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Bacteriological Screening and Antibiotic – Heavy Metal Resistance Profile of the Bacteria Isolated from Some Amphibian and Reptile Species of the Biga Stream in Turkey

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***Abstract*—**In this article, the antibiogram and heavy metal resistance profile of the bacteria isolated from total 34 studied animals (*Pelophylax ridibundus* = 12; *Mauremys rivulata* = 14; *Natrix natrix* = 8) captured around the Biga Stream, are described. There was no database information on antibiogram and heavy metal resistance profile of bacteria from these area's amphibians and reptiles.

A total of 200 bacteria were successfully isolated from cloaca and oral samples of the aquatic amphibians and reptiles as well as from the water sample. According to Jaccard's similarity index, the degree of similarity in the bacterial flora was quite high among the amphibian and reptile species under examination, whereas it was different from the bacterial diversity in the water sample. The most frequent isolates were *A. hydrophila* (31.5%), *B. pseudomallei* (8.5%), and *C. freundii* (7%). The total numbers of bacteria obtained were as follows: 45 in *P. ridibundus,* 45 in *N. natrix* 30 in *M. rivulata*, and 80 in the water sample. The result showed that cefmetazole was the most effective antibiotic to control the bacteria isolated in this study and that approximately 93.33% of the bacterial isolates were sensitive to this antibiotic. The multiple antibiotic resistances (MAR) index indicated that *P. ridibundus* (0.95) > *N. natrix* (0.89) > *M. rivulata* (0.39). Furthermore, all the tested heavy metals (Pb^{+2}, Cu^{+2}, Cr^{+3}, and Mn^{+2}) inhibit the growth of the bacterial isolates at different rates. Therefore, it indicated that the water source of the animals was contaminated with both antibiotic residues and heavy metals.

***Keywords*—**Amphibian, Bacteriological Quality, Reptile, Antibiotic & Heavy Metal Resistance.

I. Introduction

EMERGING infectious disease of wildlife has gained a growing interest due to the increasing impact of human intrusions in wildlife habitat on human disease emergence and resurgence. An increased interface between humans, domestic animals and free ranging wildlife is a global dimension of these intrusions. However, little is known about the dynamics of this interface as a factor for antibiotic resistance as a component of disease emergence [1].

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Some members of the Gram negative bacteria (*Enterobacteriaceae, Aeromonadaceae, Pasteurellaceae* etc.) are human pathogens causing a variety of gastrointestinal and other symptomatic diseases. Some reptiles and amphibians have also been found to be potential vectors of these pathogens [2]-[4]. Baseline information on the composition of normal flora of wild animals is an extremely useful tool for the correct interpretation of bacteriological culture results and to better understand the role of bacteria as pathogenic agents in disease events among these animals. In addition, determination of whether the microbial flora in these animals had any similarity to the microbial diversity of the environment with which they interacted is also important to reveal the origin of the microbial flora existing in these animals.

The majority of pathogenic bacteria that affect amphibian and reptiles are Gram–negative organisms [5]-[7]. Knowledge about the cloacal and oral bacterial flora is limited for the majority free-living reptile and amphibian species. Most studies have concentrated on a small group of bacteria that are known to be zoonotic or on reptile species with commercial interest [7], [8]. Furthermore, it has been well established that many reptile and amphibian harbor Gram negative bacteria as part of their normal flora and that these microbes are either commensal or opportunistic [7]. The majority purpose of this study was to investigate the culturable aerobic cloacal and oral flora of three free living amphibian and reptiles.

Overdosing of antibiotics in feed and excessive use of chemicals in prophylaxis has caused bacteria to become antibiotic-heavy metal resistant. Their residue may stay in the environment and could transfer to other bacteria via antibiotic resistance genes. Many bacteria also have specific genetic mechanisms of resistance to toxic metals. In the environment metals may select these resistant variants in a manner similar to the selection of antibiotic resistant strains. Indeed, it is relatively common the association of metal and antimicrobial resistance, since both resistance genes are frequently located on the same mobile genetic elements. Consequently, it can be assumed that the selective pressure exerted by heavy metals contribute to the indirect co-selection of antibiotic resistance, particularly in environments contaminated with the two elements [9].

