Studies on Prevailing Bacterial Diseases Affecting Freshwater Crayfish (*Procambarus clarkii)* in Eygpt

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Abstract

A total of 200 freshwater Crayfish were collected separately during late summer 2011 and 2012 from different natural water resources of River Nile, Dakahlia governorate. Diseased crayfish showed focal heamorrahges on cuticle, liquifacation of hepatopancreas and/or congestion. Clinical findings and postmortem lesions of naturally infected crayfish were lethargy and erosions associated with softening and darkening of hepatopancreas. The detected isolates were *Aeromonas hydrophila* which were of higher prevalence (35%), *Pseudomonas aeruginosa* in a rate of 3%, *Escherichia coli* in a rate of 5% and Proteus spp in a rate of 5%. Molecular identification revealed that *A. hydrophila* harbor the lip gene at 760bp. Histopathological findings were varied from severe to mild degenerative changes among infected tissues.

Keywords: *Procambarus clarkii, Aeromonas hydrophila, Molecular identification, Histopathological alterations.*

Introduction

The identification and risk assessment of potential biological invaders would provide valuable criteria for the allocation of resources toward the detection and control of invasion threats. Yet, freshwater biologists have made few attempts at predicting potential invaders, apparently because such efforts are perceived to be costly and futile. Crayfish *Procambarus clarkii* is tended to be one of those invaders. They are Arthropods belonging to the order *Decapoda*, family *Astacidae* (*Potamobidae*). These animals are found to live in burrows, below river banks. They are found especially in rivers originating in chalk or lime stone areas; calcium carbonates being an essential constituent of their exoskeleton of these animals ***(Jenny, 2012 and Basilico et al, 2013)***.

Crayfish *Procambarus clarkii* is considered the most important crayfish of the 400 species known

in the world. *P. clarkii* seems to have been introduced recently in Egypt. Within the last few years, it has been successfully established in various sites of the River Nile and its branches. Bacteria are normal inhabitant of the gut of freshwater crayfish, in stressed crayfish and/or those infected with a virulent strain. The bacteria may proliferate in the forgut, midgut and in the hepatopancreas. The most frequently Gram-negative genera in crayfish were Pseudomonas, Aeromonas, Acinetobacter, Flavobacterium and Vibrio spp. ***(Scott and Thune, 1986; Wong et al, 1995*** *and* ***Madetoja and Jussila, 1996 and Noonin et al, 2010)***.

The current investigation was planned to study the clinical picture of diseased freshwater crayfish, *Procambarus clarkii*, isolation and identification of the predominant bacterial pathogens using traditional and molecular methods. Besides, histopathological alterations were observed and recorded.

Material and Methods:

Crayfish:

A total of 200 freshwater Crayfish (*Procambarus clarkii*) with an average body weight 70±10 g were randomly collected in summer (in winter seasons, no crayfish were available to be examined) from two different natural water resources of River Nile (Damietta branch) Weesh El-Hagar village and Wish-Damietta- lake Manzala, Dakahlia governorate. They were transferred alive to the laboratory for clinical and bacteriological examinations. In the laboratory, they were put in fully glass aquaria of (80X40X60cm) dimensions. Such aquaria were supplied with sufficient chlorine free water and continuous aeration using electric pump. 5 blocks of poly propylene tunnels (each block consists of 4 stacked tubes: each tube measured 5 diameter and 20cm length), were designed to simulate crayfish environment (to let crayfish hide)

Abnormalities in the behavior of crayfish and clinical alterations indicating disease conditions were recorded. Samples were examined for detection of ulcerations, body darkening, diffuse or focal haemorrhages and cuticle erosions. The examination was done according to ***Austin and Austin (2012)***

Bacteriological investigations:

Crayfish were scarified and directed for bacteriological examination. The body surface was disinfected by swabbing with 70% ethyl alcohol. 0.1 ml hemolymph was collected aseptically as soon as possible avoiding its clotting from the ventral sinus by inserting a sterile needle in the ventral aspect of the membrane between the first abdominal segment and the thorax according to ***Quaglio et al (2006)***. Under complete aseptic conditions, swabs from gills, heapatopanceas and musculature were examined.

