ORIGINAL ARTICLE

SOME VIRULENCE FACTORS OF GRAM NEGATIVE BACTERIA ISOLATED FROM SOME AMPHIBIAN AND REPTILIAN SPECIES OF THE BIGA STREAM IN TURKEY

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ABSTRACT

Background:Aquatic ecosystems contain a variety of microorganisms, of which, a limited number are able to develop in association with higher organisms and of these, a small number are pathogenic bacteria. The ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of infection. **Objectives**: The main goal of the present paper was to perform microbiological investigations to phenotypically emphasize the some virulence factors (enzymatic activity) of Gram negative bacteria isolated from Biga stream and some amphibian (*Pelophylax ridibundus*) and reptilians (*Mauremys rivulata*, *Natrix natrix*) captured around this freshwater on April-May 2014. **Methods:** 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. **Results**:A total of 431 Gram negative bacteria were successfully isolated from cloaca and oral samples of the aquatic amphibians and reptiles as well as from the water sample. The most frequent isolate was *Aeromonas hydrophila* (31.09%). The total numbers of bacteria obtained were as follows: 57 in *N. natrix*, 51 in *M. rivulata,* and 50 in *P. ridibundus*, and 273 in the water sample. The presence of enzymes that determined virulence factors for bacterial strains was identified: DNase, amylase, lipase, haemolysin and protease. **Conclusions**: With reference to, *A. hydrophila* is thought to carry a high virulence factor due to its high DNase, amylase, lipase and protease activity.
Keywords: *Virulence factor, Biga Stream, Pelophylax ridibundus, Mauremys rivulata, Natrix natrix.*

1. INTRODUCTION

Some members of the Gram negative bacteria 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 [4,10,12]. 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.

In spite of advances in treatment and prevention, bacterial pathogens still pose a major threat on public health worldwide. To understand how pathogenic bacteria interact with their hosts to produce clinical disease is a fundamental issue. A key first step in this process is the identification of novel virulence determinants that may serve as targets for vaccine and drug development [21]. However, little is known about the dynamics of this interface as a factor for bacterial virulence factor as a component of disease emergence. Bacterial virulence can be enhanced through specialized virulence factors. These include capsules, enzymes, colonization factors (adhesins), invasins, and toxins. The ability of bacteria to cause disease depends to a large extent on the expression of virulence factors which help them to invade the host, produce pathological effects and evade host defenses. In extreme cases of interaction, a bacterium could insert its own genome into the eukaryotic genome, causing alteration of genetic activity to favor the pathogen. Bacterial illness is a result of complex interactions between bacteria and the host [20].

Knowledge about the cloacal and oral bacterial flora and their virulence factors are 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 [14,15]. The aim of the present investigation was to perform microbiological investigations to phenotypically emphasize the some virulence factors (enzymatic activity) of Gram negative bacteria isolated from Biga stream and some amphibian and reptiles (*Pelophylax ridibundus, Mauremys rivulata*, *Natrix natrix*) captured around this freshwater.

2. MATERIALS AND METHODS

2.1 Collecting Animal Samples and Water Samples:

Studied amphibian (*P. ridibundus* = 12) and reptilians (*M. rivulata*= 14, *N. natrix*= 8) 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.

2.2 Microbiological Analyses:

Cloacal and oral samples of animals and fresh water samples placed buffered peptone water for enrichment for 24 hr 35-37 °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 Murray et al., (1999) [13]. For obtained isolates, verification tests were performed according to Microgen ID-A panel-Gram negative (MID-64).

2.3 Determination of Enzymatic Activities:

DNase activity: Enzyme production was tested on DNA agar by inoculating the culture as a thick band at the center of the plate. The plates were incubated at 37 °C overnight. DNase production was identified by the development of a rose-pink color around the inoculum [3].

Haemolysin activity: Haemolysin production by isolated strains was tested on sheep blood agar (5 %) with a 4-5 h culture of each organism in BHIB. After incubation for 24 h at 37 °C, the plates were examined for the presence of a- or β- haemolysis around the colonies [18].

Protease activity: Protease activity was assessed with the medium of Sokol (9), which consisted of dialyzed brain heart infusion broth, 3% skim milk, and 1.5% agar. Proteolysis, as evidenced by clearing of the medium around isolated colonies of the test organism after 48 h of incubation, was measured semiquantitatively on equivalent depth poured plates [18].

Lipolytic activity: As a result of 48-hour incubation at 30 °C on tributrin agar, the transparent zones around the colonies were evaluated as positive for lipolytic activity [3].

Amylolytic activity: As a result of 48-hour incubation at 30 °C on strach agar, colonies were treated with the lugol solution to observe the amylase activity [2].

3. RESULTS

Isolated gram-negative bacilli from total 34 studied animals (*P.ridibundus* = 12; *M.rivulata* = 14; *N.natrix* = 8) and water sample where animals captured, contains several members (total=431) of the Enterobacteriaceae, Vibrionaceae, Burkholderiaceae, Pasteurellaceae, Moraxellaceae and Aeromonadaceae familia. The most frequent isolate was *A. hydrophila* (31.09%). The total numbers of bacteria obtained were as follows: 57 in *N. natrix*, 51 in *M. rivulata,* and 50 in *P. ridibundus*, and 273 in the water sample (Table 1 and Table 2, respectively).