This study aims (1) to identify the Gram-negative bacterial flora of amphibian (*Pelophylax ridibundus*) and reptiles

(*Mauremys rivulata* and *Natrix natrix*) as well as the fresh water where the animals captured (2) to find whether there is any similarity among the microbial flora of three animal species and the bacteria isolated from water, and (3) to determine the antibiotic and heavy metal resistance of the bacteria isolated bacteria from animals.

II. Materials and Methods

A. Collecting Animal Samples and Water Samples

Studied amphibian (*P. ridibundus*) and reptiles (*M. rivulata*, *N. natrix*) were captured around the Biga Stream on April-May 2014. Only healthy and mature animals were studied, and they were collected by hand capture method, generally from the bottom and surface of the water. Their cloaca and oral samples were taken with sterile swabs, and then the animals were released to the study area. To isolate water bacterial isolates, a freshwater sample was also taken from the study area where the animal species were collected. All samples were immediately brought into the laboratory for microbiological analyzing.

B. Microbiological Analyses

Cloacal and oral samples of animals and fresh water samples placed buffered peptone water for enrichment for 24 hr 35-37^{o}C. And then plated on MacConkey agar (MAC); Thiosulfate citrate bile salts sucrose agar (TCBS), Glutamate starch phenol red agar (GSP), Inositol brilliant green bile agar (IBG); Chromogenic *E. coli* agar (CE) for isolation different gram negative bacteria species. Plates were incubated at 25–30°C for 24–48 h. Isolated colonies were identified as [10]. For obtained isolates, verification tests were performed according to Microgen ID-A panel-Gram negative (MID-64).

C. Statistical Procedure

To assess differences in the bacterial communities among water and animal samples, we calculated similarity of species composition with the Jaccard's index [11] for each samples using the software package PAST [12]. For classification (clustering), the UPGMA (unweighted pair-group method using arithmetic averages) was used considering Jaccard's dissimilarity index to identify possible affinities in flora composition among water sample and animal samples [1]. The Jaccard's index was calculated following [13]. This measure of similarity is defined as the size of the intersection divided by the size of the union of two sample sets (A, B) according to the formula $J(A, B) = |A \cap B| / |A \cup B|$.

D. Antibiotic Susceptibility

All isolates from animal samples were tested for antibiotic sensitivity by agar dilution according to Clinical and Laboratory Standards Institute guidelines to 14 antibiotics [14]. The following antibiotics were used: tobramycin (TB10 µg/mL), trimethoprim (TR10 µg/mL), kanamycin (K30 µg/mL), amoxicillin (AM10 µg/mL), oxytetracycline (O30 µg/mL), cefmetazole (CMZ30 µg/mL), gentamicin (G10 µg/mL), furazolidone (FR50 µg/mL), erythromycin (E15 µg/mL), cefoxitin (CN30 µg/mL), cephalothin (CH30 µg/mL), ampicillin (A10 µg/mL), cefotaxime (CE30 µg/mL), and chloramphenicol (C30 µg/mL). Organisms were reported as resistant, intermediate, or sensitive to each antimicrobial tested according to the Clinical and Laboratory Standard Institute [15].

E. Heavy Metal Activity

The minimal inhibitory concentration (MIC) for each bacterial isolate for four heavy metals was determined using Mueller–Hinton agar (Merck) containing Pb^{+2}, Cu^{+2}, Cr^{+3}, and Mn^{+2} at concentrations ranging from 12.5 to 3.200 µg/mL. The metals were added as $PbSO_4$, $CuSO_4 5H_2O$, $K_2Cr_2O_7$, and $MnCl_2 2H_2O$. The isolates were considered resistant if the MIC values exceeded that of the *Escherichia coli* K - 12 strain which was used as the control [16].

F. Multiple Antibiotic Resistant Indexes

The multiple antibiotic resistances (MAR) index of bacterial strains against antibiotics is calculated based on method used by [17] as follows: MAR index = X/ (Y × Z) where X is the total bacteria resistant to antibiotics, Y is the total antibiotic used, and Z is the total isolates. A MAR index value less than 0.20 indicated that the antibiotics are seldom and never used, whereas a value greater than 0.20 suggests that the antibiotics are exposed to the bacteria [18].