The collected swabs were smeared separately onto Tryptic Soya agar then streaked directly on its

selective agar plates and on blood agar to be incubated at 30ºC for 48 h. Each type of suspected colony was picked up and re-streaked on a new plate of selective media then re-incubated at the same temperature and period in order to purify culturing. To detect the type of haemolysis, sheep blood agar (5%) was used. MacConkey agar, Aeromonas Isolation Medium Base with Ampicillin supplement, Pseudomonas Base Media with CN supplement and Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS). Identification of isolated bacteria was carried out by studying morphological characterization of colonies, microscopic examination, and cultural characters according to schemes of biochemical reaction provided by ***Austin and Austin (1999)***.

Molecular identification of *Aeromonas hydrophila:*

A) Isolation of genomic DNA: according to ***Hiney et al (1992).***

B) Polymerase Chain Reaction (PCR) according to ***Cascón et al (1996).***

C) Sequences of DNA (primer) according to ***Raja, et al (2004)***

Histopathological examination:

Specimens from crayfish gills, heapatopanceas and musculature were preserved in Davidson's AFA fixative to be directed for histopathological examination according to ***Bell and Lightner (1988)***.

Table (1): *Showing primer sequence of A. hydrophila lip gene*

Microorganim	Targt gene	bp fragment	Primer sequence (5" - 3')
A. hydrophila	lip gene	760 bp	F- 5'-AAC CTG GTT CCG CTC AAG CCG TTG- 3'

Results

Clinical findings:

Crayfish showed no pathognomonic clinical signs but after they had been left for one month in dechlorinated oxygenated water; to enhance stress conditions, some red patches and cuticle erosions appeared. By the end of 4th week, only 87 among 200 showed lethargey and recumbancy with postural abnormalities associated with focal heamorrahges on cuticle, erosion of walking legs. Also, darkening and softening of hapatopanceas were observed (plate 1)

Prevalence of different bacterial isolates:

Table (2) illustrates bacteria isolated from both diseased and apparently healthy crayfish where *E. coli* isolates were identified in a rate of 5%, *Ps. aeruginusa* in a rate of 3% and *A. hydrophila* in a rate of 35% and Proteus spp. In a rate of 5%.

Histopathological findings:

Gills showed severe heamolysis filling the secondary lamellae with somewhat inflammatory cells associated with edema. As severity

of infection increased, some secondary lamellae appeared shorten and denuded with dissociation of chloride cells, others appeared longer with oedema and inflammatory cells, while, musculature showed degeneration and vacuolation of myocytes. Some inflammatory cells and splitting of muscle fibers were also showed in. Owing hepatopancrease, it showed extensive necrotic lesions with pycnotic neulei and some inflammatory lymphocytes (heamocytes) were aggregated in the hemal sinuses. Ballooning structure was common appearance of hepatopancreatic acini. Star-like appearance of acini with nearly necrobiotic changes was also noticed; while somewhat thickness of the endothelial cells of basal laminae associated with extensive vacuolation was then recorded. Some vacuoles of cell debris appeared inside or cell debris appeared in lumen of acinus (Plate, 2).

Molecular identification (Polymerase Chain Reaction):

Photo (1) illustrated 6 selective isolates of biochemically identified as *A. hydrophila* harbor the lip gene at 760bp.

