بسم الله الرحمن الرحيم

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Role of crayfish in transmission of fish diseases

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B. V. Sc Assiut University 2004

A thesis submitted for Faculty of Veterinary Medicine Assiut University for fulfillment of the requirements of the Master degree

(Fish diseases and management)

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To My Father

Thanks for being my father

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Thesis Abstract

Role of crayfish in transmission of fish diseases

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Supervised by

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The aim of this study is to investigate most common bacterial and fungal pathogens that infect red swamp crayfish, *Procambarus clarkii*, in Egypt and make it a source of infection to fish, other aquatic animals and also to human.

Red swamp crayfish, *Procambarus clarkii* was introduced to River Nile, Egypt through a commercial aquaculture in Giza (Manial-Sheiha), in the early 1980's (Fishar, 2006). They greatly spread all over the River Nile and its tributaries. Data available about these conditions and pathogens that infect this newly introduced species are scanty.

Forty eight crayfish were collected from River Nile and its tributaries in Assiut governorate subjected to clinical, postmortem and bacteriological and mycological examinations. Bacteriological examination revealed the following; *Enterobacteriaceae* (63.4%), *Vibrio mimicus* (4.95%), *pseudomonas fluorescens* (3.96%) and *Aeromonas hydrophila* (1.98%) were isolated from hemolymph and digestive gland of crayfish. The Fungal isolates obtained from gills and uropods of collected samples were *Rhizopus spp.* (51.3%), *Aspergillus niger* (25%), *Penicillium spp.* (7.9%), *Fusarium oxysporum* (6.6%), *Humicola*

spp.(5.3%), *Aspergillus flavus* (1.3%), *Aspergillus fumigatus* (1.3%) and *Fusarium proliferatum* (1.3%). Identification was done according to microscopical and biochemical characters.

Experimental infection was carried out to investigate the pathogenicity of *Aeromonas hydrophila* isolate. A dose of (1.6×10^8 CFU/ml) was injected in the hemocoel and hemolymph of red swamp crayfish. Hemocoel injection resulted in mortality of 100% within 9 hours post injection, while hemolymph resulted in 80% mortality within 12 hours.

Clinical signs and postmortem lesions of crayfish injected with *Aeromonas hydrophila* included weakness, lethargy, loss of tail reflexes, greenish coloration of digestive gland with translucent gelatinous material covering and congestion of the tail musculature. While no obvious clinical signs were observed in naturally infected crayfish. This demonstrated the harmful and the dangerous role that red swamp crayfish can play in transmission of pathogenic bacteria and fungi to wild and farmed fishes.

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Introduction

Freshwater crayfish aquaculture is a rapidly growing industry in the United States, Spain, China and Turkey and a small expanding industry in Australia. The largest producer of farmed crayfish is the United States of America, with a harvest ranging from 15,000 to 25,000 metric tons annually, mostly from the state of Louisiana. The annual farm-gate value for the Louisiana crayfish industry is between US$30 and $50 million (Edgerton *et al.*, 2002).

Two main product forms of farmed freshwater crayfish are sold on international markets; hatchling and juvenile crayfish for stocking ponds or for the aquarium trade and hard shell crayfish along with a range of products derived from hard-shell animals. Crayfish are sold alive, fresh-chilled, whole cooked crayfish or crayfish products, especially peeled “tail” meat (Edgerton *et al*., 2002).

Crayfish have high nutritional value, each 100 g serving weight contain 87 calories, 137 mg cholesterol, 17.52 g protein. They also rich in vitamins; vitamin A 50 IU, Retinol 15 mcg, vitamin C 0.5 mg, thiamin 0.047 mg, Riboflavin 0.080 mg, Niacin 1.667 mg, vitamin B6 0.134 mg, vitamin B12 3.10 mcg, Folate 11 mcg and pantothenic acid 0.512 mg (*USDA* 2009).

Crayfish are also rich in minerals; calcium 51 mg, iron 1.11 mg, magnesium 33 mg, phosphorus 241 mg, potassium 238 mg, sodium 97 mg, zinc 1.48 mg, copper 0.580 mg, manganese 0.217 mg and selenium 34.2 mcg (*USDA* 2009).

The red swamp crayfish, *Procambarus clarkii* was recently introduced to Egyptian ecosystem through a commercial aquaculture in Giza (Manial-Sheiha), in the early 1980's when first immigrants of this

species were introduced from USA and the project was shortly terminated due to administrative failure. (Fishar, 2006).

It is an autochthonous species from northeast Mexico and south central US (Hobbs *et al*.,1989). They are typically found in warm freshwater rivers, slow flowing water, reservoirs, irrigation systems and rice fields. Crayfish cause a huge economical impact when introduced into a suitable habitat as quickly become a keystone species and cause dramatic changes in native plant and animal communities (Fishar, 2006).

Procambarus clarkii also leads to great economical losses in fish aquaculture as *P. clarkii* feeds on any kind of fish depending on the facility of catching the prey (Ibrahim *et al*., 1995).

They also reduce the value of freshwater habitats due to its activity. If *P. Clarkii* presents in irrigation structures, it causes economic losses due to its burrowing activity which alters the soil hydrology causing water leakage and damaging of rice plants due to its feeding (Correia, 1993; Holdich, 1999).

In Egypt a little is known about bacterial and fungal pathogens in crayfish, therefore this work was planned to fulfill the following objectives:

- Isolation and identification of bacterial pathogens of *Procambarus clarkii*.
- Isolation and identification of fungal pathogens *of Procambarus clarkii*.
- Full description of clinical signs and postmortem lesions of naturally and experimentally infected *P. clarkii* with *Aeromonas hydrophila.*

Review of literatures

Introduction in Egypt:

The red swamp crayfish, *Procambarus clarkii* is an autochthonous species from northeast Mexico and south central US (Hobbs *et al*.,1989). They are typically found in warm freshwater rivers, slow flowing water, reservoirs, irrigation systems and rice fields.

Introduction of *P. clarkii* to River Nile, Egypt ; was through a commercial aquaculture in Giza (Manial-Sheiha), in the early 1980's when first immigrants of this species were introduced from USA and the project was shortly terminated due to administrative failure. (Fishar, 2006).

P. clarkii belongs to phylum *Arthropoda*, Subphylum *Crustacea* and family *Cambaridae*. The Size of *P. clarkii* ranges from 8 to 13 cm in length. It has a dark red or reddish brown bordering in black hard outer skeleton with a wedge shaped stripe on the abdomen (Carapace), which protects the body and makes it rigid. Pincers are narrow and long. Eyes are colored black. The rostrum has lateral spines or notches near its tip. Young are greenish brown with some faint pink pigmentation. A wide mid-dorsal faint brown strip extends on the abdomen on either side by a dark line (Fishar, 2006).

The palm of cheliped of River Nile *P. clarkii* comes with a raw of tubercles along the mesial margin. The chela is elongate. There are hooks on the ischea of male at the 3rd and 4th pereiopods. A male first pleopod terminates in four elements, and the cephalic process is strongly lobate with a sharp angle on the caudodistal margin that is lacking subapical setae. The setae have a strong angular shoulder on cephalic margin that is

quite proximal to terminal elements. The right pleopod is wrapped around the margin to appear reduced or absent (Hamdi, 1994).

Sexually mature crayfish mate in open water in late spring and early summer. The female digs burrows in dry banks to lay eggs. Each female produces from 200 to 400 young crayfish. The embryonic development is temperature dependant with an inverse relationship. The period from egg laying till hatching take ; 20 days (20-21 C) in march, 17 day (23-26 C) in April and may and 11-14 days (26-29 C) during late September and mid October (Fishar, 2006).

Procambarus clarkii feeds on any kind of fish depending on the facility of catching the prey. Plant detritus, living plant material such as Elodea and smart weeds, living animals such as earthworms and small fishes are edible (Ibrahim *et al*., 1995).

The development of the hatched young of *P.clarkii* is carried out through 7 successive stages. After the young hatch, metamorphosis take place, followed by 2-3 weeks of voracious eating. After this they molt and again assume their immature appearance. Egg production completed in 6 weeks and maturation takes 8 weeks. They molted periodically, young crayfish shed every 10 days and adult shed 2-3 times per year (Hunner and Barr, 1991).

Procambarus clarkii has a wandering phase, without any daily periodicity, characterized by short peak of high speed of locomotion and a longer stationary phase, during which crayfish hide in burrows at day, emerging only at dusk of forage. Other behaviors take place at night (Gherardi and Barbaresi, 2000).

Crayfish cause a huge economical impact when introduced into a suitable habitat as quickly become a keystone species and cause dramatic changes in native plant and animal communities (Fishar, 2006).

Procambarus clarkii also reduces the value of freshwater habitats due to its activity. If *P. clarkii* present in irrigation structures, it causes economic losses due to its burrowing activity which alters the soil hydrology causing water leakage and damaging of rice plants due to its feeding (Correia, 1993 ; Holdich, 1999).

Bacterial diseases of freshwater crayfish:

1- Asymptomatic Bacteremia and Bacterial Septicemia :

This disease is caused by bacterial species from numerous genera including both gram negative (*Acinetobacter, Aeromonas, Citrobacter, Flavobacterium, Pseudomonas* and *Vibrio*) and gram positive (*Corynebacterium, Micrococcus* and *Staphylococcus*) species (Edgerton *et al.*, 2002).

It is characterized by the presence of a mixed bacterial population in hemolymph samples collected under aseptic conditions and cultured in appropriate media, usually blood or nutrient agar (Scott and Thune, 1986; Wong *et al*., 1995; Webster, 1995 ; Madetoja and Jussila, 1996).

The most frequently reported gram negative genera were *Pseudomonas morganii, P. aerogenes* and *Proteus vulgaris* (Toumanoff, 1968) , *Pseudomonas florescens* and *P. putida* (Vey *et al*., 1975) , *Pseudomonas spp*. (McKay and Jenkin, 1969), *Vibrio mimicus* and *V. cholerae* (Thune *et al*., 1991) and *Aeromonas hydrophila* (Edgerton *et al*., 1995). They have been consistently isolated from crayfish displaying

gross clinical features of bacterial septicemia. On the other hand *Micrococcus* and *Staphylococcus* were the most often reported gram positive genera (Edgerton *et al*., 2002).