III. Results

A. Bacteriological Parameters

Isolated gram-negative bacilli from total 34 studied animals (*P.ridibundus* = 12; *M.rivulata* = 14; *N.natrix* = 8) and water sample where animals captured, several members (total=200) of the *Enterobacteriaceae*, *Vibrionaceae*, *Burkholderiaceae*, *Pasteurellaceae*, *Moraxellaceae* and *Aeromonadaceae* were isolated (Table I). The most frequent isolates were *A. hydrophila* (31.5%), *B. pseudomallei* (8.5%) and *C. freundii* (7%). The numbers of bacteria were obtained in *P. ridibundus* (total = 45), *N. natrix* (total=45) and *M. rivulata* (total = 30) and water sample (total = 80). All of amphibians and reptiles investigated were arrange for bacterial flora; *P.ridibundus* = *N. natrix* > *M. rivulata.*

The Jaccard's similarity index was also used for the cluster analysis of the bacterial isolates isolated from the station where captured and from each animal. The similarities and/or distances of the bacterial isolates from the animals and the water sample were determined and summarized with a dendrogram (Fig. 1) and a similarity matrix (Table II).

According to the results of Jaccard's cluster analysis, it was determined that the similarity among the amphibian and reptile species examined in terms of the bacterial flora was quite high, while it was different from the diversity of bacteria from the water sample. When the similarity in the bacterial flora among the amphibian and reptile species is examined in the dendrogram obtained, it is seen that there is a high rate of similarity in microbial diversity between species *P.ridibundus* and *N. natrix* (Fig. 1 and Table II).

TABLE I
LIST OF ISOLATED BACTERIA

Bacterial species	Animal			Water sample	N
	P. ridibundus (n=12)	*M. rivulata* (n=14)	*N. natrix* (n=8)		
Aeromonadaceae					
Aeromonas hydrophila	7	2	21	33	63
A. veroni bio *sobria*	2	1	-	-	3
A. caviae	2	-	-	2	4
Vibrionaceae					
Vibrio alginolyticus	-	-	1	-	1
V. carchariae	-	-	-	1	1
Pseudomonadaceae					
Ps. fluorescens	-	-	-	3	3
Ps. aeruginosa	-	-	-	7	7
Shewanellaceae					
Shewanella putrefaciens	-	-	-	2	2
Flavobacteriaceae					
Elizabethkingia meningosepticum	-	-	-	1	1
Enterobacteriaceae					
Citrobacter freundii	8	5	1	-	14
C. diversus	2	2	1	-	5
Enterobacter gergoviae	3	2	3	2	10
Klebsiella oxytoca	-	1	4	2	7
K. ozaenae	-	1	-	-	1
K. pneumoniae	-	1	-	-	1
Escherichia coli	-	2	-	11	13
E. coli inactive	-	1	-	2	3
Serratia marcescens	-	1	1	-	2
S. rubidaea	-	-	-	1	1
S. liquefaciens	1	-	-	1	2
Pantoea agglomerans	1	1	-	-	2
Providencia stuartii	-	1	-	-	1
Yersinia enterocolitica	1	-	1	-	2
Salmonella arizonae	6	-	7	-	13
Proteus mirabilis	-	-	1	-	1
Burkholderiaceae					
Burkholderia cepacia	-	1	2	-	3
B. pseudomallei	6	3	2	6	17
Pasteurellaceae					
Actinobacillus sp.	-	1	-	1	2
Pasteurella multocida	2	-	-	4	6
Moraxellaceae					
Acinetobacter haemolyticus	-	2	-	-	2
A. baumanii	1	-	-	-	1
A. lwoffi	3	-	-	-	3
Moraxella sp.	-	2	-	1	3
Total	45	30	45	80	200

n: Captured animal numbers
N: number of bacterial isolates obtained

TABLE II
BACTERIOLOGICAL SIMILARITY (JACCARD'S INDEX) AMONG THE ANIMALS AND WATER SAMPLE

	M. rivulata	*P. ridibundus*	*N. natrix*	Water sample
M. rivulata	1.000			
P. ridibundus	0.280	1.000		
N. natrix	0.304	0.368	1.000	
Water sample	0.421	0.291	0.160	1.000

