

Isolation and characterization of different bacterial agents from crayfish (*Astacus leptodactylus*) of Aras reservoir, Iran

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Received: June 2015

Accepted: November 2015

Abstract

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. This study was conducted to investigate infestation condition on freshwater crayfish regarding the bacterial agents by culturing from haemolymph in aseptic condition on Blood Agar, Trypton Soya Agar (TSA) and Cytophaga Agar and was performed by biochemical tests. In this study *Aeromonas hydrophila*, *Staphylococcus aureus*, *Micrococcus luteus* and *Flavobacterium johnsoniae* were isolated and determined. According to the results which was performed for first time on Aras reservoir freshwater crayfish, the percentage of crayfish which carried the bacteria in haemolymph were variable (20.0-58.5%). Also, the gram negative bacteria particularly *Aeromonas hydrophila* were dominant among the bacterial isolates from crayfish.

Keywords: bacterial infestation, *Astacus leptodactylus*, Aras reservoir, West Azarbaijan, Iran

Introduction

Freshwater crayfish (*Astacus leptodactylus*) of Aras

reservoir is considered as one of the important economic 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 (Unestam 1973). 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 (Vogt 1999). Bacteria are usually secondary or opportunistic pathogens, however some strains cause disease when host exposed to unsuitable environmental and biological condition such as crowding, inadequate nutrition and water pollution. Both gram negative and gram positive bacteria with or without clinical signs have been isolated from haemolymph of *A. leptodactylus* from natural habitat or cultural ponds. *Pseudomonas* sp., *Aeromonas* sp., *Acinetobacter* sp., *Flavobacterium* sp. and *Vibrio* sp. have been reported, as well as gram positive bacteria including *Micrococcus* sp., *Staphylococcus* sp., *Bacillus* sp. (Scott & Thune 1986; Fowler & Desmarchelier, 1995; Webster 1995; Edgerton, Owens, Harris, Thomas & Wingfield 1995). Rod shaped gram negative bacteria were prevalent in freshwater crayfish. In *Astacus astacus*, *Cherax quadricarinatus*, and *C. albidusdestructor*, 50 %, 35 % and 77% of observed bacteria were gram negative rod bacteria, respectively (Wong, Fowler & Desmarchelier 1995; Madetoja & Jussila 1996). Bacterial infection in freshwater crayfish can occur with septicemia or

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without any clinical sign. The latter does not show clinical signs or even pathologic injuries and detection is based on sampling from haemolymph and culture in bacterial culture media. The etiology and pathologic importance of the ones without any clinical sign is unclear and have a prevalence between 41 to 100 % (Scott & Thune 1986; Webster 1995; Wong, Fowler & Desmarchelier 1995; Medetoja & Jussila 1996). The major bacterial diseases reported in freshwater crayfish included bacteraemia, rickettsiosis, enteric infection, shell disease, eye necrosis syndrome and gill disease. Clinical signs of bacterial septicemia in freshwater crayfish are included lethargy, decrease in response to stimuli, decrease in muscle tonicity, muscle hardness, bacteriemia, small nodules or granuloma and haemocyte gathering due to defense reaction against bacteria, side standing and histopathologically gathering of haemocytic granules in heart, gills, hepatopancreas, antennal glands, abdomen muscles and connective tissue (Evans, Fan & Finn 1992; Edgerton, Owens, Harris, Thomas & Wingfield 1995). The aim of present study is to determine the isolated bacterial agents from freshwater crayfish (*Astacus leptodactylus*) of Aras reservoir, Iran.

Materials and Methods

During the year 2010, 70 juveniles and adult *A. leptodactylus* samples with different length and weight captured from Aras reservoir with conical traps, randomly. Also some physicochemical water parameters such as temperature, dissolved oxygen, pH, total hardness and E.C. of Aras reservoir water were determined. 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 mediums linearly under sterile condition. Cultured mediums were incubated in 22-25°C 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 and sugar fermentation tests.

Results

Biometrical characteristics

Biometrical characteristics of 70 adult and juvenile *A. leptodactylus* samples from Aras reservoir during 2010, revealed that the mean weight of males and females of samples were 31.39 ± 12.21 and 28.97 ± 11.07 g and the mean length of them were 82.9 ± 11.67 and 103.6 ± 10.56 mm, respectively (Table 1).

Physicochemical water parameters

Some physicochemical water parameters of Aras reservoir were summarized in Table 2. The range of temperature, dissolved oxygen, pH, total hardness and E.C of water were fluctuated in the range of 9.3-23.9 °C, 9.4- 14 mgL⁻¹, 7.4- 8.96, 284- 580 mgL⁻¹ and 247- 1560 $\mu\text{mos/cm}$, respectively.