The bacteria were not normally present in crustacean hemolymph, but as a result of exposure to environmental stressors (Bang, 1970; Lee and Pfeifer, 1975 ; Johnson, 1976). The prevalence of bacteremia in healthy, pond reared crayfish has been shown to increase during periods of elevated temperature and low dissolved oxygen (Scott and Thune, 1986 ; Thune, 1994). It varied from 41-100% (Scott and Thune, 1986; Webster, 1995; Wong *et al*., 1995; Madetoja and Jussila, 1996).

Increasing the time of holding crayfishes in laboratory tanks under suboptimal conditions, increased the prevalence of bacteremia in tested animals. So that the samples of hemolymph for bacterial culture in apparently healthy animals were usually taken after the crayfishes were held in aquaria for extended periods to evoke stress (Madetoja and Jussila ,1996).

Bacterial septicemia is an opportunistic infection by mildly pathogenic strains of ubiquitous bacteria that enter the hemocoel through the oral route or through wounds, proliferate in the hemolymph and then multiply in body tissues (Vey *et al*., 1975; Johnson, 1983 ; Alderman and Polglase, 1988).

The bacteria inhabited the ecosystem in which the crayfish live and were found in water and sediments, resident on the exoskeleton or in the gut. They enter the hemocoel through minor wounds, gastrointestinal tract and other different routes (Tubiashi *et al*., 1975; Davis and Sizemore, 1982).

Wound infection was also recorded; pathogenic *pseudomonads, Pseudomonas florescens* and *P. putida* were used; wounded crayfish were putted in water containing the bacteria and infection was induced successfully (Vey *et al*., 1975). Infections via the oral route were also achieved; *Vibrio mimicus* isolates found in the hemolymph of crayfish in asymptomatic bacteremia were primary pathogens, capable of causing disease in unstressed, healthy aquatic animals (Wong *et al*., 1995).

There have been no reports of mass mortalities in crayfish culture from bacterial septicemia in well managed ponds although significant mortalities when culture conditions were suboptimal were observed (Thune *et al*., 1991; Eaves and Ketterer, 1994).

Vibrio, Acinetohacter, Aeromonas, Arthrobacter, Bacillus, Corynebacterium, Flavobacterium, Pseudomonas were isolated from the hemolymph of apparently healthy red swamp crawfish *Procambarus clarkii* exposed to temperatures exceeding 24 °C. Below this temperature the prevalence of bacteria was low, but at temperatures above 28°C the prevalence and numbers of bacteria increased significantly due to temperature effect on the crawfish's ability to control bacterial growth in the hemolymph (Scott and Thune, 1986). Bacterial septicemia in the red swamp crawfish associated with infection with a single bacterial species identified as either *Vibno mimicus* or *V. cholera* was observed (Thune *et al.,* 1991). Crayfish from 13 of the 15 cases investigated, had been held at temperatures of 25°C or higher, with low dissolved oxygen concentrations. *V, mimicus* appeared to be an opportunistic pathogen causing a systemic infection following stress (Lorraine and Peter, 1994).

Vibrio mimicus were isolated from mortalities in cultured red claw crayfish *Cherax quadricarinatus* died due to systemic infection. Two

different strains of *Vibrio mimicus* were isolated (Lorraine and Peter, 1994).

Thier experiment was done in November 1989; three dead red claws from a commercial crayfish enterprise in northern New South Wales containing a hatchery and ponds were frozen and submitted for examination. The crayfish were necropsied and hemolymph and hepatopancreas were sampled for bacteriological examination. Blood agar (containing 5% sheep blood) and Trypticase Soy Agar plates incubated aerobically at 25 C. The cultures were checked for purity and an oxidase test was done on them. The oxidative/fermentation media was inoculated. API 20E and API 20NE was used for identification. Comprehensive identification of *Vibrionaceae* was made.

The authors isolated two strains of *Vibrio species*. The strains were motile, grow on media containing no NaCl and did not grow in media containing 6.5% NaCl, oxidase positive, ferment glucose without production of gas, sucrose negative, indole positive, Vogus proskour negative, hemolyse sheep RBCs in blood agar. A mixed culture of *Escherichia coli* and *Enterobacter intermedium* were also isolated from the hemolymph, and *Aeromonas hydrophila* and *Citrobacter freundii* from the hepatopancreas of one crayfish. The hemolymph and hepatopancreas of the other 2 crayfish yielded pure cultures of an organism initially identified as *Vibrio cholera*. 20NE. However, on comprehensive characterization, these isolates were subsequently identified as *V. mimicus.*

Histologically the heart of one of the crayfish (yielding a pure culture of *V. mimicus*) exhibited severe inflammation of the pericardium and foci of small gram negative rods.

In July 1990, 3 live red claws were submitted from a commercial crayfish farm in southeast Queensland. The farm had a history of low-level mortalities over a 2 years period which increased to 20 % when stocks were transferred to cement tanks. Heaviest losses occurred during hot weather and were initially observed following the addition of hydrated lime to the ponds to raise the pH. Affected crayfish were unable to walk and appeared to be stuck in the residue on the bottom of the ponds. Many had a white powdery coating over their bodies. Two of the submitted crayfish had blistering on the end of the telson and one of them also had blisters on the uropod. The third crayfish exhibited erosion at the end of the telson. Hemolymph from the heart of each submitted crayfish was cultured bacteriologically.

The hemolymph of each crayfish yielded a pure culture of a Gram-negative organism identified as *Vibno mimicus* using API 20E test kits. On histology, different degrees of inflammation were noticed, indicated bacterial septicemia, in the gills and hearts, and also in the antenna1 gland of 1 crayfish were involved. The tail integument exhibited epithelial necrosis and cuticular erosion with evidence of bacterial invasion (Lorraine and Peter, 1994).

Aeromonas spp. was isolated from hemolymph of apparently healthy red swamp crayfish (Scott & Thune, 1986). *Aeromonas hydrophila* was isolated from the hepatopancreas of cultured red claw crayfish *Cherax quadricarinatus* (Lorraine and Peter, 1994). *A. hydrophila* also caused mortality in 80% of control group of crayfish *Pacifastacus leniusculus* within 50 hours at 20±2 C injected by (200 µl of 4 x 10^6 CFU/ml) in crayfish saline buffer (CFS) via the base of fourth walking leg (Liu *et al*., 2007).

Aeromonas hydrophila endotoxins can induce behavioral fever in crayfish *Cambarus bartoni* (Casterlin and Reynolds, 1980). A group of crayfish *C. bartoni* was injected into the abdomen with 10^9 killed *A. hydrophila* was recorded to be pyrogenic to *C. bartoni* (Casterlin and Reynolds, 1977b).

During the summer and early autumn of 2003 (June-October), a total of 102 white clawed crayfish, *Austropotamobius pallipes complex*, from wild populations were collected from streams in the regions of Emilia Romagna and Friuli Venezia Giulia (Northern Italy). The specimens were collected from five different watercourses; the Bidente River (Forlì-Cesena Province, Emilia Romagna), Chiarò, Orvenco, Rieca and Zamlin Creeks (Udine Province, Friuli Venezia Giulia).

During the capture period, only three crayfish were found dead in three different streams (Bidente River, Orvenco and Rieca Creek). Fifty one of the 102 crayfish were placed in four tanks. The density was 12-13 subjects for each tank. The crayfish were maintained in the laboratory in well-aerated water at 15°C and fed twice a week with fragments of vegetables (potatoes and carrots) and fish pellets. Disease outbreaks occurred in two tanks at 2 and 4 weeks respectively.

Affected crayfish were found to be anorexic, lethargic and having a weak tail-flick escape response. In one tank 4 crayfish (33%) were affected and in the second tank, 8 crayfish (61%) were affected.

Bacteriological analysis was performed on a total of 22 crayfish. Samples of 0.1 ml of hemolymph were taken from the pericardial sinus of each crayfish using 1 ml sterile syringes, inoculated on Blood Agar and incubated at 25°C for 24-48 hours.

Subcultures were then incubated on Blood Agar, MacConkey Agar and TCBS (Thiosulphate Citrate Bile Salts) plates, respectively. The identification of the isolates was carried out using the commercial miniaturized API 20E and API 20 NE Systems, and also motility, oxidase, catalase and O/F tests.

Aeromonas hydrophila was present in the hemolymph of 50% of the healthy crayfish taken from Zamlin Creek. *Citrobacter freundii* was found in 87.5% of the moribund crayfish from the second tank; in these specimens, histological examination revealed bacteria in the lumen of the hepatopancreatic tubules, often adhering to the surface of the epithelium. Hepatopancreocytes were more cuboidal than columnar. Multifocal desquamation of degenerated epithelial cells was also observed. Similarly, in some samples the midgut and midgut caecum had focal necrosis. Focal haemocytic infiltrates were found in the haemal spaces adjacent to these necrotic areas and were accompanied by the melanization of the necrotic areas in more advanced stages. In later stages, multifocal granulomas were observed, characterized by central debris inclusive of bacteria, a surrounding amorphous melanin capsule and an outer cellular capsule composed of concentric haemocytes and fibroblasts (Quaglio *et al.,* 2006).

Pseudomonas spp. was isolated from an experimental group of crayfish *Parachaeraps bicarinatus* treated with endotoxin. Many of them became sick and died 2-9 days after infection, the infection was transmissible only by injection , the crayfish in the same tanks with infected ones didn't become sick and the bacteria was isolated from their blood. Pathogenic *Pseudomonas spp*. produce an acute bacteraemic infection, the number of circulating blood cells was reduced and shortly before death there was almost complete absence of circulating

haemocytes. An important point was observed that passage of *Pseudomonas spp.* in crayfish didn't increase the virulence and neither did the repeated subcultures in broth or on agar slopes decrease it (McKay and Jenkin, 1969).

The general clinical signs characterized this diseases are lethargy, reduced response to stimuli and loss of muscle tone or postural abnormalities as they tend to lie on their sides. In mild cases clinical signs were lacked and the condition was diagnosed through histopathological examination (Edgerton *et al*., 2002).

Histopathological studies on bacterial septicemia revealed small nodules or granulomas with hemocyte aggregations and encapsulation reactions. As the disease progressed; hemocyte aggregations were seen in heart, hemal sinuses and spaces, hepatopancreas, gills, antennal gland and the Y organ (Vey *et al*., 1975 ; Johnson, 1976).