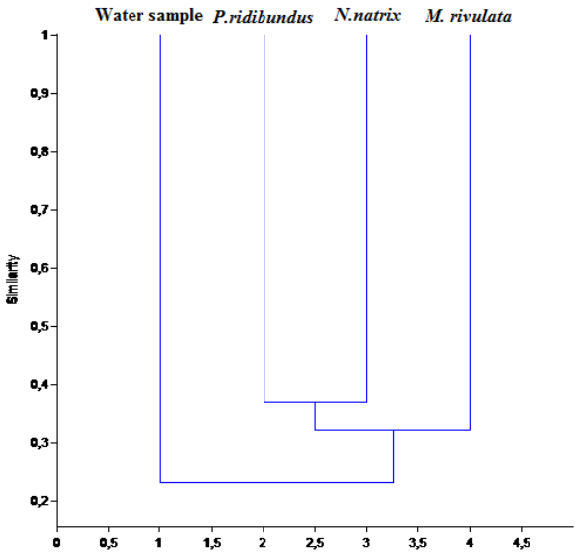

Fig. 1 Dendrogram of the bacterial isolates similarity among the animals and water sample

B. Antimicrobial Resistance of Bacteria

The results of antibiotic susceptibility test revealed that 13.33 – 93.33% of the isolated gram-negative Bacilli were resistant to CMZ30 (93.33%), CN30 and CH30 (91.11%) (Fig. 2). All bacterial isolates showed antibacterial resistance to all antibiotics at different rates; however, it was determined that none of the bacteria isolated from *M. rivulata* developed any resistance to TB10. The MAR index value of isolated bacteria was found *P. ridibundus* (0.95) > *N. natrix* (0.89) > *M. rivulata* (0.39).

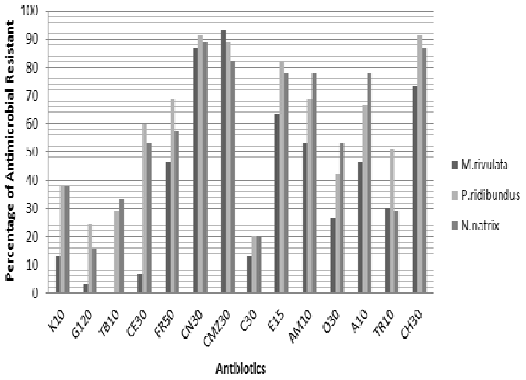

Fig. 2 Antimicrobial resistance profiles of gram-negative bacteria against antibiotics

C. Heavy Metal Resistance

In the present study, resistance to four heavy metals (Zn^{+2}, Cu^{+2}, Cr^{+3}, and Pb^{+2}) was investigated for all isolates. The trends in heavy metal resistance are shown in Table III; Mn (58.33%) > Cu (55.00%) > Cr (33.33%) > Pb (22.50%).

TABLE III
HEAVY METAL TOLERANCE IN BACTERIA FROM CLOACA AND ORAL SAPMLES OF AMPHIBIAN AND REPTILES

Heavy metal	Total bacteria N	Metal concentrations (µg/ml) with number of tolerant isolates							Resistant isolates	
		100	200	400	800	1600	3200	>3200	n	%
Chromium	120	80*	25	5	5	5	-	-	40	33.33
Manganese	120				50*	60	5	5	70	58.33
Copper	120	54*	36	22	6	1	1	-	66	55.00
Lead	120	93*	25	2	-	-	-	-	27	22.50

* Minimal inhibition concentration of standard strain *E. coli* K12

IV. DISCUSSION

Freshwater ecosystem is recognized as a natural habitat of some pathogenic microorganisms. The infectious diseases caused by these pathogens are dangerous to freshwater animal's health and can potentially affect several species, including endangered freshwater animals. Freshwater animals are probably the best sentinel organisms in aquatic and coastal environments, because many species have long life spans and are at the top of food chain. Therefore, some pathogens microorganisms isolated from these animals could be used as indicators of disturbance in the freshwater ecosystem.

In our study, the microbial flora of reptiles and amphibians has a considerably wide microbial spectrum, including potential pathogens. In Turkey, investigations about the reptile and amphibian microbial flora are very limited [18], [19]. The findings we have obtained reveal that the animals studied were richer in microbial diversity as compared with the values of microbial diversity obtained in the previous studies.

