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Identification of Antibiotic and Heavy Metal Susceptibility, Bacterıa Isolated from Crayfish (*Astacus Leptodactylus*) of Aras Dam

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ABSTRACT

The purpose of this study was to investigate the presence of Aeromonas hydrophila in freshwater crayfish on the river Aras Dam and to determine the in-vitro antimicrobial susceptibility and Heavy metal resistance of isolates. In total, 150 Crayfish samples were collected and 22.66% of them were positive for A. hydrophila. The A. hydrophila were recovered from Crayfish, cultured on TSA and cytophaga agar and identified by biochemical tests. Detection of drug susceptibility was determined using disc diffusion method to Ciprofloxacin (5 µg), Trimethoprim (5 µg), Chloramphenicol (30 µg), Amikacin (30 µg), Clindamycin (2 µg), Oxacilin (1 µg), Ampicillin (10 µg), Gentamicin (10 µg), Tetracycline (30 µg), Vancomycin (30 µg), Ofloxacin (5 µg), Streptomycin (µg), Kanamycin (30 µg), Imipenem (10 µg) and Cefazolin (30 µg). Most of the isolates showed multi-drug resistance to two or more antibiotics. Trimethoprim, Imipenem and Tetracycline, were the most sensitive drugs with 100% efficacy whereas Oxacilin, Ampicillin and Vancomycin were the most resistant drugs having 94.12%, 47.05% and 32.35% resistance, respectively. There was low resistance against Ofloxacin (14.71%) and gentamicin (5.88%). Most isolates were tolerant to different concentrations of various heavy metals, as evidenced by their MICs ranging from 6.25 µg/mL to >3200 µg/mL.

Keywords: Antibiotic and heavy metal Susceptibility, *bacteria*, crayfish

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INTRODUCTION

Freshwater crayfish (*Astacus leptodactylus*) is an important economic fisheries resource of Aras reservoir, Iran. The infection of freshwater crayfish by gram negative and gram positive bacteria are common in natural and cultural environments. These bacteria are considered as secondary disease agents or opportunities. Freshwater crayfish (Astacus leptodactylus) of Aras reservoir is considered as one of the important economy aquatic animal resources of Iran. It provides a luxury and delicious but expensive meal in most countries. Such as other aquatic animals, A. leptodactylus is treated with a variety of biotic and abiotic factors [1]. Also, harmful biotic factors to A. leptodactylus are classified as viruses, fungi, bacteria, rickettsia like organisms, protozoa and metazoan. Among these, Fungi (specially, *Aphanomyces astasi* which cause plaque) and viruses are the most harmful groups. In spite of long-term research on A. leptodactylus pathogens and other symbionts and or commensals, the pathology as well as geographic distribution has remained unclear [2]. Many pathogens can impact this species in natural habitat. Among these pathogen bacteria *Aeromonas, hydrophila, Citobacter spp. Flavobacterium spp*. plays important roles

in susceptibility of disease in Crayfish [3-55 presented a significant effect of bacterial pathogens on losing the yields in aquaculture. The research indicated that the numerous genera of bacterial species including gram negative (Acinetobacter, Aeromonas, Citrobacter, Flavobacterium, pseudomonas and Vibrio) and gram–positive (Corynebacterium, Bacillus, Micrococcus and staphylococcus) species isolated from freshwater Crayfish [3]. These bacteria are responsible for heavy economic losses (high mortality and deterioration of quality) in throughout of the world [6, 7]. Studies demonstrated that disease is only occurred when the host affected by the stressor factors, such as high density, poor nutrition and so on [8]. Among Aeromonas spp. A. hydrophila is an important bacterial pathogen as it infects both aquatic animals and humans [9]. Under poor conditions, such as high density, lack of foods or poor water quality, Aeromoniasis appears in Crayfish [10]. Aeromonas can be found in soil, fresh and saline water, drinking water and animal faeces [11]. Currently, there is limited information regarding the prevalence of A. hydrophila in Crayfish in Iran.