The presence of perivascular cuffing of hepatopancreatic hemolymph vessels and granulocytic hemocyte aggregations in the heart, gills, hepatopancreas, antennal gland, abdominal muscle and connective tissue was recorded (Evans *et al*., 1992 ; Edgerton *et al*., 1995).

The bacteria were demonstrated in the hemolymph, phagocytic hemocytes , fixed phagocytes of the hepatopancreas , focal areas of necrotic muscle , the heart and abdominal muscle (Vey *et al*., 1975). Edgerton *et al* (1995) did not observe bacteria or bacterial colonies in histological preparations but follow-up bacterial isolation of hemolymph from crayfish from the same pond revealing prolific growth of *Pseudomonas spp*.

Clinical signs and histopathology were done in case of progressive form of disease (Edgerton *et al*., 2002). Crayfish with asymptomatic

bacteremia typically exhibited no gross or histopathological signs of disease. The condition was diagnosed by standard bacterial culture and identification techniques for aquatic bacteria. Hemolymph was collected aseptically, usually from the ventral sinus, through insertion of a needle in the ventral aspect of the membrane between the first abdominal segment and the thorax or Collection by insertion of needle into the posterior aorta or by severing an antenna or from the sinuses at the base of the third walking leg; pre-branchial blood; (Lucía *et al*., 2003).

2- Enteric bacterial infection

The disease is caused by *Citrobacter freundii*, *Citrobacter spp*., *Acinetobacterium spp*. and *Pseudomonas spp*. (Edgerton *et al*., 2002).

The mortalities were associated with bacteria normally found in the crayfish gut (Toumanoff, 1968; Amborski *et al.*, 1975a; Vey *et al.*, 1975; Boemare and Vey, 1977 ; Mickeniene, 1983).

Infectivity trials with one bacterial species isolated from affected animals were done; a strain of *Citrobacter freundii* caused similar lesions in normal crayfish force-fed large inoculums but overall infectivity of bacterial isolates was low (Boemare and Vey, 1977). These observations suggested that the disease was only occurring when the host immunity was weakened by exposure to unfavorable environmental conditions. This condition was diagnosed by histopathological examination of midgut and hepatopancreatic tissues. Large numbers of bacteria were observed in the lumen of the midgut and the hepatopancreas of moribund animals. Bacteria were usually absent from epithelial cells. Where the bacteria presented, a massive hemocytic reaction was observed; Necrotic

hepatopancreatic tubules were encapsulated with layers of hemocytes (Edgerton *et al*., 2002).

Deposition of melanin pigments was rarely observed, but it was reported in milder form in *C. tenuimanus* and *C. albidus-destructor* (Evans *et al.*, 1992) and also was reported in *Cherax quadricarinatus* (Edgerton, 1996) but in both studies no gross signs of the disease or mortalities were reported.

Fungal diseases of freshwater crayfish:

- **Fusarium infection (Fungus disease)**

The main causative agent is Fusarium species including *Fusarium solani, Fusarium oxysporum, Fusarium tabacinum, Fusarium roseum* var. *culmorum* (Edgerton *et al*., 2002).

Species of *Fusarium* are widespread and often are found in soil and plants. In freshwater crayfish the fungus is considered to be an opportunistic pathogen which infects freshwater crayfish after stressors such as wounding or water pollution. High losses were recorded (Maestracci and Vey, 1987). The losses were caused by *F. oxysporum* (Alderman, 1985) and *Fusarium solani* (Chinain and Vey, 1987a; 1987b; 1988).

Other species of *Fusarium* such as *F. roseum* var. *culmorum* had been found to cause gill disease in freshwater crayfish. A *Fusarium* fungus had also been described in the North American crayfish; *Procambarus simulans simulans* but no pathology was performed in that study (Lahser, 1975 ; Vey, 1979).

Death in most fungal infections occurred up to several months due to physiological disturbances resulting from interference with molting, exotoxin production by the fungus and disturbances of osmotic pressure and sodium and chloride ion concentration in the haemolymph. Mortality under experimental conditions had been up to 100% (Alderman, 1985; Maestracci and Vey, 1987; Chinain and Vey, 1987a; 1987b; 1988).

Fusarium solani was first reported in freshwater crustaceans and described the cuticular infections in Atlantoastacus pallipes Lereboullet; Crustacea, Decapoda; (Vey and Vago, 1972). Similar infection in *Astacus*

leptodactylus Escholtz was reported in which large brown patches developed, particularly on the dorsal abdomen, but sometimes also extending to the rest of the abdomen; so the disease had its name "brown abdomen disease" (Vey, 1981). The appearance of these brown patches is a result of a melanisation process which is controlled by a complex enzyme activation cascade (prophenoloxidase activating system = ProPo system) (Soderhall and Smith, 1986). Both thin soft intersegmental areas and thicker calcified areas of the exoskeleton are susceptible to attack by fungal hyphae. The lesions in the cuticle, gills and haemocoel of freshwater crayfish due to melanisation reaction occurred around the fungal hyphae leading to the presence of large brown patches in the cuticle and an intense cellular defense reaction in underlying tissues. The hemocyte aggregations lead to formation of large encapsulations or granulomas. In gills brownish spots were present and there were widespread changes in the gill epithelium (Maestracci and Vey, 1987).

Surface disinfection of infected tissue with 5% sodium hypochlorite and antibiotic solutions followed by incubation on malt agar at 25°C were used to isolate *F. solani.* The colonies were white and later developed a rose pink color. RGY agar with streptomycin sulphate and penicillum G incubated at 16° were used to isolate *F. tabacinum* (Alderman, 1985 ; Chinain and Vey, 1988).

Experimental infection of *Astacus leptodactylus* crayfish was done via hemorrhagic cuticular wounds by superficial dorsal or ventral scratches produced using the tip of a scalpel blade applied to the 4th and 5th abdominal segments after surface disinfection of the area to be wounded. Crayfish were exposed for 36 hours to a known concentration of a suspension of spores. Confirmation of successful infection was made by examination of fresh mounts of tissues in which the characteristic

brown markings of infection were apparent. Four months after exposure to spore challenge, wounded individuals increasingly weakened and mortalities reached 85% (Chinain and Vey, 1988).

Materials and Methods

Materials

Crayfish:

a- Crayfish used for bacteriological and fungal isolation :

Forty eight *Procambarus clarkii* crayfish (Fishar, 2006) were randomly obtained from the River Nile of Assiut governorate for bacterial examination. They were transported in a plastic tank (25 width X 40 length) containing a very little amount of water (about 1 cm hight). Twenty five crayfish (10 female and 15 male) were collected from April to June 2007 and 23 crayfish (3 female and 20 male) from April to June 2008.

b- Crayfish used for experimental infection :

Forty apparently healthy *P. Clarkii* crayfish were collected from the River Nile of Assiut governorate. The crayfish were acclimated to laboratory conditions (Assiut University, Faculty of Veterinary Medicine, Department of Animal Medicine, Fish Diseases and Management lab).

Bateriological media and reagents used for primary isolation:

1- Brain heart infusion, (BHI) agar media (SUVCHEM Laboratory chemicals AR 5009, India).
2- Cytophaga agar media (Kusuda, 1982).
3- Nutrient agar with 6.5 and 10 % sodium chloride (Difco laboratories – Detroit Michigan USA) .

4- Aeromonas agar media (Bile salt irgasan brilliant green agar) (LAB M, LAB 167).
5- Blood agar (Biolife , Milano Italy,5009).
6- Triple sugar iron media (TSI) (Idg, International diagnostic group Plc.,Uk)
7- Vogus proskour (Acetoin production). (Mackie and McCartney, 1989).
8- Methyl red medium (Mackie and McCartney, 1989).
9- Indole test medium (Mackie and McCartney, 1989).
10- Gram stain (Monica, 1985).
11- Oxidase reagent 1% N,N,N'N' tetramethyl-p-phenylenediamine (Washington *et al*., 2005).
12- Catalase reagent 3% hydrogen peroxide (Luis *et al.*, 1997).
13- Carbohydrate Utilization test (Mackie and McCartney, 1989):
    a- Glucose oxidative fermentation test.
    b- Sucrose fermentation test.
14- Urease test (Mackie and McCartney, 1989).
15- Citrate utilization test (Mackie and McCartney, 1989).

Media used for isolation of fungi :

- Sabouraud dextrose agar (Biolife, Milano, Italy).

Methods

Isolation of bacteria:

Procambarus clarkii crayfish were collected from River Nile of Assiut governorate. Bacteria were isolated from hemolymph, from the sinuses at the base of the third walking leg (pre-branchial blood) (Lucía *et al.,* 2003). A loopful was taken aseptically from ovary and digestive gland. Then samples were cultured on Brain heart infusion agar and cytophaga agar at 28 C for 24 to 48 h. The isolated bacterial colonies were picked up from the culture media and subcultured on the same type of media for purification and the single colony was inoculated on two slant of brain heart infusion. One was used for identification by specific media and biochemical tests and the other was kept as a stock for further experimental procedures.

Identification of the isolated bacteria:

Purified bacterial isolates were identified by culture behavior, colony morphology, microscopic examination and biochemical tests according to the methods described by Buller (2004) as the following:

A- **Microscopic examination:**

Smears from suspected colonies were stained with gram stain and examined microscopically for morphology. (Monica, 1985).

B- **Motility test :**

Motility was detected by microscopical "hanging drop" method. (Mackie and McCartney, 1989).

C- **Biochemical examination:**

Several biochemical tests were done according to Buller (2004).

1- Oxidase test :

Using Filter paper and 1% N,N,N'N' tetramethyl-p-phenylene-diamine dihydrochloride .Pre-wet filter paper with oxidase reagent (N,N,N',N'-p-phenylene-diamine dihydrochloride) was allowed to dry. Picked bacterial colony with a sterile toothpick was gently scratched onto the filter paper. A blue color was produced (positive). (Washington *et al*., 2005).

2- Catalase test :

A drop of 3% hydrogen peroxide was placed on picked colony (Luis *et al*., 1997).

3- Carbohydrate Utilization test :

Acid production from carbohydrate was determined according to Mackie and McCartney (1989) by inoculating the suspected gram negative bacteria into peptone water with 0.01% phenol red as indicator and 1% sugar tested. Carbohydrates used in this test were glucose and sucrose. Change in color of bromothymol blue to yellow in acidic pH 6.8 consider positive of acid production and to green in alkaline pH considered negative of acid production .