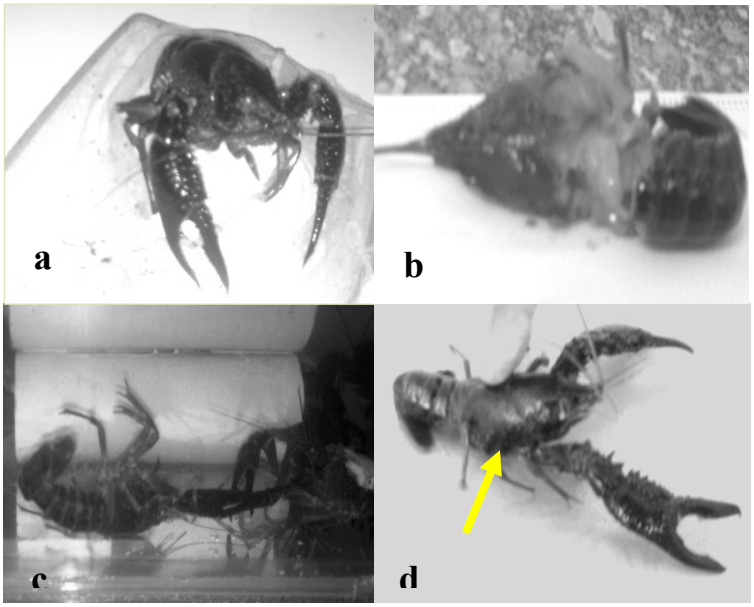

Plate (1): showing clinically diseased *P. clarckii* naturally infected with *A. hydrophila* a: erosion of articulated legs, b: darkening and softening of hepatopancrease, c: lethargy and recumbent on one side, d: red patches on cuticle

Table (2): *Prevalence of different isolated pathogens during late summer*

Type of pathogen	Total No. of crayfish	Diseased (87)		Apparently healthy(113)		Total No. of isolates	
		No.	%	No.	%	No.	%
E. coli	200	2	2.29	8	7.07	10	5
Ps. aeruginosa		3	3.44	3	2.65	6	3
A. hydrophila		25	28.73	45	39.82	70	35
Proteus spp.		2	2.29	8	7.07	10	5
Total		32	36.78	64	56.63	96	48

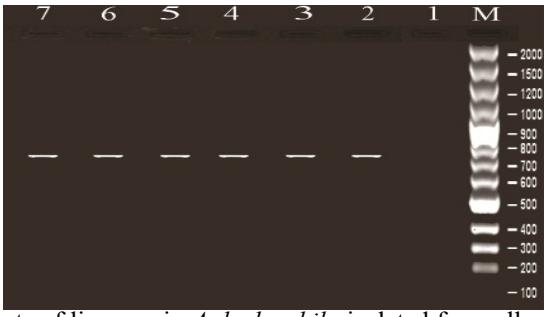

photo (1) PCR fragments of lip gene in *A. hydrophila* isolated from all examined strains.(a): Lane M: 100bp ladder marker, lane 1: control negative, lanes 2-7: *A. hydrophila* isolated from crayfish.

