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SUSCEPTIBILITY RATE OF NILE TILAPIA, *(OREOCHROMIS NILOTICUS)* AND RED SWAMP CRAYFISH, *(Procambarus clarkii)* to *PROTEUS VULGARIS* INFECTION

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ABSTRACT

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The aim of the present study was to investigate *Proteus vulgaris* infections in Nile tilapia, and red swamp crayfish and homologous and heterologous-species susceptibility of both Nile tilapia and Red swamp crayfish. Eight isolates of suspected *Proteus vulgaris* were recovered from 6 fish out of 50 Nile tilapia collected from fish pond in Assiut Governorate. While, 12 isolates were recovered from 12 red swamp crayfish. Suspected isolates were identified as *P. vulgaris*. The pathogenicity of *P. vulgaris* to Nile tilapia and red swamp crayfish were experimentallly studied and revealed that an increase in the susceptibility to infection was observed in Nile tilapia experimentally infected with the *P.vulgaris* isolated from fish when compared to those infected with a strain isolated from red swamp crayfish. The mortality rates were 53.33and 33.33% respectively, by the end of the challenge. Equivalent experiments demonstrated that *P.vulgaris* isolated from the Nile tilapia was more virulente to red swamp crayfish than that isolated from crayfish. The mortality rates were 66.67% and 33.3% respectively.

Key words: *Proteus vulgaris, Nile tilapia, hetero-species susceptibility, red swamp crayfish.*

INTRODUCTION

Bacterial diseases were considered the main cause of high mortalities and economic losses among fish and fish farm (Austin and Austin, 2007). Enterobacteriaceae is a large family of some 25 genera and more than 100 species of facultatively anaerobic and gram- negative rods. The most important members of the family Enterobacteriaceae that are pathogenic to fish are *Yersinia ruckeri*, and *Edwardsiella* sp. However, recent reports showed that *proteus* has been implicated as potential fish pathogens. Proteus species are small Gram-negative rods, catalase positive, oxidase negative and produce acid from glucose by both oxidative and fermentative metabolism (Daly and Aoki, 2011). *Proteus vulgaris* is a common inhabitant of the human gut and a urinary tract pathogen; (Senior, B. W., 1979), but it was reported to produce active infections in fish. Previous studies reported that *Proteus vulgaris* was isolated from external ulcers of the fresh water fish Channa punctatus (Mandal *et al.,* 2002). *Proteus vulgaris* isolate*d* from diseased and apparently healthy fish (*Clarias gariepinus, Clarias lazera, Oreochromis niloticus, Lates niloticus, Heterotis niloticus, Heterobranchus bidorsalis*) and crabs Obiajuru and Ogbulie (2006) and Ahmed and Elkamel, (2006). Moreover, Manikandan *et al.* (2012) isolated *P.vulgaris* from freshwater fish *channa punctatus* Salihu *el al.* (2012) could isolate *P.vulgaris from* freshwater fishes caught from Sokotp river, Sokoto, Nigeria.

Bacterial infections of crayfish are common and widespread and many consist of opportunistic infections. Typically bacteria isolated from crayfish include representatives of the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Citrobacter*, *Corynebacterium*, *Micrococcus*, *Pseudomonas*, *Staphylococcus* and *Vibrio* (Alderman and Polglase, 1988; Edgerton *et al.,* 2002a). Furthermore *Proteus vulgaris* was isolated from freshwater crayfish, *Procambarus clarkii*, Toumanoff (1968) and Saad El-deen (2009) was isolated *P. vulgaris* from *Procambarus clarkii* and *Orconectes timosus.* Bacterial Infections of crayfish lead to mortalities in both farmed and wild fish, particularly in combination with other underlying poor conditions (Edgerton *et al.,* 2002a).

This study was done to investigate the prevalence of *Proteus vulgaris* in Nile tilapia, and red swamp crayfish, in Assiut. Clinical picture and postmortem lesions associated with infections in naturally infected fish and crayfish were monitored. Possibilities of heterologous and homologous species susceptibility between them were also studied.