4- Vogus Proskauer (Acetoin production test):

Glucose phosphate broth culture was incubated at 28C for 48 hours. 1 ml of 40% potassium hydroxide solution and 3 ml of 5% alcoholic solution of alpha-napthanol was added to each tested tube and mixture was thoroughly shaked. A positive reaction is indicated by the development of

pink color in 2-5 min and become crimson in 30 min. The tube was shaken at intervals to ensure the maximum aeration (Mackie and McCartney, 1989).

5- Methyl red test:

Glucose phosphate peptone water liquid medium was inoculated with colony from a young agar slope culture and incubate at 28 C for 48 hr. Add about 5 drops of the methyl red reagent and mix and read immediately. Positive test were bright red and negative were yellow. (Mackie and McCartney, 1989).

6- Indole test:

Peptone water was inoculated and incubated for 96 h at 28 C. 0.5 ml of Kovac's reagent was added and shaked gently. A red color in the alcohol layer indicated a positive reaction (Mackie and McCartney, 1989).

7- Urease test :

Over the entire slope surface was heavily inoculated and incubate at 28 C examined after 4 hours and overnight incubation. No tube being reported negative until after 4 days of incubation. Urease positive cultures change the color of indicator to purple pink. (Mackie and McCartney, 1989).

8- Citrate utilization test :

Simmon citrate medium was inoculated with the suspected organism and incubated 96h at 28C. Blue color indicated the positive result. (Mackie and McCartney, 1989).

D- **Growth at different media:**

The isolated bacteria were tested for growth on Triple sugar iron media (TSI) at 28 C and examined after 24 and 48 hours , Nutrient agar with 6.5 % and 10 % soduim chloride at 28 C for 24 hours for identification of *Vibrio mimicus* , on Aeromonas agar media at 28 C for 24 hours for identification of *Aeromonas hydrophila*, pink translucent colonies and on Blood Agar to determine the virulence of strains according to hemolysis.

Experimental infection of *Aeromonas hydrophila*:

- Broth was inoculated from the pure culture and incubated for 14 hours at 28 C.
- Optical density was measured at 600 using spectrophotometer (Labomed. Inc. 2411. La Cienegu Blud, suileA.culver City Ca 9023 USA).
- Bacterial count was determined by ten fold serial dilution and plate count.

Preparation of bacterial inoculants:

1- Bacterial preparation :

Selected *Aeromonas hydrophila* strain was grown in brain heart infusion broth (BHI). Identified strain was kept in BHI with 15% (Vol. /Vol.) glycerol at -20 C for further investigations.

Before pathogenicity trails started, the strain was passed 1 time via injection through hemolymph of *P. clarkii* to regain its virulence , re-isolated and used directly after identification.

Growth culture:

By stand plate count method. Briefly 3 samples of 200 µl each from identified strain were 10 fold serially diluted in microtiter plate (96 well plate) using normal sterilized saline, by transferring 20 µl from each well to next one that contain 180 µl saline. After thoroughly mixing, tips of micropipette were changed and the process was repeated up to dilution of 10^{-9}. From last raw a drop from 20 µl from each well was dropped on BHI plates at 28 C for 24 hours. Counts were taken at mean number of bacteria and calculated in 1 ml by reversing dilution (Collins *et al*., 1991). Relation between bacterial concentration and bacterial broth optical density was investigated.

Calculation of the dose:

Broth was inoculated from the pure culture and incubated for 12 h at 28 C, O.D = 0.906, washed by centrifugation (Hermle z230 A) at 5000X for 10 minutes (Maalej *et al*., 2004). The supernatant was discarded and equal amount of sterilized saline was added then mixed thoroughly.

Bacterial solution was diluted according to the O.D. was taken to be 1.24×10^7 CFU/ml.

Isolation of Fungi:

Samples were taken from gills and uropods, planted on Sabouraud's dextrose agar media and were incubated up to 1- 2 week at 28 C.

Identification of the isolated fungi:

Purified fungal isolates were identified by colony morphology, microscopic examination. According to Deacon (1997); Luis *et al*. (1997) and John *et al*. (2006).

Results

1- Clinical examination:

Clinical and postmortem examination of examined crayfish revealed no gross lesion where all crayfish appeared to be clinically healthy.

2- Bacteriological isolation:

Bacteriological examination of crayfish revealed 101 bacterial isolates from the digestive gland, hemolymph, ovary and calcified organ of 48 examined crayfish on (BHI) agar media and cytophaga agar media.

3- Isolation and identification of isolates:

The isolates were identified according to culture morphology and biochemical characters as 64 (63.4%) *enterobacteriaceae*, 5 (4.95%) *Vibrio mimicus*, 4 (3.96%) *Pseudomonas fluorescens*, 2 (1.98%) *Aeromonas hydrophila* and 26 (27.7%) unidentified strains. These results are shown in table (1), (2) and figure (1).

3.1. Identification of *Aeromonas hydrophila*:

The colonies were 1–3 mm in diameter buff in color, with buttery consistency, after 28 hours of incubation at 28 C were smooth, circular and convex on (BHI) agar media. The pure culture was subcultured on Aeromonas agar media. Pink translucent colonies were obtained after 24 hours of incubation at 28 C. Beta hemolysis were occurred when the isolates

grown on blood agar. Biochemical characters of *Aeromonas hydrophila* isolates were present in the table (2).

a- Frequency distribution of *Aeromonas hydrophila* (n= 101 isolates):

Aeromonas hydrophila was isolated in a mixed infection from naturally infected crayfish with a percentage of isolation of 1.98% compared to the total number of the isolates (n=101), table (1) and figure (1).

b- Percent of infection with *Aeromonas hydrophila* and organ susceptibility:

The percentage of infection with *Aeromonas hydrophila* was 4.2% compared to the total number (n=48) of examined crayfish, Table (3). *Aeromonas hydrophila* was isolated from hemolymph of examined crayfish.

3.2. Identification of *Vibrio mimicus* isolates:

Vibrio colonies were 2–5 mm in diameter with entire margin and smooth, buff to cream colored colonies, grown after 24 hours of incubation at 28 C. The pure culture was subcultured on Nutient agar media containing 6.5 and 10 % NaCl colonies grow after 24 hours of incubation at 28 C. Four from five isolates caused beta hemolysis when grown on sheep blood agar media. Biochemical characters of *Vibrio mimicus* isolates were present in the table (2).

c- Frequency distribution of *Vibrio mimicus* (n= 101 isolates):

Vibrio mimicus was isolated with a percent of 4.95% compared to the total number of the isolates (n=101), table (1) and figure (1).

d- Percent of infection with *Vibrio mimicus* and organ susceptibility:

The percentage of infection with *Vibrio mimicus* was 10.41% compared to the total number (n=48) of examined crayfish, Table (3). *Vibrio mimicus* was isolated from digestive gland, hemolymph and calcified organ of examined crayfish specimens.

3.3. Identification of *Pseudomonas fluorescens* isolates:

Pseudomonas colonies were 3- 5 mm in diameter, whitish grey in color , cultured media fluorescent in the vicinity of colony and, butter like in consistency after 24 hours of incubation at 28 C. Two from four isolates caused beta hemolysis when grown on sheep blood agar media. Biochemical characters of *Pseudomonas fluorescens* isolates were present in the table (2).

a- Frequency distribution of *Pseudomonas fluorescens* in crayfishes (n= 101 isolates):

Pseudomonas fluorescens was isolated in a mixed infection from naturally infected crayfish with a percentage of 3.96% compared to the total number of the isolates (n=101), table (1) and figure (1).

b- Percent of infection with *Pseudomonas fluorescens* and organ susceptibility of :

The percentage of infection with *Pseudomonas fluorescens* was 8.33% compared to the total number (n=48) of examined crayfish, Table (3). *Pseudomonas fluorescens* was isolated from digestive gland and hemolymph of examined crayfish samples.

Pathogenicity of injected Aeromonas hydrophila to crayfish:

The experimentally infected crayfish were weakened and fatigued. When the crayfish lied on their back, they could not return to their normal upright position. Some of them lost their tail flip reflex. Greenish coloration of the digestive gland was observed and it was covered by translucent gelatinous material. Tail muscle congestion was observed on some injected crayfish as shown in figure (2). Mortality rate, time of death and survival rate are shown in table (4).

4- Fungal examination and isolation:

Primary isolation of fungi was done on sabouraud's dextrose agar media. Fungal examination of 48 examined crayfish revealed 76 fungal isolates from gills and uropods. The percentage of isolated fungi from gills and uropods were recorded in the table (5).

The isolates were identified to 19 *Aspergillus niger*, 1 *Aspergillus flavus*, 1 *Aspergillus fumigatus,* 39 *Rizopus spp.,* 5 *Fusarium oxysporum*, 1 *Fusarium proliferatum,* 4 *Humicola spp.* and 6 *Penicillium spp.* according to

culture morphology and microscopical characters. These results are shown in table (6) and figure (3).

5- Identification of recovered fungal isolates:

5.1.1. Identification of *Aspergillus niger* isolates:

Aspergillus niger colony was white becoming black with conidial production. The reverse was pale yellow, grown after 12 days of incubation at 28C.

a- Frequency distribution of *Aspergillus niger* (n=76) :

Nineteen isolates of *Aspergillus niger* were isolated from examined crayfish at a percent of 25% compared to the total number of isolates (n=76), table (5) and figure (4).

b- Percent of infection with *Aspergillus niger* and organ susceptibility:

The percentage of infection with *Aspergillus niger* was 31.25% compared to the total number (n=48) of examined crayfish, Table (6). *Aspergillus niger* was isolated from gills and uropods of examined samples. Eight isolates were recovered from gills and 11 from uropods.

5.1.2 Identification of *Aspergillus flavus* isolates:

Aspergillus flavus colony was olive to lime green with a cream reverse. Texture was woolly to cottony, grown after 12 days of incubation at 28C.

a- Frequency distribution of *Aspergillus flavus* (n=76):

The percentage of isolation of *Aspergillus flavus* was 1.3% compared to the total number of isolates (n=76), table (5) and figure (5).

b- Percent of infection with *Aspergillus flavus* and organ susceptibility:

The percentage of infection with *Aspergillus flavus* 2.08% was compared to the total number (n=48) of examined crayfish, table (6). *Aspergillus flavus* was isolated from uropods of examined samples.