In the study, bacteria were also isolated from the water sample collected from the Biga Stream in order to determine the origin of the bacterial flora obtained from the reptile and amphibian species and to understand whether it had any correlation with the microbial species obtained from the Biga Stream, which constituted the habitat of the animals. For this purpose, the method of the Jaccard's index was employed to establish whether there was any similarity between the bacteria obtained from the animals and from the water sample.

In a numerical taxonomy studies and variation of microbial flora of animals and their habitats, the Jaccard's index is much used primarily because of their clear conceptual bases and an algorithm called UPGMA (unweighted pair-group method using arithmetic averages) is commonly used to find the average of the resemblance coefficients when clusters are merged [20]. Furthermore, [21] used the simple matching coefficient (Ssm) for a numerical taxonomy study of lactic acid bacteria isolated from freshwater fishes and their surrounding environments. Blanco et al. [1] also studied a numerical taxonomy of cloacal bacterial flora isolated from threatened avian scavenger in four areas of Spain using Jaccard's similarity coefficient. In the Jaccard's index analysis performed for the bacterial isolates obtained from the water sample and from the animals in our study, it was determined that water formed some branching which was different from that of the animals and that the species isolated from *N. natrix* and *P. ridibundus* had a quite high rate of similarity. This constitutes some essential proof of the fact that the habitat of animals alone cannot be influential on the formation of the microbial flora of the animals and therefore all biotic and abiotic factors should be examined for this purpose.

Several studies have isolated *Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae* etc. from reptilian and amphibian oral and cloacal samples [2], [3], [5], [18], [19]. *A. hydrophila, B. pseudomallei, S. arizonae* and *C. freundii* were the most common microorganism identified in the animal and water samples. This bacterium has been documented to cause dermatitis, stomatitis, rhinitis, pneumonia, osteomyelitis, septicaemic cutaneous ulcerative disease and septicaemia, skin disease and red leg syndrome in reptiles and amphibians [3], [6], [22]-[24]. Especially, infection in animals and humans with *Salmonella* may result in serious disease or give rise to a reservoir for other species and contacts within that environment. The interaction of S*almonella* with a host gives rise to a number of clinical presentations including inapparent infection, recovered carrier state, enteritis, septicemia, and combinations of disease syndromes [2]. However, due to the absence of clinical symptoms, these species are still likely to originate from a physiological commensal population (especially in gut) [7]. On the other hand, the failure to detect species *S.arizonae* in the water sample while it was isolated from *P. ridibundus* and *N. natrix* at a significant rate constitutes some important proof of the information that these bacteria will not originate from water. This overlaps the information in a study by Schmidt et al. [7] that no correlation could be found between the habitat of capturing and the isolated bacteria, so that an environmental influence is unlikely as cause of the difference of the cloacal flora of free-living reptile species. Moreover, these findings show that the presences of these microorganisms, which may be pathogens for humans, in amphibians and reptiles will be a risk for public health. Thus, the people who live near this areas, fishermen and aquarists should be more careful when being in contact with these animals.

The antibiotic-heavy metal resistance and multiple drug resistance found are fairly consistent with what has been found in various studies of reptile and amphibian pathogens and aquaculture environments [3], [9], [16], [19], [20], [24]. This may be due to the captured water sources of these animals that

were highly contaminated with antibiotic and heavy metal residues. This was supported by the MAR values obtained in this study which showed that animal's microorganisms were highly exposed to the tested antibiotics. The heavy metal resistance detected in the present study could be the result of heavy metal contamination from untreated sewage, industrial wastes, and agricultural activities. Although Cu, Cr, and Mn etc. have an important part of biological systems of all living organisms, it is known that continuous exposure to high doses causes toxic effects [25].

In addition, a number of authorities plan and conduct reintroduction or population recovery programs for reptilian and amphibians, especially in protected areas along rivers [19]. According to these results, a significant occurrence of bacteria in the internal organs of amphibian and reptiles, with a high incidence of resistance against antibiotics and heavy metals, may risk aquatic animals and the public health. These data appoint the importance of epidemiological surveillance and microbiological monitoring and reinforce the need to implement environment protection programs for these free-living animals.

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