Table 1: List of isolated bacteria from captured animals.

Isolated bacteria			Animal species					
			P. ridibundus		***M. rivulata***		***N. natrix***	
Phylum/Class	**Familia**	**Bacterial species**	**Oral**	**Cloacal**	**Oral**	**Cloacal**	**Oral**	**Cloacal**
Proteobacteria/ γ	Enterobacteriaceae	*E.coli*	-	-	2	1	-	2
		E.gergoviae	2	2	2	1	3	1
		K.pneumoniae	-	1	2	-	1	1
		K.oxytoca	-	-	4	3	5	1
		E.coli-inactive	-	-	1	-	-	-
		C.diversus	1	1	1	3	-	1
		C.freundii	5	4	3	6	3	1
		S.marcescens	-	-	2	1	1	1
		P.mirabilis	-	-	-	-	1	-
		S.liquefaciens	-	1	-	-	-	-
		S.arizonae	3	3	-	-	5	2
		Y. enterocolitica	-	1	-	-	-	1
		Providencia stuartii	-	-	1	-	-	-
		Pantoea	1	-	-	1	-	-

Proteobacteria		*agglomerans*						
	Moraxellaceae	*A.baumanii*	-	1	-	-	-	-
		A.lwoffi	3	-	-	-	-	-
		A.haemolyticus	-	-	-	2	-	-
		Moraxella sp.	-	-	1	1	-	-
	Aeromonadaceae	*A.hydrophila*	**3**	**5**	**3**	**3**	**15**	**7**
		A.caviae	2	-	-	-	-	-
		A.veroni bio sobria	-	2	1	-	-	-
	Vibrionaceae	*Vibrio alginolyticus*	-	-	-	-	1	-
	Pseudomonadaceae	*Ps. aeruginosa*	-	1	-	-	-	-
	Pasteurellaceae	*Actinobacillus sp.*	-	-	-	1	-	-
		P. multocida	1	1	-	-	-	-
Proteobacteria/β Proteobacteria	Burkholderiaceae	*B.pseudomallei*	3	4	-	4	2	-
		B.cepacia	-	-	-	1	-	2
		Total	**23**	**27**	**23**	**28**	**37**	**20**

Table 2: List of isolated bacteria from Biga stream stations.

Phylum/Class	Familia	Bacterial species	Stations				
			I (n=64)	II (n=82)	III (n=64)	IV (n=63)	Total
Proteobacteria/ γ Proteobacteria	Enterobacteriaceae	*Escherichia coli*	5	12	11	7	35
		E.coli-inactive	2	-	-	1	3
		E.gergoviae	1	2	-	2	4
		Klebsiella pneumoniae	1	2	3	3	8
		K.oxytoca	-	3	2	4	9
		Citrobacter diversus	-	-	-	1	1
		Serratia liquefaciens	1	-	-	1	2
		S.rubidaea	-	1	-	-	1
		Salmonella arizonae	-	-	-	1	1
		Proteus mirabilis	1	1	-	-	2
	Moraxellaceae	*Acinetobacter baumanii*	-	-	-	1	1
		Moraxella sp.	-	1	-	-	1
	Shewanellaceae	*Shewanella putrefaciens*	-	2	-	-	2
	Pasteurellaceae	*Pasteurella multocida*	3	8	6	3	20
		Actinobacillus sp.	-	2	-	-	2
	Aeromonadaceae	*Aeromonas hydrophila*	27	26	29	16	98
		A.caviae	3	2	3	7	15
	Vibrionaceae	*Vibrio carchariae*	2	-	-	-	2
	Pseudomonadaceae	*Pseudomonas fluerescens*	1	-	-	-	1
		P.fluerescens 25^{0}C	2	1	-	2	5
		Ps. aeruginosa	9	9	3	2	23
Proteobacteria/β Proteobacteria	Burkholderiaceae	*Burkholderia pseudomallei*	6	9	7	11	33
		B.cepacia	-	-	-	1	1
Bacteroidetes/ Flavobacteria	Flavobacteriaceae	*E.meningosepticum*	-	1	-	-	1

n: Number of isolated bacteria

The presence of enzymes that determined virulence factors for bacterial strains was identified: DNase, amylase, lipase, haemolysin and protease were shown in Table 3. With reference to, *A. hydrophila*, *A.caviae* and *B. pseudomallei* are thought to carry a high virulence factor due to their high DNase, amylase activity in both animal and water samples. However *S. rubidae, Y. enterocolitica, V. carcharia, P.fluorescens 25°C, S. putrefaciens, E.meningospeticum* which were isolated from all samples (animal and water) have no enzymatic activity.

Table 3: Enzymatic activity of Gram negative bacteria isolated from animal and water samples.