5.1.3. Identification of *Aspergillus fumigatus* isolates:

Aspergillus fumigatus colony was smoky gray green with a slight yellow reverse. Texture was woolly to cottony, grown after 12 days of incubation at 28C.

a- Frequency distribution of *Aspergillus fumigatus* (n=76):

The percentage of isolation of *Aspergillus fumigatus* was 1.3% compared to the total number of isolates (n=76), table (5).

b- Percent of infection with *Aspergillus fumigatus* and organ susceptibility:

The percentage of infection with *Aspergillus fumigatus* was 2.08% compared to the total number (n=48) of examined crayfish, table (6). *Aspergillus fumigatus* was isolated from gills of examined samples.

5.2. Identification of *Rhizopus spp.* isolates:

Rhizopus spp. texture was typically cotton candy like. The color of the colony is white turned into grey by time. The reverse was white, grown after 7 days of incubation at 28C.

a- Frequency distribution of *Rhizopus spp.* (n=76):

The percentage of isolation of *Rhizopus spp.* was 51.3 % compared to the total number of isolates (n=76), table (5) and figure (6), (7), (8) and (9).

a- Percent of infection with *Rhizopus spp*. and organ susceptibility:

The percentage of infection with *Rhizopus spp.* was 50% compared to the total number (n=48) of examined crayfish, table (6). *Rhizopus spp.* was isolated from gills and uropods of examined samples.

5.3.1. Identification of *Fusarium oxysporum* isolates:

Fusarium oxysporum white tinged with pink color at maturity. The reverse was lavender in color. The texture was wooly, grown after 10 days of incubation at 28C.

a- Frequency distribution of *Fusarium oxysporum* (n=76):

The percentage of isolation of *Fusarium oxysporum* was 6.6% compared to the total number of isolates (n=76), table (5) and figure (10) and (11).

b- **Percent of infection with *Fusarium oxysporum* and organ susceptibility:**

The percentage of infection with *Fusarium oxysporum* was 10.42% compared to the total number (n=48) of examined crayfish, table (6). *Fusarium oxysporum* was isolated from gills and uropods of examined samples.

5.3.2. Identification of *Fusarium proliferatum* isolates:

Fusarium proliferatum colony was wooly white yellowish. The texture was wooly, grown after 10 days of incubation at 28C.

a- **Frequency distribution of *Fusarium proliferatum* (n=76):**

The percentage of isolation of *Fusarium proliferatum* was 1.3% compared to the total number of isolates (n=76), table (5) and figure (12) and (13).

b- **Percent of infection with *Fusarium proliferatum* and organ susceptibility:**

The percentage of infection with *Fusarium proliferatum* was 2.08% compared to the total number (n=48) of examined crayfish, table (6). *Fusarium proliferatum* was isolated from uropods of examined samples.

5.4. Identification of *Humicola spp*. isolates:

Humicola spp. colony was golden brown. Texture was cottony and grown after 2 weeks of incubation at 28C.

a- Frequency distribution of *Humicola spp.* (n=76):

The percentage of isolation of *Humicola spp.* was 5.3% compared to the total number of isolates (n=76), table (5).

b- Percent of infection with *Humicola spp.* and organ susceptibility:

The percentage of infection with *Humicola spp.* was 8.33% compared to the total number (n=48) of examined crayfish, table (6). *Humicola spp.* was isolated from gills and uropods of examined samples.

5.5. Identification of *Penicillium spp*. isolates:

The colony was white became blue green by time, wooly in texture, grown after 12 days of incubation at 28C.

a- Frequency distribution of *Penicillium spp.* (n=76):

The percentage of isolation of *Penicillium spp.* was 7.9% compared to the total number of isolates (n=76), table (5).

b- Percent of infection with *Penicillium spp.* and organ susceptibility:

The percentage of infection with *Penicillium spp.* was 12.5% compared to the total number (n=48) of examined crayfish, table (6). *Penicillium spp*. was isolated from gills and uropods of examined samples.

Table (1): Types of isolated bacterial strains and its percentage.

Bacterial spp.	Total number of isolates	Number of Bacterial isolates	%
Aeromonas hydrophila	101	2	1.98
Vibrio mimicus		5	4.95
Pseudomonas fluorescens		4	3.96
Unidentified strains		90	89.1

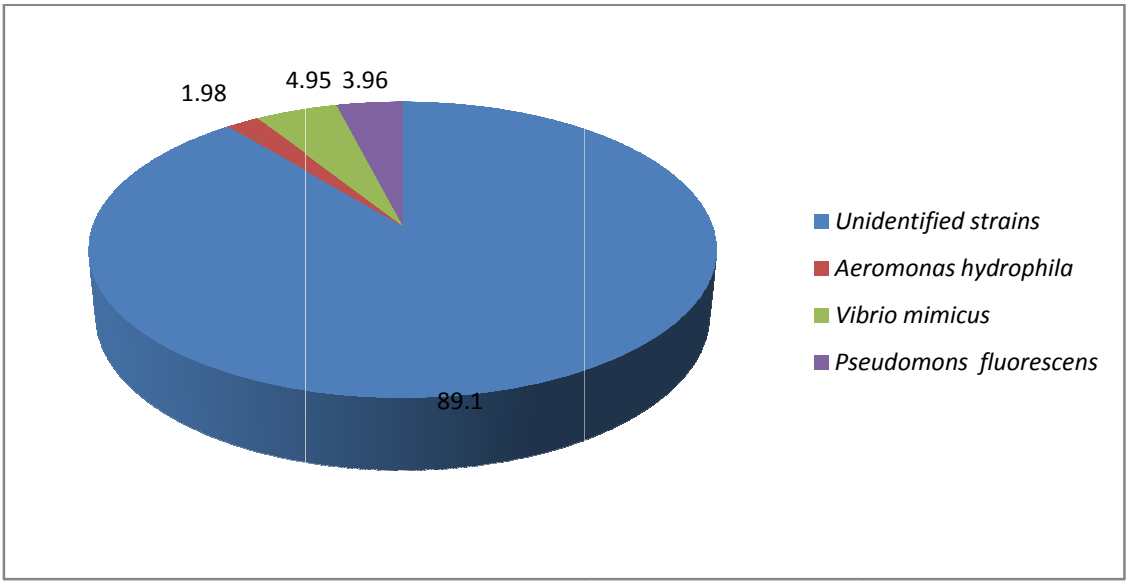

Figure (1) : Types of isolated bacterial strains and Its percentage

Table (2): Cultural and biochemical characters of isolated bacteria (+ve /No. of isolates)

Biochemical reactions		*Pseudomonas fluorescens*	*Vibrio mimicus*	*Aeromonas hydrophila*	Enterobacteriaceae
Gram stain		-ve	-ve	-ve	-ve
Motility		4/4	5/5	2/2	15/64
Catalase		4/4	5/5	2/2	64/64
Oxidase		4/4	5/5	2/2	0/64
o/f		0/0	5/5	2/2	-
Nutrient agar 6.5% Nacl		2/4	5/5	0/2	-
Nutrient agar 10% Nacl		2/4	5/5	0/2	-
Vogus Proskaur		0/4	1/5	2/2	-
Triple sugar iron	K/A	0/4	4/5	2/2	-
	Gas	0/4	4/5	1/2	-
	H_2S	0/4	3/5	0/2	-
Methyl red		0/4	4/5	2/2	-
Indole		0/4	1/5	2/2	-
Urease		4/4	5/5	2/2	-
Sucrose		1/4	5/5	2/2	-
Koser's media		0/4	0/5	0/2	-
Aeromonas agar		2/4	5/5	2/2	-
Blood Agar		2/4	4/5	2/2	-

Table (3): Percentages of infection of crayfish (n = 48)

Bacterial isolates	Infected crayfish	
	No.	%
Aeromonas hydrophila	2	4.2
Vibrio mimicus	5	10.41
Pseudomonas fluorescences	4	8.33

Table (4): Pathogenicity of injected Aeromonas hydrophila to crayfish

groups	No. of injected crayfish	Site of injection	No. of dead crayfish post inoculation				Mortality rate	No. of survived crayfish
			3 hours	6 hours	9 hours	12 hours		
Saline control groups	**10**	**hemocoel**	**-**	**-**	**-**	**-**	**-**	**10**
	10	**hemolymph**	**-**	**-**	**-**	**-**	**-**	**10**
Aeromonas hydrrophila injected groups	**10**	**hemocoel**	**-**	**6**	**4**	**-**	**100%**	**0**
	10	**hemolymph**	**2**	**2**	**2**	**2**	**80%**	**2**

Figure (2): Congestion of tail muscle due to *Aeromonas hydrophila* Injection.

Table (5): Types of isolated fungal strains and its percentage.