MATERIALS and METHODS

1-Fish

Naturally infected fish:

A total number of 50 Nile tilapia were collected from fish pond treated with chicken manure as organic fertilizer in Assiut Governorate, Egypt. Fish were transferred alive or freshly dead as soon as possible to

the Aquatic Animals Wet Lab., Veterinary Hospital Clinic, Faculty of Veterinary Medicine, Assiut University. Fish were subjected to full clinical, PM and bacteriological examinations (Noga, 1996).

Experimental fish:
Apparently healthy juvenile Nile tilapia (50±5g) were collected from a private fish farm at Assiut Governorate. Fish were acclimated to laboratory condition for 2 weeks. Random specimens from fish were taken for diseases examinations to ensure that fish were healthy and none infected (Austin and Austin 2007). Fish were fed on pelleted ration at rate of 3% of their body weight a twice daily.

2-Red swamp crayfish, *P. clarkii.*
-Naturally infected crayfish:
A total of 50 a live red swamp crayfish *Procambarus clarkii*, were collected from the small tributaries of El-Ibrahemia canal, Assiut city. The body weight ranged from 10 to 30 g with total length of 9-16 cm. They were transported to the Aquatic Animals Diagnostic Laboratory, Faculty of Veterinary Medicine, Assiut University, where clinical and postmortem examination were conducted according to Melba *et al.* (2001).

Experimental Crayfish:
Sixty apparently healthy red swamp crayfish, with average body weight of 25±5g and total length 11±3cm were obtained from tributaries of El-Ibrahemia Canal, Assiut City, of which 10 were randomly examined to exclude infections as described above. Remaining crayfish were acclimated to laboratory conditions for 3 weeks.

-Bacteriological examination and identification:
1-Fish:
Bacterial sampling was taking from liver, kidney, spleen. Samples were streaked on (BiBG) (Lab M) and brain heart infusion agar (BHI) (Biolife) then incubated at 28 ºC for 24-48 hr. Pure cultures of the isolates were identified based on cultural, morphological, and biochemical characters according to (Brenner, 1984 and Austin and Austin, 2007).

2- Red swamp crayfish:
Bacterial samplings were taken from haemolymph and digestive gland and subjected to the same bacteriological examination as reported.

Pathogenicity of Proteus vulgaris:
Bacterial strains:
The isolated Bacterial strains were kept in BHI broth with 15% glycerol (Vol / Vol) (El-Gomhurrhia, Cairo, Egypt) at -20ºC. A *Proteus vulgaris* strain isolated from Nile tilapia (N.1) was passed through Nile tilapia via intrapretoneal (I/P) injection three times. A bacterial strain isolated from hemolymph of infected red swamp crayfish and identified as *Proteus vulgaris* (N.2) was passed 3 times through crayfish via hemocoel injection and used for determination pathogenicity. Strains (N.1, N.2) were grown on BHI agar and suspended in sterile distilled water to be diluted for experimental infection. Colony forming unit (CFU) counts of the bacterial suspensions were determined using spectrophotometery optical density at 600 values , ten fold serial dilution and plate count method (Elkamel and Thune, 2003).

Experimental challenge to O. niloticus:
In a preliminary challenge, an intraperitoneal (I/P) injection of 1ml of bacterial suspension of $1X10^7$ cfu/ml , $1X10^8$ cfu/ml ,or $1X10^9$ cfu/ml proved to be lethal within 7 days to all Nile tilapia. Thus, lower concentrations of the bacterial suspensions were used for experimental challenge.

Acclimated Nile tilapia were divided into three groups with 15 fish each. The first group was intraperitoneally injected with 0.06 ml of bacterial suspension of $1.5X10^9$ cfu/ml, while the second group was I/P injected with 0.06 ml of distilled water and the third group remained un-injected. The experiment was repeated three times.