MATERIALS AND METHODS

Bacteria Isolation

During the year 2014, 150 *Astacus leptodactylus* samples captured from Aras Dam with conical traps randomly. All live samples were transported to laboratory and maintained in plastic vans with aeration for microbial studies. Haemolymph of *A. leptodactylus* samples were inoculated to bacterial culture media. Antenna or 5th thoracopods were disinfected with 70% alcohol and sliced and 1-2 drop of infiltrated haemolymph was cultured on blood agar, TSA and cytophaga agar (Merck) mediums linearly under sterile condition. Cultured mediums were incubated in 22-25ºC for 36-72 h and controlled for bacterial growth daily and primary identification was carried on gram staining of prepared slides. Then, grown bacteria were purified with secondary cultures and finally bacteria were identified based on biochemical (Motility, Nitrate, Gelatin, Citrate, Haemolysis, H_2S, Oxidase, Catalase, Indol, Urease, OF, MR, VP, O/129 and Sugar fermentation) tests [12-13].

Antimicrobial susceptibility of A. hydrophila isolates by disc diffusion test

Antibiotic susceptibility was performed according to the national Committee for Clinical Laboratory Standards (CLSI, 2007) method on Mueller–Hinton Agar (Difco) by the disc diffusion method [14]. Resistance to the following antibiotics (Rosco-DK) of *A. hydrophila* strains (10^6 CFU/ml) was tested with discs containing Ciprofloxacin (5 µg), Trimethoprim (5 µg), Chloramphenicol (30 µg), Amikacin (30 µg), Clindamycins (2 µg), Oxacilin (1 µg), Ampicillin (10 µg), Gentamicin (10 µg), Tetracycline (30 µg), Vancomycin (30 µg), Ofloxacin (5 µg), Streptomycin (µg), Kanamycin (30 µg), Imipenem (10 µg) and Cefazolin (30 µg). Isolates were identified as susceptible, intermediate or, resistant according to the CLSI (2006) guidelines

Determination of the MIC of heavy metals

The MIC for three heavy metals was determined for *Aeromonas hydrophila* strains. The inoculates were prepared and used for agar dilution testing on Mueller–Hinton agar containing Cd^{2+}, Co^{2+} and Mn^{2+} at concentrations ranging from 3.12 µg/mL to 3200 µg/mL. The metals were added as $CdCl_2$, $CoCl_2$, $MnCl_2.4H_2O$ and $ZnCl_2$ (Sigma-Aldrich) then plates were incubated. The MIC was determined as the lowest concentration of metal ion that completely inhibited growth. *Escherichia coli* K-12 was used as the control strain [15].

RESULTS

Bacterial isolation and Antibacterial susceptibility resistance

In investigation of hemolymph of Crayfish (*Astacus leptodactylus*) 58% (87 of 150) samples indicated bacterial growth. Of 87 bacterial genera isolated included *Aeromonas hydrophila, Staphylococcus aureus, Micrococcus luteus* and *Flavobacterium johnsoniae*. (Table 1)

Table 1: Prevalence (%) of isolated bacteria from Crayfish samples

Number of cultured sample	Bacterial growth (%)	Non - growth	*Aeromonas hydrophila*	*Staphylococcus aureus*	*Micrococcus luteus*	*Flavobacterium johnsoniae*
150	87 (58%)	63 (42%)	34 (22.66%)	25 (16.66%)	21(14%)	7 (4.66%)

As indicated in Table 1 the prevalence of bacterial agents in different sites of Aras Dam-Iran. The results of antibiotic susceptibility of the test strains for the various antibiotics and drugs are shown in Table 2. Most of the isolates were resistant to Ampicillin, Oxacilin (ß-Lactams) and Vancomycin but were sensitive to Clindamycins,

Ofloxacin, Cefazolin, Chloramphenicol, quinolone and aminoglycosides. All the isolates were sensitive to Trimethoprim, Imipenem and Tetracycline. The latest agent (Trimethoprim, Imipenem and Tetracycline) showed excellent activity against almost all the isolates of the aeromonas. The most potent Aminoglycosides showing activity against most of the isolates of the major species (*A. hydrophila*) were Amikacin, Gentamicin, Kanamycin and Streptomycin (Table 2).