Fungal spp.	Number of Fungal isolates	%	Gills		Uropods	
			No.	%	No.	%
Rhizopus spp.	39	51.3	20	26.3	19	25
Aspergillus niger	19	25	8	10.5	11	14.5
Aspergillus flavus	1	1.3	-	-	1	1.3
Aspergillus fumigatus	1	1.3	1	1.3	-	-
Fusarium oxysporum	5	6.6	2	2.6	3	3.9
Fusarium proliferatum	1	1.3	-	-	1	1.3
Penicillium spp.	6	7.9	4	5.3	2	2.6
Humicola spp.	4	5.3	3	3.9	1	1.3
Total number of isolates	76	100	38	50	38	50

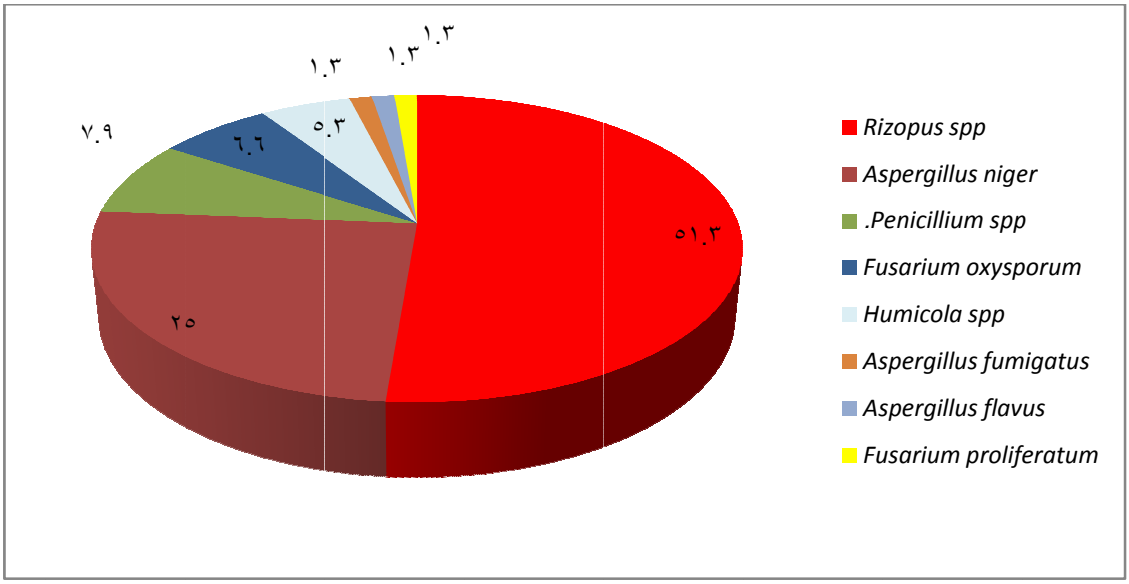

Figure (3) : Types of isolated fungal strains and Its percentage.

Table (6): Percentages of fungal infection of crayfish (n = 48)

Fungal isolates	Infected crayfish	
	No.	%
Rhizopus spp.	39	50
Aspergillus niger	19	31.25
Aspergillus flavus	1	2.08
Aspergillus fumigatus	1	2.08
Fusarium oxysporum	5	10.42
Fusarium proliferatum	1	2.08
Penicillium spp.	6	12.5
Humicola spp.	4	8.33

Figure (4) : White Colonies with black conidia of *Aspergillus niger* on sabouraud's dextrose agar media.

Figure (5) : Colonies of *Aspergillus flavus* on sabouraud's dextrose agar media were velvety and olive to lime green in color.

Figure (6) : The white Colonies of Rhizopus spp. (turned into grey by time).

Sabouraud ‘s dextrose agar media .

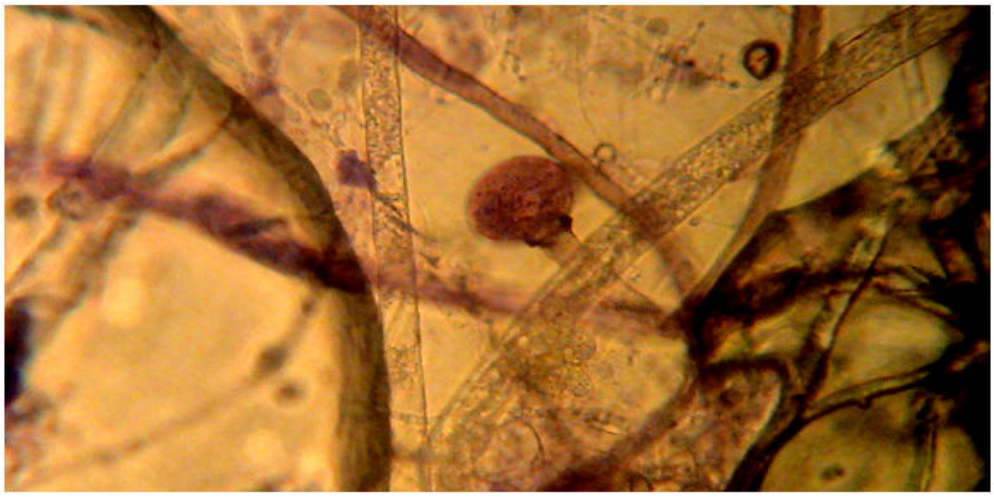

Figure (7) : *Rhizopus spp*. showing brown, non branched and non septated sporangiophores carrying round Sporangia with flattened base (wet mount).

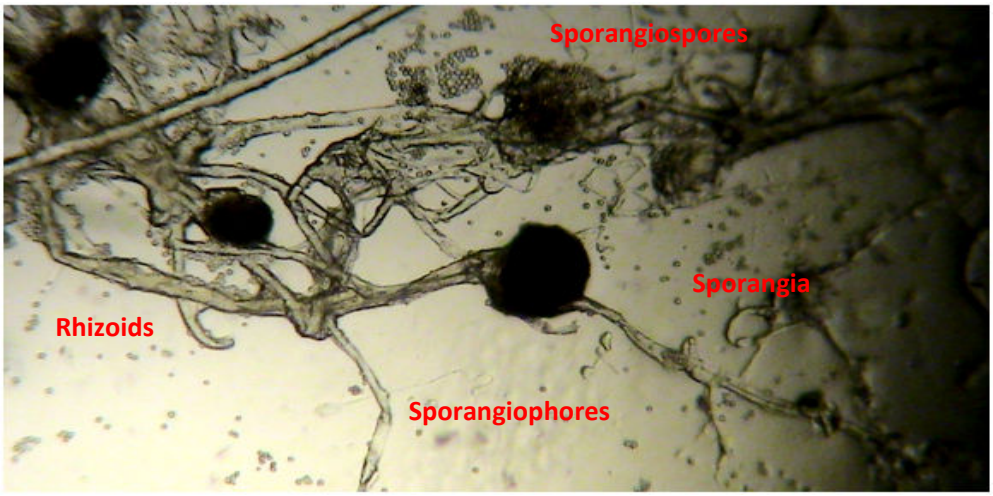

Figure (8) : *Rhizopus spp*. showing (root-like hyphae) giving rise to sporangiophores carrying sporangia. Sporangiospores were visualized. Microscopical examination of wet mount

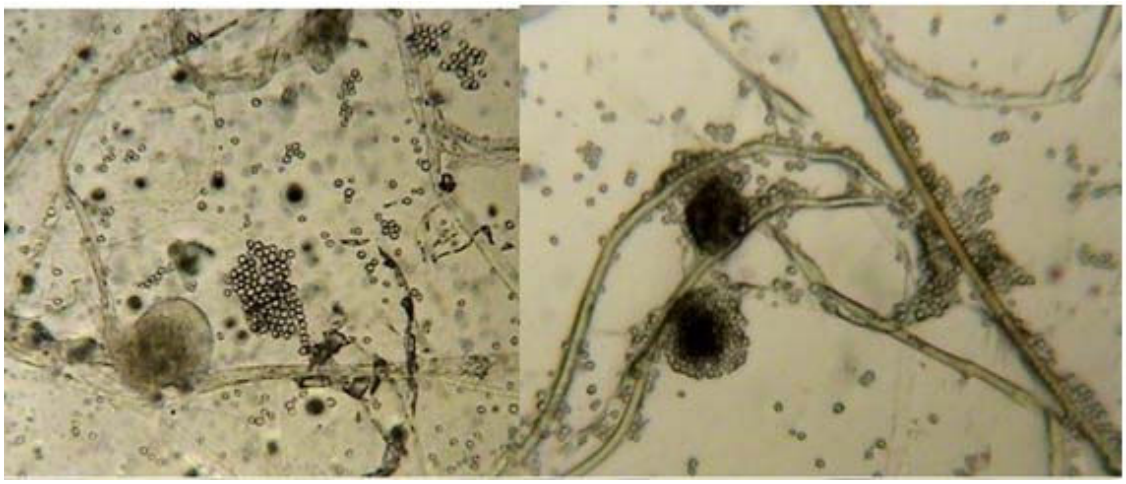

Figure (9) : *Rhizopus spp*. Sporangiospores were unicellular, round to ovoid in shape, hyaline brown in color. Microscopical examination of wet mount .

Figure (10) : Wooly pink colonies of *Fusarium oxysporum* on sabouraud's dextrose agar.

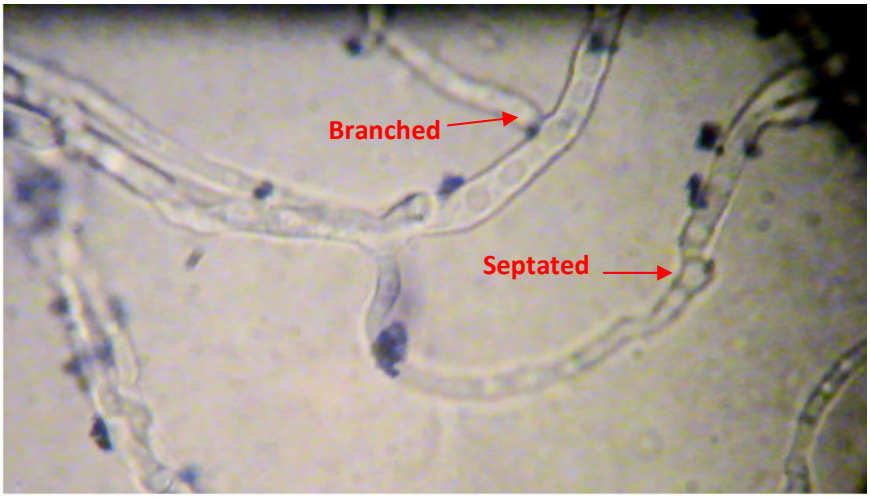

Figure (11) : *Fusarium oxysporum* showing hyaline branched septated hyphae. Microscopical examination of wet mount

Figure (12) *:* Wooly white yellowish colonies of *Fusarium proliferatum* on sabouraud 's dextrose agar.