Bacteriological examinations

The results showed that *Aeromonas hydrophila*, *Staphylococcus aureus*, *Micrococcus luteus* and *Flavobacterium johnsonae* were determined (Fig. 1) that the percent of infested *Astacus leptodactylus* varied between 20.0- 58.5 % which gram negative bacteria especially *Aeromonas hydrophila* was prevalent (Table 3). Also, some biochemical characters of isolated bacteria were summarized in Table 4.

Discussion

Bacterial infestation in freshwater crayfish is common and usually considered as a secondary and opportunistic factor. In any case, different species of both gram negative and gram positive bacteria were identified and isolated from haemolymph of apparently healthy and without any clinical signs freshwater crayfish. The most common isolated bacteria were included *Aeromonas* spp., *Pseudomonas* spp., *Acinitobacter* spp., *Flavobacterium* spp., *Vibrio* spp., *Citrobacteria* spp., *Staphylococcus* spp., *Micrococcus* spp. and *Bacillus* spp., that ethi-

Table 1 Biometrical characteristics of *Astacus leptodactylus* samples.

<i>Astacus leptodactylus</i>		Weight (g)				length (mm)			
Sex	Number	Maximum	Minimum	Mean	SD	Maximum	Minimum	Mean	SD
Male	45	118	4	31.39	12.21	155	60	82.9	11.67
Female	25	78	7	28.97	11.07	150	75	103.6	10.56

Table 2 Some physicochemical water factors of dam reservoir.

Number	Water Factors	Spring	Summer	Autumn	Winter
1	Temperature °C	19.4	23.9	14.0	9.3
2	Dissolved .Oxygen (mg L-1)	9.4	9.8	10.3	14.0
3	Ph	7.4	8.2	8.2	8.9
4	Total Hardness (mg L-1)	286	284	580	424
5	E.C. (µmos/cm)	750	247	280	1560

Table 3 Prevalence of isolated bacteria from studied samples.

Number of cultured samples	Bacterial growth (%)	Non – growth	<i>Aeromonas hydrophila</i>	<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>	<i>Flavobacterium johnsoniae</i>
70	41(58.50%)	29(41.50%)	16(22.85%)	12(17.14%)	10(14.28%)	3(4.28%)

Table 4 Some biochemical characters of isolated bacteria

Bacteria	Gram	Mo-tility	Ni-trate	Gela-tin	Ci-trate	Hae-moly-sis	H2S	Oxi-dase	Cata-lase	Indol	Ure-ase	OF*	MR*	VP*	O/129*
<i>A. hy-drophila</i>	-	+	+	+	+	+	-	+	+	+	-	F	+	+	R*
<i>M. luteus</i>	+	-	-	+	-	+	-	+	+	-	-	O	-	-	
<i>S. aureus</i>	+	-	+	+	+	+	-	-	+	+		F		+	
<i>F. john-soniae</i>	-	-	+	+	-	-	-	+	+	--	+	F	-	-	S*

O/F= Oxidative / Fermentative. MR= Methyl Red. VP= Voges Proskauer. =* O/129 = 2, 4-diamino-6,7-diisopropylpteridine
R= resistant. S= susceptible.

ology and pathologic importance of most of them is unknown. It has been reported that gram negative rod bacteria are prevalent in freshwater crayfish, as in *Astacus astacus*, *Cherax quadricarinatus* and *C. albidusdestructor*, 50, 35 and 77% of observed bacteria were appertained to gram negative rod bacteria, respectively (Alderman & Polglase 1988; Roy 1993; Webster 1995; Edgerton, Owens, Harris, Thomas & Wingfield 1995). Similarly, in this study the identified bacteria were included gram negative bacteria of *Aeromonas hydrophila* and *Flavobacterium johnsoniae* and gram positive bacteria were included *Staphylococcus aureus* and *Micrococcus luteus* which isolated from haemolymph of apparently healthy and without clinical signs *A. leptodactylus* that among them *A. hydrophila* was preva-

lent. Although the cause of bacterial infestation in apparently healthy freshwater crayfish is not clear, it seems that presence of bacteria in aquatic environments and environmental stress were the predominant factors for this condition. Thune (1994) and Medetoja & Jussila (1996) showed the bacterial infestation incidence in healthy cultured freshwater crayfish and intensified the infestation in unsuitable condition such as high temperature, low dissolved oxygen and long-term maintenance on harmful condition.