Isolated bacteria	Enzymatic activity				
	DNase W/A-R (%)	**Amylase W/A-R (%)**	**Lipase W/A-R (%)**	**Protease W/A-R (%)**	**Hemolysis W/A-R (%)**
E.coli	14,70/20	-/20	-/-	-/20	25/18,18
E.gergoviae	-/9,09	25/18,18	-/18,18	-/36,36	-/7,69
K.pneumoniae	-/-	33,3/-	-/20	-/-	-/-
K.oxytoca	-/7,69	-/30,76	-/46,15	-/38,46	25/18,18
E.coli-inactive	-/-	-/-	-/100	-/100	46,15/-
C.diversus	100/14,28	-/28,57	-/14,28	-/-	-/14,28
C.freundii	-/9,09	-/9,09	-/13,63	-/18,18	-/13,63
S.marcescens	-/-	-/20	-/40	-/-	-/-
S. rubidae	-/-	-/-	-/-	-/-	-/-
P.mirabilis	-/100	-/-	/100	/100	-/-
A.baumanii	-/-	-/-	-/-	-/-	-/7,69
A.lwoffi	-/-	-/-	-/33,33	-/-	-/33,33
A.haemolyticus	-/-	-/-	-/-	-/-	-/53,84
P. stuartii	-/-	-/-	-/-	/100	-/-
P.agglomerans	-/50	-/-	-/50	/50	-/-
S.liquefaciens	-/-	-/-	-/100	/-	-/23,07
S.arizonae	-/7,69	-/7,69	-/53,84	-/23,07	-/-
Y. enterocolitica	-/-	-/-	-/-	-/-	-/-
P.multocida	20/-	-/50	-/50	-/-	-/-
A.hydrophila	**49,23/27,77**	**36,92/52,77**	**-/58,33**	**-/36,11**	-/-
A.caviae	**14,28/100**	**14,28/50**	**-/100**	**-/50**	-/-
A.veroni bio sobria	-/33,33	-/33,33	-/100	-/66,66	-/-
B.pseudomallei	**28,57/61,53**	**42,85/53,84**	**-/92,30**	**-/38,46**	-/-
B.cepacia	-/-	-/66,66	-/33,33	-/33,33	-/-
Actinobacillus sp.	-/-	-/100	-/100	-/-	-/-
V. alginolyticus	-/-	-/100	-/100	-/-	-/-
V. carcharia	-/-	-/-	-/-	-/-	-/-
Moraxella sp.	-/-	-/100	/50	-/-	-/-
Ps. aeruginosa	-/100	13,04/100	/100	/100	-/-
P.fluorescens	-/-	10/-	-/-	-/-	-/-
P.fluorescens 25°C	-/-	-/-	-/-	-/-	-/-
S. putrefaciens	-/-	-/-	-/-	-/-	-/-
E.meningospeticum	-/-	-/-	-/-	-/-	-/-

(-): Not found any bacteria; **W**: Water samples; **A-R**: Amphibian-Reptilian samples.

4. 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 [9]. In our study, the microbial flora of reptilian and amphibian has a considerably wide microbial spectrum, including potential pathogens. In Turkey, investigations about the reptilian and amphibian microbial flora are very limited [7,8,9]. 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.

Several studies have isolated Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae etc. from reptilian and amphibian oral and cloacal samples [4,7,8,12]. *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, septicemic cutaneous ulcerative disease and septicemia, skin disease and red leg syndrome in reptiles and amphibians [4,5,6,11,17]. 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 *Salmonella* with a host gives rise to a number of clinical presentations including inapparent infection, recovered carrier state, enteritis, septicemia, and combinations of disease syndromes [12]. However, due to the absence of clinical symptoms, these species are still likely to originate from a physiological commensal population (especially in gut) [14]. 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. [14] 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.

Some strains from the water and animal samples with high microbiological contamination levels were isolated and they were seeded on different enzymatic substrates to emphasize the virulence factors. It has been reported that the pathogenicity of the bacteria is correlated with some virulence factors and there is a positive correlation between disease formation and some extracellular products (hemolysin, enterotoxins, etc.) [19]. For this reason, DNase, hemolytic, proteolytic, lipolytic, amylase activities were investigated in order to reveal the pathogenicity of some isolated bacterial species. Most of the studies on amphibian-reptilian species have focused gastrointestinal microbiota, pathogenic or potentially pathogenic species, and knowledge of the enzymatic activities of these bacteria are very limited.

Bacterial pathogenicity is considered as biochemical mechanism by which microorganisms determine the appearance of pathology. Not all pathogens have this capability as a multifunctional complex. The presence of enzymes involved in the detection of the integrity of tissue invasiveness and DNase, in some strains analysed, may explain their ability to invade eukaryotic cells, to escape from fagosomi and to outsource in infected cells, thereby producing systemic infections [1].

5. CONCLUSION

The data obtained in our study show that bacterial strains isolated from amphibian-reptilian strains have much higher enzymatic activity than those isolated from water samples. These results suggest that under any biotic or abiotic stress amphibian and reptile species in the region are facing high risk of microbial disease.

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