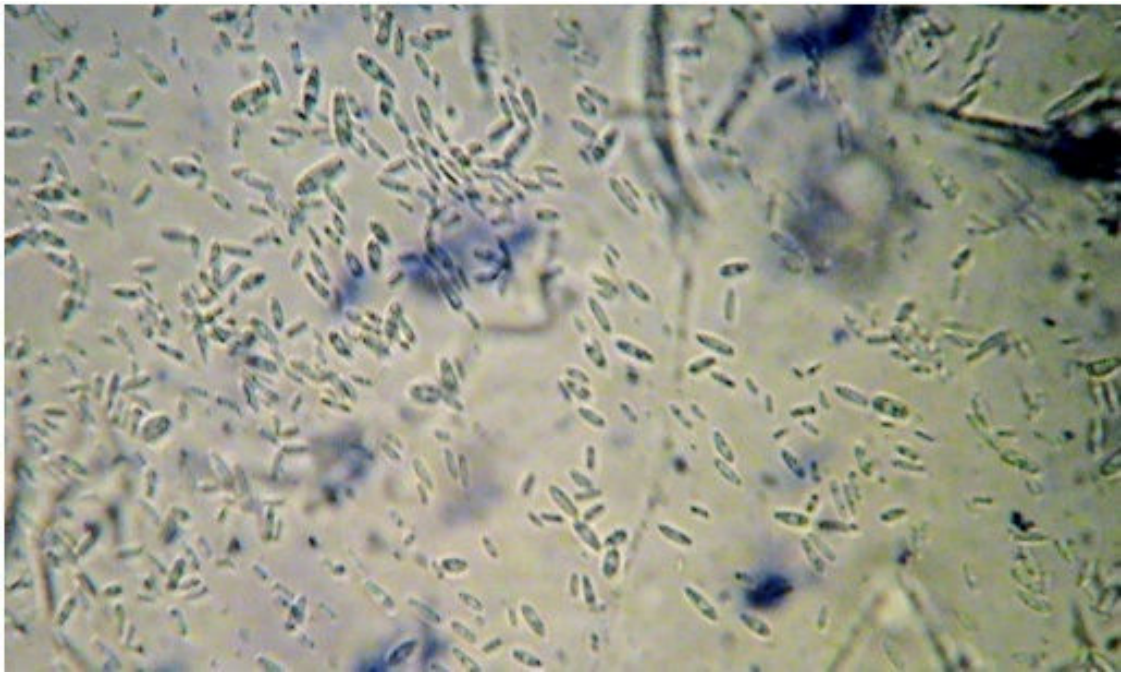

Figure (13) *: Fusarium proliferatum* showing single-celled microconidia. Microscopical examination of wet mount

Discussion

Typical clinical and gross signs of infection were not obvious in clinical and postmortem examination of naturally infected red swamp crayfish, *Procambarus clarkii*, suggesting that the infection was during the stage of asymptomatic bacteremia. Although bacteria were successfully isolated from the hemolymph, external and internal clinical signs of infection were not apparent. This could be due to lack of bacterial multiplication and toxin secretion as was described by Edgerton *et al*. (2002) in case of asymptomatic bacteremia.

Asymptomatic bacteremia had been reported in apparently healthy freshwater crayfish. It is characterized by the presence of a mixed bacterial population in hemolymph samples (Scott and Thune, 1986; Webster, 1995; Wong *et al.,* 1995; Madetoja and Jussila, 1996). It is caused by bacterial species from numerous genera including numerous gram negative bacteria such as *Acinetobacter, Aeromonas, Citrobacter, Flavobacterium, Pseudomonas and Vibrio* and gram positive bacteria such as *Corynebacterium, Bacillus, Micrococcus and Staphylococcus* (Edgerton *et al.,* 2002). The most frequently reported bacteria genera were *Pseudomonas, Aeromonas, Acinetobacter, Flavobacterium*, *Vibrio*, *Micrococcus* and *Staphylococcus*. The bacteria were not normally present in crustacean hemolymph, but as a result of exposure to environmental stressors (Bang, 1970; Lee and Pfeifer, 1975; Johnson, 1976). This condition has been demonstrated in numerous crayfish species including *Astacus astacus, Cherax albidus-destructor, Cherax quadricarinatus* and *Procambarus clarkii* (Edgerton *et al.,* 2002).

Prevalence of asymptomatic bacteria was 100% in investigated apparently healthy *Procambarus clarkii*. The prevalence of asymptomatic

bacteremia in freshwater crayfish varied according to crayfish species and environmental conditions. It was 41% in *Procambarus clarkii*, 100% in *Cherax albidus and Cherax destructor,* varied from 98 to 100% in *Cherax quadricarinatus* and 41 to 79% in *Astacus astacus* (Scott and Thune, 1986; Webster ,1995; Wong *et al*., 1995; Madetoja and Jussila, 1996).

Bacteriological examination of collected crayfish resulted in recovery of 101 bacterial isolates from the digestive gland, hemolymph, ovary and calcified organ. The isolates were identified according to culture morphology and biochemical characters as enterobacteriaceae 63.4%, *Vibrio mimicus* 4.95%, *Pseudomonas flurescence* 3.96%, *Aeromonas hydrophila* 1.98% and unidentified strains 27.7%. Scott and Thune (1986) isolated *Acinetobacter spp., A. lwoffi, A. antitratum, A. calcoaceticus, Aeromonas hydrophila, Enterobacter aerogenes, E. cloacae, Flavobacterium spp., F. dormitator, Pseudomonas alcaligenes, P. mendocina, P. putrefaciens, P. stutzeri, Vibrio spp.*and *V. alginolyticus* and isolated gram positive bacteria such as *Arthrobacter spp., Bacillus spp., Corynebacterium spp., Micrococcus luteus, M. roseus , Staphylococcus epidermis* and *Streptococcus spp* .from the hemolymph of apparently healthy *Procambarus clarkii* obtained from crayfish pond. Also from hemolymph of apparently healthy *Cherax albidus* and *C. destructor* obtained from ponds Wong *et al*. (1995), isolated bacteria including *Aeromonas sobria, Acinetobacter calcoaceticus var. lwoffi, Alcaligenes spp., Citrobacter freundii, Coliform-like spp., Plesiomonas shigelloides, Shewanella putrefaciens, Vibrio cholera, Vibrio mimicus* and gram positive bacteria such as *Corynebacterium spp., Kurthia spp., Micrococcus spp.* and *Staphylococcus spp.* The same authors isolated *Aeromonas sobria, Acinetobacter spp., Flavobacterium spp.,*

Pseudomonas cepacia, Pseudomonas maltophila, Vibrio cholera, Vibrio mimicus and gram positive bacteria *Bacillus spp., Micrococcus luteus, Micrococcus spp., Staphylococcus cohnii* and *S. epidermis* from apparently healthy *Cherax quadricarinatus*. In addition**,** Madetoja and Jussila (1996) recovered *Acinetobacter spp., Aeromonas spp., Comamonas acidovorans, Flavobacterium spp., Pasteurella spp., Pseudomonas florescens, P. putida, P. cepacia, P. vesicularis* and *Sphingomonas paucimobilis* from the hemolymph of apparently healthy *Astacus astacus* lived in laboratory tanks. On the other hand, *Pseudomonas morganii, P. aeroginosa, Proteus vulgaris*, *Pseudomonas florescens, P. putida, Aeromonas spp., Citrobacter spp., Flavobacterium spp., V. cholerae* and *V. mimicus* were isolated from diseased *Procambarus clarkii*, *Orconectes limosus, Austropotambius pallipes* and *Atlantoastacus pallipes* (Toumanoff, 1968; Amborski *et al*., 1975a; Vey *et al*., 1975; Boemare and Vey, 1977 ; Thune *et al*., 1991) during septicemic stage.

The percentage of infection with *Aeromonas hydrophila* was 4.2% of total 48 crayfish. The organism was isolated from hemolymph of examined crayfish. Quaglio *et al*. (2006) isolated *Aeromonas hydrophila* from hemolymph of one white-clawed crayfish *Austropotamobius pallipes* with 4.5% of total 22 crayfish. On the other hand, Quaglio *et al.* (2001) isolated *A. hydrophila* from all examined 15 *Pacifastacus leniusculus* crayfish (100%). *Aeromonas hydrophila* and *Aeromonas sobria* were regularly isolated from the eye lesions and the hemolymph Edgerton *et al*. (1995). Vice versa Eaves and Ketterer (1994) isolated *Aeromonas hydrophila* from digestive gland of one crayfish.

The result of clinical and postmortem examinations of experimentally infected crayfish revealed that the crayfish were

weakened, lethargic, loss of their tail reflex, greenish coloration of digestive gland was observed and covered with translucent gelatinous material. Congestion of the tail muscle was observed in some injected crayfish. Nearly similar clinical signs were described by Roy (1993) and Edgerton *et al*. (1995) during experimental infection of the crayfish with *Aeromonas hydrophila*. Quaglio *et al*. (2006) described crayfish naturally infected with *Aeromonas hydrophila* to be anorexic, lethargic and having a weak tail-flick escape response.

In case of infection of crayfish with *Aeromonas hydrophila* through hemolymph and hemocoel injection with 1.6×10^8 CFU/ml, the mortality rate was 80% within 12 hours in case of hemolymph injection and 100% within 9 hours in case of hemocoel injection. The differences in mortality rate and time of death between hemolymph and hemocoel injection may be attributed to rapid transmission of microorganism to all tissues of body in case of injection in hemocoel. Jiravanichpaisal *et al*. (2009) recorded a mortality rate 100% within 6hours after injection of *Pacifastacus leniusculus* with dose 1.24×10^7 CFU/ml of *Aeromonas hydrophila*. The mortality rate was 80% in the same species of crayfish when injected with 4×10^6 CFU/ml of *Aeromonas hydrophila* within 50 hours at 20±2 C (Liu *et al*. 2007).The difference of mortality rate and time of death in the present investigation and previous authors may be attributed to virulence of injected strain, injected doses, crayfish species and environmental condition of experiments.

Based on results in the present investigation, *A. hydrophila* is a highly virulent and pathogenic bacterium to crayfish. When this bacterium enters the crayfish body they proliferate and multiply and during multiplication they produce several kinds of toxins and these toxins are cytotoxic to the host cells. As a result, no hemocytic reactions

such as nodular formation, encapsulation and phagocytosis were found in the tissues of infected crayfish. This led to that the immune system was destroyed by the pathogen and as a consequence caused death of crayfish rapidly after an infection (Jiravanichpaisal *et al*. 2009).

Vibrio mimicus was isolated from digestive gland, hemolymph and unidentified calcified organ of examined crayfish .The percentage of infection with *Vibrio mimicus* was 10.41% of total 48 crayfish.