Some workers have isolated a complex of bacterial species from haemolymph of freshwater crayfish and other crustaceans, especially spiny crab, Penaidae shrimps (Edgerton, Evans, Stephens & Overstreet 2002). Also, some isolated bacteria were

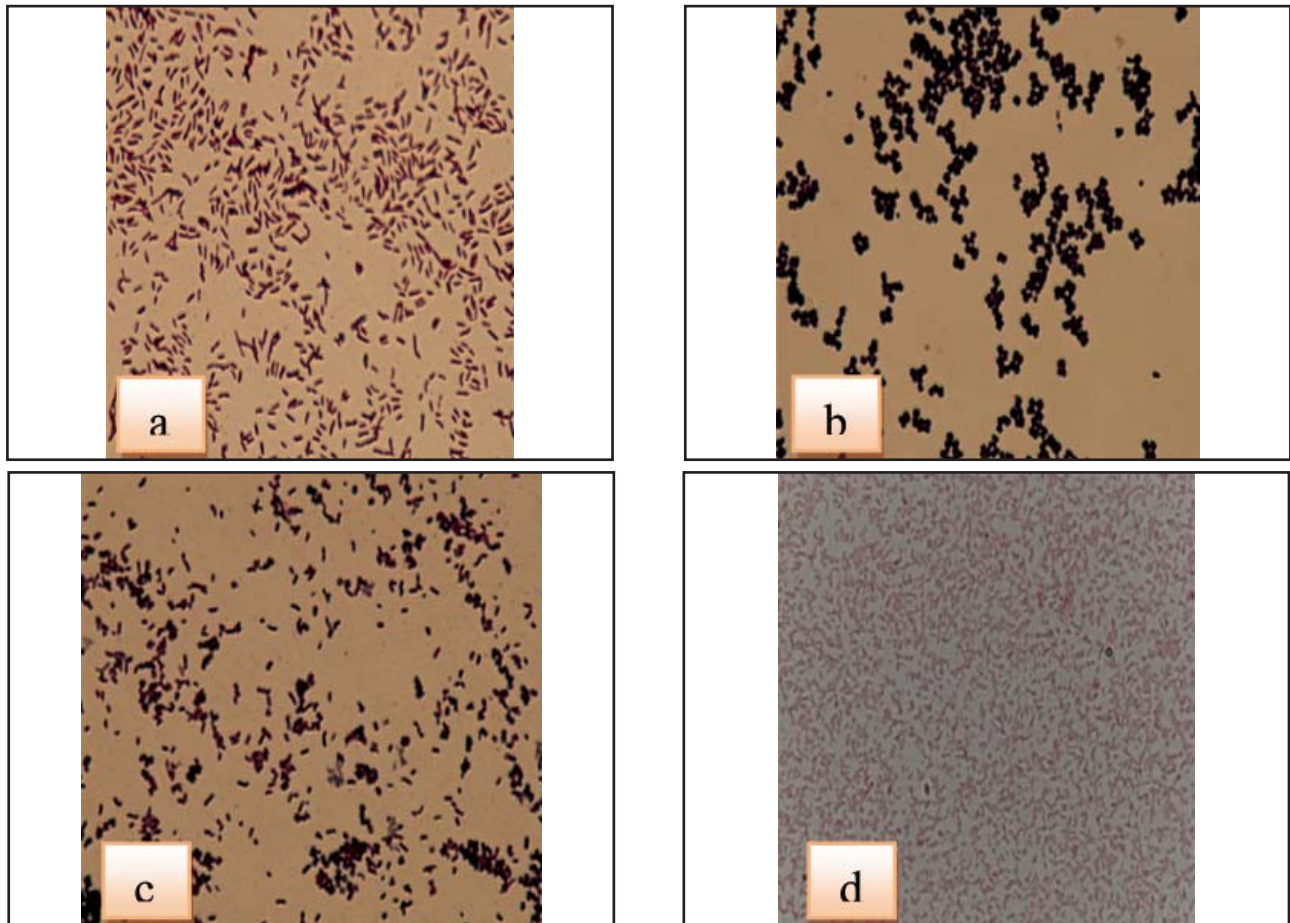


Figure 1 Isolated bacteria from crayfish samples from Aras Reservoir. a) *Aeromonas hydrophila*. b) *Staphylococcus aureus*. c) *Micrococcus luteus*. d) *Flavobacterium johnsoniae*.

reported as *Flavobacterium* spp. in freshwater crayfish with experimental infection (Scott & Thune 1986; Wong, Fowler & Desmarchelier 1995; Madetoja & Jussila 1996; Edgerton, Evans, Stephens & Overstreet 2002). Also, *Micrococcus luteus* and *Staphylococcus* spp. were reported from apparently healthy freshwater crayfish (Wong, Fowler & Desmarchelier 1995). However, it's believed that the presence of bacterial agents in freshwater crayfish haemolymph necessarily is not the sign of disease and crustaceans can tolerate them without harmful effects while disease occurs at unsuitable and stress conditions (Jiravanichpaisal, Roos, Edsman, Liu & Sderhall 2009). In certain cases mortality has reported in unsuitable cultured systems or natural environments from bacterial infections of freshwater crayfish with clinical signs and or even without clinical signs (Edgerton, Evans, Stephens & Overstreet 2002; Quaglio, Morolli, Galuppi, Tampieri, Bonoli, Marcer, Rotundo & Germinara 2006b). *Aeromonas*