Table 2: Percentage susceptibility of 34 *Aeromonas hydrophila* isolates from crayfish samples

Antibiotics and interpretation	**Disk diffusion**		
	S	**I**	**R**
	Quinolones		
Ciprofloxacin	52.9	29.46	17.64
	Aminoglycosides		
Amikacin	47.07	38.23	14.70
Gentamicin	79.41	14.71	5.88
Kanamycin	76.48	11.76	11.76
Streptomycin	61.77	17.65	20.58
	Sulfonamides		
Trimethoprim	100	-	-
	Carbapenems		
Imipenem	100	-	-
	ß-Lactam		
Ampicillin	32.37	20.58	47.05
Oxacillin	38.23	29.42	32.35
	Cephalosporins		
Cefazolin (cephazolin)	91.18	8.82	-
	Tetracycline		
Tetracycline	100	-	-
	Lincosamides		
Clindamycin	32.35	50	17.65
	Others		
Ofloxacin	79.41	5.88	14.71
Vancomycin	2.94	2.94	94.12
Chloramphenicol	58.82	17.64	23.54

Heavy metal resistance

Resistance to four heavy metals (Cd2+, Co2+, Mn2+ and Zn2+) was investigated among aeromonas isolates (Table 3). The study showed that Cd resistance was present in 32.5% of aeromonas isolates whereas for Co the level was 41.17%. Resistance to Mn in aeromonas isolates was detected in 41.17% of isolates, furthermore Zn resistance in *Aeromonas* isolates was found in 81.8% of them. Isolates were found to be tolerant to different concentrations of heavy metals, as evidenced by their MICs ranging from 50 µg/mL to >3200 µg/mL. In assessing the range of MICs obtained, a maximum MIC (>3200 µg/mL) was observed for Mn, with a minimum for cadmium (50 µg/mL). Aeromonas strains showed a maximum MIC of 3200 µg/mL for Mn, 1600 µg/mL for Mn, 800 µg/mL for Co, Mn and Zn although 200 µg/mL for Cd, and Zn (Table 3). In Aeromonas isolates a heavy metal resistance pattern was Zn> Mn >Co >Cd (Table 3).

Table 3: Incidence of metal resistance in *Aeromonas* strains isolated from Crayfish in Aras Dam

	n=34	**No. of isolates with MIC (µg/mL)**												Resistant isolates	
		3.12	6.25	12.5	25	50	100	200	400	800	1600	3200	>3200	**n**	**%**
Cadmium							*								
						10	**13**	**1**	**10**					**11**	**32.5%**
Cobalt									*						
									21	**13**				**13**	**38.23%**
											*				
Manganese									**7**	**9**	**4**	**14**		**14**	**41.17%**
Zinc								*							
						1	**10**	**8**	**2**	**13**				**15**	**44.11%**

DISCUSSION

The recent changes in environmental factors combined with the ability of *A. hydrophila* to exist in a diverse set of environments may have allowed *A. hydrophila* to adapt to and occupy a previous non-existing ecological niche.

Faecal contamination of the river from both animal and human sources may have led to the hyper-eutrophication of water [16] This study conducted in three field of study includes isolation of bacteria; investigation of antimicrobial pattern and heavy metal resistance in *A. hydrophila* isolates. The results of 15 different antimicrobial susceptibility test (AST) and susceptibility against 4 heavy metal methods using thirty four A. hydrophila isolates [isolated from 150 crayfish (*A. leptodactylus*)]. In field of isolation, result of our study showed similar results proportional of isolates number) [12].

Antibiotic resistance of Aeromonas species to multiple antibiotics is becoming a serious public health concern in this study. High resistance of Aeromonas to ampicillin and Oxacilin was observed in this study which may be attributed to β-lactamase activity in the resistant isolates. Resistance was observed against Clindamycin amongst isolates. Tetracycline resistance has been reported in Aeromonas species isolated from a river that receives wastewater discharge [17] but there is no any similarity since there was not Tetracycline resistance in related tested strains although among the strains tested, most of the strains were resistant to Vancomycin this is partially supported by Isoken et al [18] and G. Vivekanandhan et al [19]. Different studies have reported the incidence of antibiotic and heavy metal resistance in bacteria from clinical sources [20, 21] and fish from various metal-contaminated environments [22, 23], but there is little specific information on commercial aquaculture and environmental source. Although the tolerance to different concentrations of heavy metals has been reported in various studies is not too similar to our findings [24] but in another study are similar [25]. The heavy metal tolerance observed in our study could be the result of heavy metal contamination with fertilizers and wastewaters since the Aras River passing from different country in case of our study are in agricultural and industrial areas. A substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance [26, 27]. In conclusion, our findings confirm that wastewater from aquaculture contributes to the antibiotic and metal resistance found in the environment and thus may be affecting water quality and thus pose a risk to biodiversity and, in the long run, human health.

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