The behavioral and clinical abnormalities were recorded daily, for 15 days. The visible morphological changes of the internal organs were also recorded after autopsy of the moribund fish. Percent mortality was calculated after the fish kill. To confirm if the disease was caused by the injected pathogen only, re-isolation of the injected pathogens from the internal organs was carried out by sacrificing moribund fish.

The same Experimental challenge was made to Nile tilapia with the same dose but with strain (N.2) which isolated from red swamp crayfish.

Experimental challenge *to P.clarkii*:
In a preliminary challenge study, a hemocoel injection of 1 ml of the higher concentration of bacterial suspension ($1X10^5$, $1X10^6$, $1X10^7$,$1X10^8$ cfu/ml) proved to be lethal to all crayfish within 7 days. Thus, the lower concentration was used for experimental challenge throughout the experiment. Acclimated crayfish were divided into three groups with 15-crayfish each. One group remained un-injected as a control, while crayfish of the other two groups were hemocoel injected with either 0.02 ml of distilled water and the later injected with 0.02 ml of diluted bacterial suspension ($1X10^8$ cfu/ml), and the whole experiment was repeated 3 times.

The same experiment was made with strain (N.1) with dose 0.02ml of diluted bacterial suspension ($1 X10^8$cfu/ml). All crayfish were observed daily up to 15 days post infection where Clinical signs and mortalities were recorded. Bacterial re-isolation from

the injected crayfish was performed as previously described.

RESULTS

Clinical signs and lesions of naturally infected *O. niloticus* and *P.clarkii:*

A-Fish:
The naturally infected fish, showed haemorrhages in the body surface especially in buccal cavity and at the base of the fins; fin rot, protruded hemorrhagic anus and congested gills in 21 of examined fish. Congestion of the internal organs were also detected in 9 fish .In 4 cases , showed focal hemorrhage in the liver surface.

B-Red swamp crayfish:
Examined crayfish (n= 50) did not show specific clinical signs of proteus vulgaris infection. While 5 crayfish showed necrosis at telsons and uropods and congested gills. 7crayfish showed hemorrhage and brown pigmentation on the hepatopancrease, while the heart and gonads were apparently healthy in all cases.

2-Isolation and identification of P.vulgaris:
A-Fish:
Bacteriological examination resulted in recovery of 15 isolates, 8 of which were suspected to be *P.vulgaris* based on morphological and biochemical characteristics. *P.vulgaris* isolates were recovered from 6 fish out of 50 (12%) examined fish. Primary isolates grew well on BHI agar giving thin, colorless, transparent highly swarming colonies. On BiBG, colonies were pink, convex with characteristic swarming. Results of the biochemical characters of the suspected isolates were demonstrated in Table (1).

B-Red swamp crayfish:
Bacteriological examination resulted in recovery of 12 isolates out of 50 crayfish examined. Isolates recovered from 12 crayfish out of 50 (24%) examined crayfish.. Isolates were recovered from the digestive gland and haemolymph. *Proteus vulgaris* strains isolated from red swamp crayfish appeared morphologically and biochemically identical to those isolated from Nile tilapia.

Experimental infection:
A-Fish:
5 Moribund Nile tilapia challenged with *P. vulgaris* that were isolated from fish exhibited irregular hemorrhage at the ventral part of abdomen (Fig.1). In 3 examined fish, scales detachment, rejected pectoral fin (Fig.2), haemorrhage in the body surface and congested gills were observed in 8 examined fish (Fig.3). Internally, there were congested kidney and ovary (n=5). Spleens were congested and enlarged in 1fish. Liver was pale or greenish in 2 cases and congested in 5 cases. Gall bladder was enlarged and distended with bile. Intestine of 3 fish was filled with bloody serous fluid (Fig.4). The mortality rate was 53.33% by the end of experiment. Re- isolation of the bacteria in pure culture was done from freshly dead and moribund fish. There was no mortality or clinical signs of infection in both of the control groups.