hydrophila is a ubiquitous gram negative bacterium of aquatic environment which can cause a large number of diseases in various aquatic animals, crustaceans and human being (Tulsidas, Ong & Chan 2008). It can be the cause of morbidity and mortality in freshwater and marine fishes as a secondary agent with hemorrhagic septicemia. This bacterium is usually isolated from apparently healthy crayfish haemolymph or moribund fish and crustacean (Sung, Hwang & Tasi 2000; Nielsen, Hoi, Schmidt, Qian, Shimata, Shen & Larsen, 2001; Edgerton, Evans, Stephens & Overstreet 2002; Jiravanichpaisal, Roos, Edsman, Liu & Sderhall 2009). As well as, it has a potential to create disease in freshwater crayfish especially at cultural and unsuitable environments (Quaglio, Morolli, Galuppi, Bonoli, Marcer, Nobile, De Luise & Tampieri 2006a). *Aeromonas hydrophila* and some other bacteria were isolated from experimental re-infected *Pasifastacus leniusculus*, that the most mortality rate was due to

A. hydrophila occurred at 22°C and 6 h after bacterial injection (Jiravanichpaisal, Roos, Edsman, Liu & Sderhall 2009). Therefore regarding the direct entrance of bacteria via environment, injury, gastrointestinal apparatus and haemolymph into body, crayfish containing bacterium can act as a carrier and reservoir of bacterium and change to pathogenic from opportunistic state at unsuitable environmental conditions, molting, stress and immunodeficiency and this can cause a serious threat for freshwater crayfish.

This study carried out on *A. leptodactylus* from Aras reservoir for the first time, revealed that interaction between parasitic, bacterial or commensal agents with host (*A. leptodactylus*) may depend on environmental condition, host immune defense and the intensity of invasion.

This interaction may collide regarding the presence of parasitic and bacterial agents in aquatic environments and the *A. leptodactylus* body surface, impact of human activities, including overfishing, industrial pollution and urban agriculture on aquatic ecosystems. However, recent studies by Mohsenpour Azari (2010) showed the eutrophic condition of Aras reservoir. On the other hand, unfavorable environmental condition, weakened crayfish, reduction of immune defenses, along with other factors, can cause viral, bacterial, parasitic and fungal diseases and damage to the aquatic resources such as crayfish *A. leptodactylus*.

Therefore, long-term monitoring together with good management of water resources can play an important role in decreasing of ongoing challenge.

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آلودگی به عوامل باکتریایی در شاه میگوی دراز آب شیرین سد ارس (*Astacus leptodactylus*)

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چکیده

شاه میگوی دراز آب شیرین سد ارس از منابع آبی مهم اقتصادی کشور محسوب می‌شود. آلودگی به عوامل باکتریایی گرم منفی و گرم مثبت در شاه میگوهای دراز آب شیرین در محیط‌های پرورشی و طبیعی شایع و متداول بوده و بیشتر به عنوان عوامل بیماری‌زای ثانویه و فرصت طلب مورد توجه قرار می‌گیرند. این پروژه با هدف بررسی وضعیت بهداشتی شاه میگوی ارس از لحاظ آلودگی به عوامل باکتریایی بر روی ۷۰ قطعه شاه میگو بطریق کشت از همولنف شاه میگوها در شرایط استریل در محیط‌های کشت میکروبی بلاد آگار، تریپتون سویا آگار و محیط آگار سایتو فاگا و انجام تست‌های بیوشیمیایی صورت گرفت و باکتری‌های *Aeromonas hydrophila*، استافیلوکوکوس اورئوس (*Staphylococcus aureus*)، میکروکوکوس لوتئوس (*Micrococcus luteus*)، فلاوباکتریوم جونسونا (*Flavobacterium johnsonae*) شناسائی و جداسازی گردیدند. براساس نتایج بدست آمده در این پژوهش که برای اولین بار بر روی شاه میگوی دراز آب شیرین سد ارس صورت می‌گیرد تعداد شاه میگوهای حامل باکتری در همولنف متفاوت و متغیر بوده و بین ۲۰ الی ۵۸/۵ درصد شاه میگوها را شامل می‌شد. همچنین در بین تعداد شاه میگوهای حامل باکتری و باکتری‌های جداسازی شده باکترهای گرم منفی بویژه *Aeromonas hydrophila* غالب بود.

واژه‌های کلیدی: شاه میگو، آلودگی باکتریایی، سد ارس، آذربایجان غربی.

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