In spite of isolation of *Vibrio mimicus* from digestive gland, hemolymph and unidentified calcified organ, there was no obvious clinical signs can be recorded on investigated samples. Thune *et al.* (1991) isolated *Vibrio mimicus* and *V. cholera* from systemic infection of *Procambarus clarkii*. The infection was favored by elevated temperature and low dissolved oxygen**.** Mortality rates between 5 and 25% were observed, and affected crawfish were lethargic but exhibited no gross clinical signs. Eaves and Ketterer (1994) described two cases of mortalities in cultured red claw crayfish *Cherax quadricarinatus* associated with systemic *Vibrio mimicus* infections and considered *V.mimicus* as a secondary invader of red claw crayfish (*Cherax quadricarinatus*), which were weakened by poor management, overcrowding and/or adverse water quality. Wong *et al*. (1995) isolated *Vibrio mimicus* from hemolymph of 14% *C. albidus* samples over 3 months. Three of seven *V. mimicus* isolates were virulent and produced rapid mortalities when 10^5 viable cells were inoculated into crayfish by intramuscular injection. They confirmed that strains of *V. mimicus* present in the aquaculture environment can produce systemic disease in commercially cultured species of crayfish**.**

In the present investigation *p. fluorescens* were recovered with other bacterial strains from digestive gland and hemolymph of examined crayfish with percentage of 3.96% compared to total number of isolates. Different strains from *pseudomonas spp*. were isolated from hemolymph of apparently healthy different species of freshwater crayfish (Vey *et al.,* 1975; Boemare and vey, 1977; Scott and Thune, 1986; Edgerton *et al*.,1995; Wong *et al*., 1995).

Mycological examination of investigated samples revealed 76 fungal isolates from gills and uropods. According to morphological and microscopical examination, isolates were identified as 19 *Aspergillus niger*, 1 *Aspergillus flavus*, 1 *Aspergillus fumigatus,* 39 *Rizopus spp.,* 5 *Fusarium oxysporum*, 1 *Fusarium proliferatum,* 4 *Humicola spp.* and 6 *Penicillium spp.*. No clinical or gross lesions were recorded .This observation demonstrated the fact that crayfish are carrier to these fungi as a normal contaminant in aqua system. Quaglio *et al*. (2006) mentioned that in healthy crayfish *Acremonium, Alternaria, Aspergillus* and *Trichoderma genera* can be considered naturally occurring saprophytes associated with poor water quality. *Fusarium spp.* was the most frequently found on healthy crayfish without apparent lesions as saprophyte or a superficial contaminant. *Fusarium spp.* are also affecting marine shrimp, lobster and freshwater relatives (Johnson, 1983). In freshwater crayfish the fungus is considered to be an opportunistic pathogen which infects freshwater crayfish after stressors such as wounding or water pollution have decreased the resistance of the animal. Conditions which favor the infection of crayfish are common in aquaculture situations and high losses can be experienced (Maestracci and Vey, 1987).

The present study is considered preliminary investigations of diseases causing organisms in the exotic species of crayfish in Egypt. Further studies must be done to investigate the pathogenicity of all isolated organisms and environmental conditions that favor the occurrence of infections in red swamp crayfish.

This study proved that red swamp crayfish *Procambarus clarkii* can act as a carrier for many bacterial pathogens that are known to be pathogenic to fish, as *Aeromonas hydrophila, Vibrio mimicus, Pseudomonas fluorescens* and *enterobacteriaceae* bacterial group and also they may harbor many fungal agents, like *Aspergillus, Penicillium, Rhizopus, Fusarium* and *Humicola* species. This suggest the harmful and the dangerous role that red swamp crayfish can play in transmission of pathogenic bacteria to wild and farmed fishes. Its high resistance turned it into a dangerous carrier capable of spreading infection in a wide zone as its borrowing activity in river banks and muddy dams makes it able to reach fish farms easily especially those raised in rice fields.

Conclusion

1. *Procambarus clarkii* is a natural carrier for the following bacteria *Aeromonas hydrophila, Vibrio mimicus, Pseudomonas fluorescens* and *some member of enterobacteriaceae* group.
2. Prevalence of *Vibrio mimicus* infection was (10.41%) and isolated from digestive gland, hemolymph and calcified organ.
3. Prevalence of *Aeromonas hydrophila* infection was low (4.2%) and isolated from hemolymph.
4. Prevalence of *Pseudomonas fluorescens* infection is (8.33%) and isolated from digestive gland and hemolymph.
5. Experimental infection proved that *Aeromonas hydrophila* was a highly virulence bacteria to red swamp crayfish causing death and the injection in the hemocoel was more determental than hemolymph.

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الملخص العربي

دور استاكوزا المياة العذبة في نقل أمراض الأسماك

يسرا محمد إبراهيم محمد الشري

بكالوريوس العلوم الطبية البيطرية جامعة أسيوط 2004

أطروحة مقدمة إلى كلية الطب البيطري جامعة أسيوط لاستكمال متطلبات الحصول على درجة الماجستير في العلوم الطبية البيطرية

(أمراض الأسماك و رعايتها)

تحت إشراف

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استهدفت هذه الدراسة استقصاء أكثر الأنواع الشائعة للبكتريا و الفطريات التي تصيب استاكوزا المستنقعات الحمراء في مصر و تجعل منها مصدرا للعدوى للأسماك و الكائنات البحرية و كذلك الإنسان.

أدخلت استاكوزا المستنقعات الحمراء إلى نهر النيل في مصر عن طريق مزرعة أسماك في منيل شيحة بالجيزة سنة 1980 (فيشار 2006) و إنتشرت على طول نهر النيل و توابعه ، و لا زالت المعلومات المتوفرة قليلة حول الظروف و المسببات المرضية التي تصيب هذا النوع من الإستاكوزا المدخله حديثا.

تم جمع 48 استاكوزا من نهر النيل و توابعة في محافظة أسيوط و تعريضهم للفحص الظاهري و التشريح المرضي و الفحص البكتيري و الفطري .

أظهر الفحص البكتيري أن نسبة عزل البكتريا من السائل الدموي الليمفاوي و الغدة الهضمية كانت كالتالي :

الإنتيروبكتيريسي (63.4%) و الفيبريو ميميكس (4.95%) و السودومونس فلوريسينس (3.96%) الآيرومونس هيدروفيلا (1.98%). أما الفحص الفطري من الخياشيم و الطرف الذيلي من العينات المجمعة فأدى إلى عزل التالي: فطر الريزوبس (51.3%) و الأسبيرجيلس نيجر (25%) و البنسليوم (7.9%)، و الفيوزيريم أوكسيسبورم (6.6%) و الهوميكولا (5.3%) و الأسبيرجيلس فليفس (1.3%) و الأسبيرجيلس فيوميجيتس (1.3%) و الفيوزيريم بروليفيراتم (1.3%). تم التصنيف تبعا للفحص بالمجهر الضوئي و إختبارات البيوكيميائية.

تمت العدوى التجريبية على عدد 40 استاكوزا لتقصي قدرة عترة بكتيريا الأيرومونس هيدروفيلا المعزولة على الإمراض. تم حقن جرعة (1.6×10^8 وحدة تكوين المستعمرات / مللي ليتر) في السائل الدموي الليمفاوي و التجويف الدموي للاستاكوزا. تسبب حقن البكتريا في التجويف الدموي في نفوق بنسبة 100% خلال 9 ساعات بينما تسبب الحقن في السائل الدموي الليمفاوي في نفوق بنسبة 80% خلال 12 ساعة

اشتملت الأعراض الظاهرية و الإصابات أثناء التشريح المرضي للأستاكوزا المعداه ببكتيريا الإيرومونس هيدروفيلا على إعياء ، خمول ، فقدان الإنعكاسات الذيلية ، إخضرار الغدة الهضمية مع تغطيتها بمادة شفافة جيلاتينية و إحتقان عضلات الذيل ، بينما لم يتم ملاحظة أي أعراض واضحة في الإستاكوزا المصابة طبيعيا ، و هذا يوضح الدور الضار و الخطير الذي يمكن أن تلعبه استاكوزا المستنقعات الحمراء في نقل البكتريا و الفطريات القادرين على إحداث العدوى في الأسماك البرية و أسماك المزارع.

دور استاكوزا المياة العذبة في نقل أمراض الأسماك

يسرا محمد إبراهيم الشري

بكالوريوس في العلوم الطبية البيطرية جامعة أسيوط 2004

أطروحة مقدمة إلى كلية الطب البيطري جامعة أسيوط لاستكمال متطلبات الحصول على درجة الماجستير في العلوم الطبية البيطرية

(أمراض الأسماك و رعايتها)

تحت إشراف

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بمعهد بحوث صحة الحيوان

أسيوط

أسيوط

جمهورية مصر العربية

2009

جامعة أسيوط
كلية الطب البيطري

بسم الله الرحمن الرحيم

قـــــرار لجنـــة الحكـــــم

على رسالـــــة الماجستير

المقدمة من ط.ب. يسرا محمد إبراهيم محمد الشري

للحصول على درجة الماجستير في العلوم الطبية البيطرية

(أمراض الأسماك و رعايتها)

قررت لجنة الحكم و مناقشة رسالة الماجستير المقدمة من السيدة ط.ب. يسرا محمد إبراهيم محمد الشري ترشيح سيادتها للحصول على درجة الماجستير في العلوم الطبية البيطرية (أمراض الأسماك و رعايتها) .

اللجنـــــــــة	التوقيـــــع
ا.د. إســـــــماعيل عبد المنعـــــم عيسى أستـــــاذ أمـــراض الأسمـــاك و رعايتـــها كليـــة الطب البيطري – جامعـــة قنـــاة السويس	
ا.د. عـــــادل عـــبد العـــليم شاهيـــــن أستاذ و رئيس قسم أمراض الأسماك و رعايتها كليـــة الطب البيطري - مشتـــهر - جامعة بنـــها	
ا.د. شـــــعبان محمـــد أحمـــد شحاتـــــة أستـــــاذ أمـــراض الأسمـــاك و رعايتـــها كليـــة الطب البيطري – جامعـــة أســـيوط و المشـــــــرف على الرسالـــــــة	
ا.م.د. أحـــمد عبـــد الهـــادي الكامـــل أستـــــاذ مساعد أمـــراض الأسماك و رعايتـــها كليـــة الطب البيطري – جامعـــة أســـيوط و المشـــــــرف على الرسالـــــــة	

تمت بحمد الله