Nile tilapia challenged with *P. vulgaris* that were isolated from red swamp crayfish showed petechial hemorrhage on the body surface, congested gills (n= 4) and in 2 cases showed dark coloration of the body. Postmortem examination showed pale liver. Ovary was congested with hyperemia of the blood vessels of 2 fishes (Fig.5). By the end of the experiment, the mortality rate was 33.33%. Fish of both control groups remained a live and showed no signs of infection by the end of the challenge. *P. vulgaris* was re-isolated from freshly dead and moribund fish to verify the specificity of deaths.

B-Red swamp crayfish:
Moribund red swamp crayfish, challenged with *P. vulgaris* isolated from fish were weakned, lost their tail flip reflex, easily caught by hand and lied on their back (n=11).Hemorrhage in the musculature (Fig.6) and brown coloration (melanization) on the digestive gland were observed in 5 crayfish (Fig.7) while, in one crayfish showed hemorrhage on the digestive gland. The mortality rate was 66.67 % (10 crayfish). Pure cultures of *Proteus vulgaris* were re-isolated from the hepatopancrease of moribund and dead crayfish after challenge.

Crayfish inoculated with 0.02ml of $1X10^8$cfu/ml of *P.vulgaris* isolated from red swamp crayfish were weak and easily caught by hand. Two weeks post inoculation, the main number of dead crayfish was 5 (33.3%), while other crayfish survived till the end of the experiment. Dead crayfish showed haemorrhages on musculature in one crayfish, also rudimentary muscle was observed in one case. There was gelatinous material cover the heart. Browne in one case or greenish coloration and hemorrhage on digestive gland (n= 1) (Fig.8). Pure cultures of *P. vulgaris* were recovered from the digestive gland of the dead and moribund crayfish after challenge. No lesions were seen in control groups throughout the experiment.

Table 1: Cultural and biochemical characteristics of the suspected *Proteus vulgaris.*

Test	Result
Oxidase	**-**
Catalase	+
Motility	+
Gram-stain	**Gram negative rode shap**
indole	+
urease	**+ within 6 hours**
Swarming on BHI	+
Methyl red	+
Vogus proskauer	**-**
Simmon citrate	**-**
TSI H2s	+
Gas	+
Arginine decarboxylase	**-**
Lysine decarboxylase	**-**
Ornithine decarboxylase	+
Glucose	+
Lactose	**-**
Sucrose	**-**
Maltose	**-**
Sorbitol	**-**
Arabinose	**-**
xylose	**-**

(+) positive (-) Negative

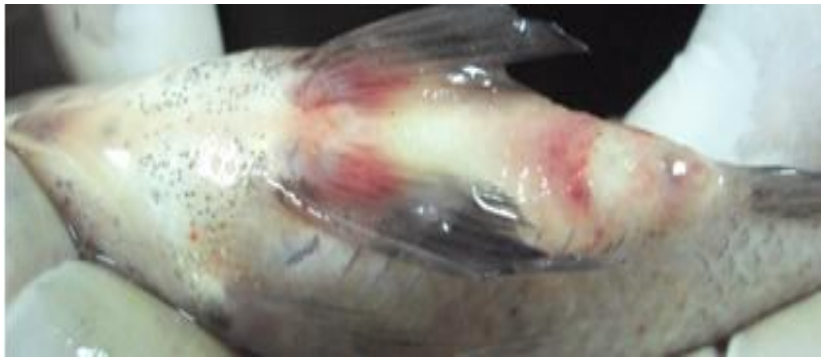

Fig. 1: Nile tilapia, experimentaly infected with *Proteus* vulgaris showing hemorrhage at the ventral part of abdomen.

Fig. 2: Nile tilapia, experimentaly infected with *Proteus* vulgaris showing scales detachment, rejected pectoral fin.

Fig. 3: Nile tilapia, experimentaly infected with *Proteus* vulgaris showing haemorrhage on body surface and congested gills.

