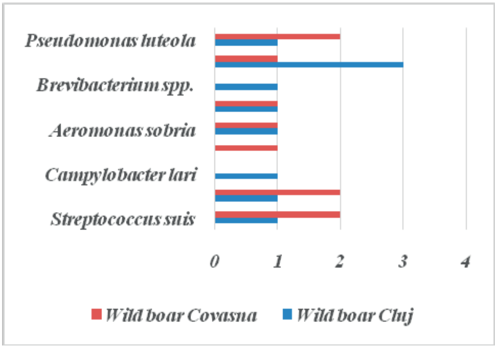

Figure 1. Isolated pathogens form wild boar from Covasna and Cluj counties

Similar to other studies (Costa & Iraola, 2019; Ruiz-Roldán et al., 2020) conducted on samples collected from wild boars, our study identified bacterial species belonging to the genus Pseudomonas such as *P. aeruginosa* and *P. luteola* and the genus Campylobacter namely *Campylobacter coli* and *Campylobacter lari.*

Other bacterial strains, such as *Brevibacterium* spp., *Aerococcus viridans,* and *Aeromonas sobria*, were also isolated. The presence of *Brevibacterium* spp. can be explained by the nutritional habits of wild boars, which often consume insects, birds, and other small mammals, from which strains of *Brevibacterium* spp. are frequently isolated (Giorgio et al., 2018). *Aerococcus viridans* is frequently isolated from domestic animals (Liu et al., 2015; Moreno et al., 2016; Nguyen et al., 2021), therefore the proximity of human settlements explains the identification of this bacterial species. *Aeromonas sobria* was isolated from freshwater, stagnant water, and wastewater (Miyagi et al., 2016), so contamination of wild boars could be achieved by consuming water from these sources (Fernández-Bravo & Figueras, 2020).

The isolated strains were evaluated for their level of antibiotic resistance, the results are indicated in Tables 1 and 2. Of the total isolated strains, 35% showed resistance to amoxicillin/clavulanic acid, penicillin, and tylosin, 40% to tetracyclines, 30% to neomycin, 20% to cephalexin and florfenicol, and 15% to Enrofloxacin.

In the present study, the resistance to Tetracyclines was the highest (40%) followed by penicillin (35%), Amoxicillin/Clavulanic acid, (35%), and Tylosin (35%), lower percentages were observed for Enrofloxacin (15%), Cephalexin and Florfenicol both with 20%. Five of the isolates were susceptible to all tested antimicrobials, and three of the isolates indicated resistance to a single antibiotic.

A MAR index value ≥0.2 was observed in 100% of the resistant pathogens. The MAR index calculated for isolated strains was between 0.33 and 0.88. The highest value of the MAR index was obtained in *P. aeruginosa, S. aureus*, and *Streptococcus suis* strains isolated from both countries. The lowest value of the MAR index was obtained in *P. luteola, Campilobacter coli, A. viridans, A. sobria* strains, predominantly isolated from Cluj County (Table 3). The most obvious similarity is the increased resistance of bacterial strains to antimicrobials such as, amoxicillin/clavulanic acid, penicillin, and tyrosine, which have been used for a long time and excessively in both human and veterinary medicine.

Table 1. The antimicrobial sensitivity patterns of the isolated strains from wild boars from Covasna County

Drugs	***P. luteola***	***P.luteola***	***P. aeruginosa***	***S. suis***	***S. suis***	***C. coli***	***A. sobria***	***A. viridans***	***S. aureus***	***S. aureus***
AMC	R	S	R	R	R	S	S	S	S	R
FFC	S	S	S	S	R	S	S	S	S	S
CEP	S	S	R	S	S	S	S	S	I	I
DO	S	S	S	S	S	S	S	S	S	S
ENR	S	S	S	S	S	I	S	S	S	S
N	R	S	S	S	R	S	S	S	S	I
OT	R	S	S	S	S	S	S	S	S	S
P	S	S	R	R	S	S	S	S	S	R
TY	S	R	R	R	S	S	S	S	S	S

AMC - amoxicillin/clavulanic acid, DO – doxycycline, ENR -enrofloxacin, FFC- florfenicol, P- penicillin, O – oxytetracycline, CEP – cephalexin, N- neomycin, TY- tylosin; R-resistant, I-intermediate, S- sensitive