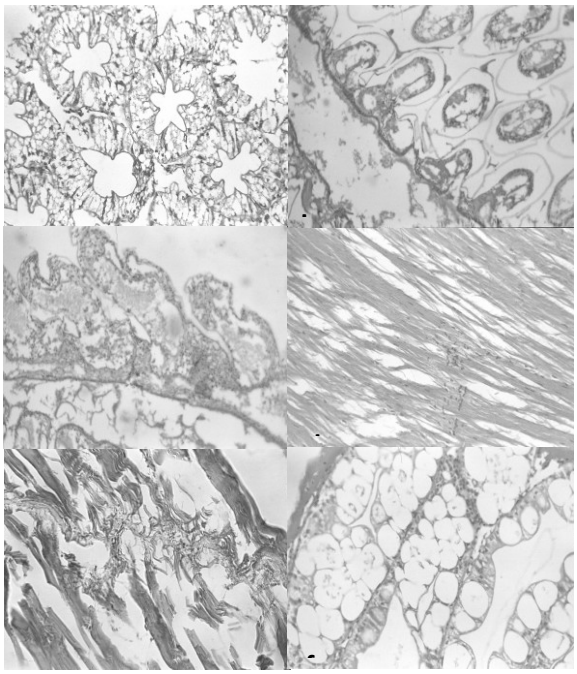

Plate (2): a:hepatopancreas showing star-like appearance with severe vacuolar degeneration. H&E X400. b: gills showing edema and inflammatory cells. H&E X200. c: tips of secondary lamellae of gills showing bifurcation, inflammatory cells and heamolysis. H&E X200. d: musculature showing splitting of its fiber. H&E X400.e: musculature showing disorganization and oedema in between muscle fibers. H&E X400**.** f: hepatopancreas showing ballooning structure of its acinus. H&E X400**.**

Discussion

The most abundant crayfish is *P. clarkii* which had been introduced to Egyptian Nile water; this species is a polytrophic crustacean that can serve as an effective organism in controlling species composition of freshwater ecosystem ***(Ibrahim et al, 1996).*** Bacteria are frequently considered to be secondary or opportunistic pathogens of freshwater Crayfish ***(Lindqvist and Mikkola 1979; Johnson, 1983)***. However, a number of species or strains have been associated with serious mortality, often in holding or purging tanks ***(Thune 1994 and Madetoja and Jussila, 1996)***.

The clinical picture of naturally infected crayfish was anorexic, lethargic and having a weak tail-flick escape response associated with hemorrhagic septicemia, congestion of liver and liquefaction, softening of hepatopanceas with absence of external lesions that agreed with ***Edgerton et al (1995)*** and ***Quaglio et al (2006)***. The most predominant bacterial species isolated from such lesions was *A. hydrophila* (35%). Nearly similar clinical signs were described by ***Roy (1993)*** during experimental infection of the crayfish with *Aeromonas hydrophila*.

In the present study, 70 isolates of *A. hydrophila* were identified from crayfish *Procambarus clarkii* in a rate of 35%, while it was with low rate for *E.coli,* Proteus spp and *Ps. aeruginosa* as 5, 5 and 3% respectively. These results were nearly similar to that found by ***Brett et al (2002)*** *and* ***Khalil and Saad (2013).*** Interestingly, the prevalence of infection in apparently healthy crayfish is higher than that of diseased ones. This points to the great risk of such crayfish to human and other neighbering aquatic animals.

Owing to molecular identification of *Aeromonas hydrophila*, a product size of 760 bp was found to be specific for the detection of *A. hydrophila* by polymerase chain reaction (PCR), *A. hydrophila* was detected by amplification of lip gene, which codes for a thermostable extra cellular lipase of *A. hydrophila*. This result agreed with ***Raja et al (2004) and Eissa et al (2015)***.

Regarding histopathological findings, gills of crayfish were ranging from somewhat lymphocytic infiltration (hemocytic) with degradation of necrosis and degenerated epithelial lining lamellae were seen that can be due to virulence of bacteria. The swelling and fusion lamellae, abnormal gill tips, degeneration and necrosis and clavate, globate lamellae of gills that is due to toxins of *Aeromonas hydrophila*. Our results agreed with ***Saravona and Geraldine (2000)***. Crayfish heapatopancreas showed, slight heamocytic infiltration and heamolysis in the interstitial sinuses, thickening of basal laminae. Severe vacular degeneration of the acini tubular

cells associated with necrosis of the hepatopancreatic acinus was also noticed. Such lesions could be contributed to bacterial toxins. These results were in agreement with the results obtained by ***Jiravnichpaisal et al (2009)***. Musculature of crayfish showed zinker necrosis, disorganization of muscle fibers with edema between muscle bundles and splitting between muscle fiber were the same observations obtained by ***Robert (2001)***. Studies obtained by ***Jiravnichpaisal et al (2009)*** showed that the decreasing number of lymphocytes (heamocytes) in crayfish due to destruction by toxin of *Aeromonas hydrophila* could lead to subversion of immunological function in host and finally got diseased or dead.

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