Table 2. The antimicrobial sensitivity patterns of the isolated strains from wild boars from Cluj County

Drugs	***P. luteola***	***P. aeruginosa***	***P. aeruginosa***	***P. aeruginosa***	***A. viridans***	***A. sobria***	***C. lari***	***Brevibacterium spp.***	***S. aureus***	***S. suis***
AMC	S	R	R	S	S	S	S	S	S	S
FFC	R	R	R	S	S	S	S	S	S	S
CEP	S	S	R	S	S	S	S	S	S	S
DO	S	I	I	I	S	S	S	S	S	S
ENR	I	S	S	S	S	S	I	S	S	S
N	S	S	S	R	S	S	I	S	I	S
OT	S	I	I	I	S	S	S	S	S	I
P	S	R	R	R	S	S	S	S	R	R
TY	R	R	S	R	S	S	S	S	S	R

Table 3. Multiple antibiotic resistance (MAR) index of antibiotics against isolated

Antimicrobial class	Drug	Total number of resistant strains	MAR index of the tested antibiotics
Beta lactamase	Amoxicillin + Clavulanic acid	7	0.7
Cephalosporins	Cephalexin	4	0.44
Fluoroquinolones	Enrofloxacin	3	0.33
Phenicols	Florfenicol	4	0.44
Penicillins	Penicillin	7	0.77
Aminoglycosides	Neomycin	6	0.66
Tetracyclines	Doxycycline Oxytetracycline	8	0.88
Macrolide	Tylosin	7	0.77

At the same time, in the case of samples collected from both counties, low resistance to enrofloxacin was observed. The differences between the results obtained from the two counties appeared for the other antimicrobial substances such as florfenicol, neomycin, and oxytetracycline. These differences can be explained by the irrational use of antibiotics and the induction of different antibiotic resistance depending on the region but also by the natural resistance of some bacterial species to the mechanisms of action of some antimicrobials. Thus, we can state that the groups of antibiotics to which resistance has been identified (beta-lactamase, penicillin, macrolide, tetracyclines, and aminoglycosides) and those with efficacy (phenicols, cephalosporins, and fluoroquinolones) are the same for the isolated bacterial strains from the two counties. The values of the MAR index show a higher level of antibiotic resistance in Cluj County compared to Covasna County. The reason for this result can be correlated with the larger space development of human settlements, which implicitly increases the possibility of contamination with antibiotic residues leading to easier spread in the environment of bacterial strains resistant to antimicrobials. Identifying possible sources of the spread of antimicrobial-resistant bacterial strains in wildlife is of immeasurable importance to public health. Wild animals have a major implication in the biological mechanisms of the spread of antibiotic resistance genes (Radhouani et al., 2014).

The patterns of antibiotic resistance identified in our study indicate that the prevalence of antimicrobial resistance is increased in bacteria isolated from wildlife in the two areas studied. This reflects a high level of exposure of these wild species to antimicrobials, and an increased percentage of resistant bacteria in the areas where these animals live and feed. These results can be interpreted in terms of anthropogenic impact in these areas. The results of the study reflect the presence of antimicrobial-resistant bacterial strains that are considered with the highest priority of critical importance by the World Health Organization (WHO, 2019).

CONCLUSIONS

Continuous monitoring of the prevalence and antimicrobial resistance of bacterial strains isolated from wild boar is important for the purpose of epidemiological surveillance of different territories. These assessments are also important for public health. Our study confirms the role of wild boars as natural reservoirs of resistant bacterial strains. The value of the MAR index indicates the existence of bacterial species resistant to several groups of antimicrobial substances in the natural environment. The results of the antibiotic resistance pattern together with the MAR index reveal a higher antibiotic resistance level in the samples collected near the developed human settlements.

In order to prevent the spread of antibiotic resistance, rational use of antibiotics is extremely important, only for therapeutic purposes, at the doses and intervals necessary to ensure maximum effectiveness, both in human and veterinary medicine. In order to monitor antibiotic resistance, it is important to periodically investigate bacterial susceptibility on samples collected from the human population, domestic and wild animal populations, and the environment.

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