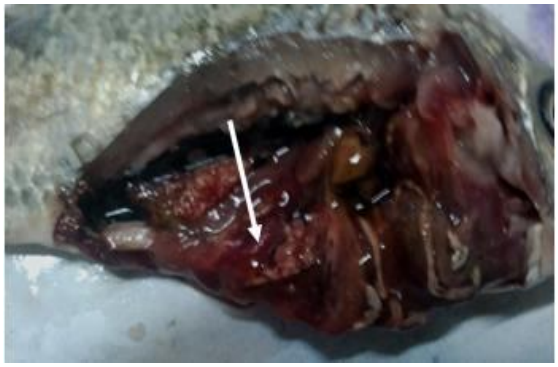

Fig. 4: Nile tilapia, experimentaly infected with *Proteus* vulgaris showing bloody serous exudate in the intestine**.**

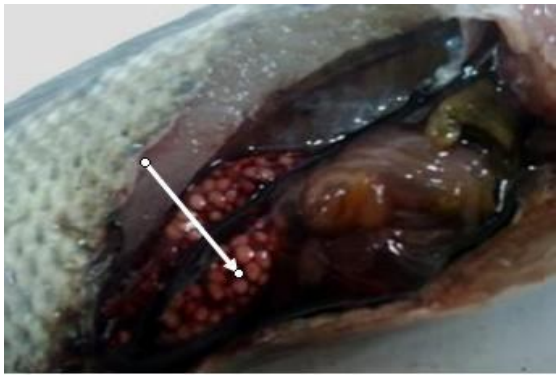

Fig. 5: Nile tilapia, experimentaly infected with *Proteus* vulgaris showing congested ovary.

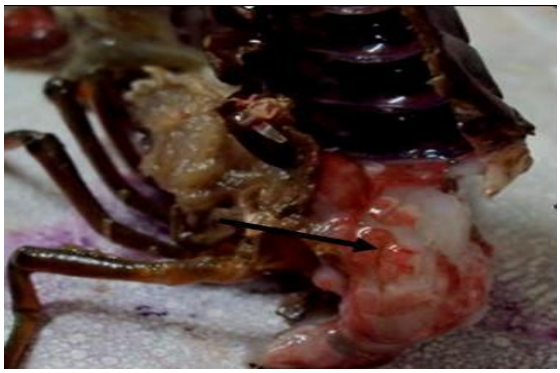

Fig.6: Red *swamp cray*fish experimentaly infected with *Proteus* vulgaris showing haemorrhages on musculature.

Fig.7: R*ed swamp cray*fish experimentaly infected with *Proteus* vulgaris showing brown coloration (melanization) on the hepatopancrease.

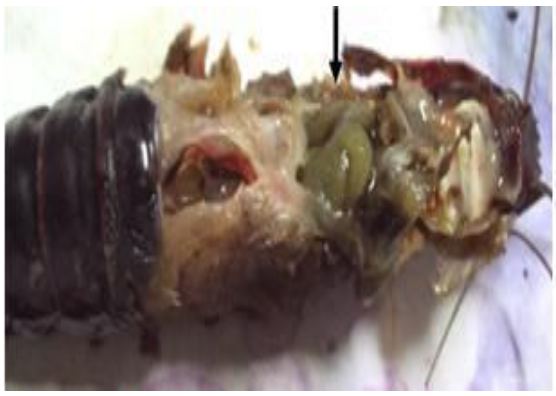

Fig.8*: Red swamp cray*fish experimentaly infected with *Proteus* vulgaris showing green coloration on the hepatopancrease.

DISCUSSION

Proteus vulgaris infection is an emerging disease in Egypt (Ahmed and Elkamel 2006) and responsible for blotch disease (Bullock *et al.,* 1971), Red Spot disease in *Channa puctatus* and *Trichogaster fasciatus* (Conroy and Herman 1970) and spottiness of skin (Van Duijn Jr 1973). The current study revealed that naturally infected fishes with *P.vulgaris* showed various signs and lesions of infection. The results were similar to findings recorded by Ahmed and Elkamel (2006). The phenotypic and culture characters of the colonies and the staining properties of the bacteria isolated from Nile tilapia, *O. niloticus*, and red swamp crayfish ,P.clarkii, suggested that the suspected isolates are *P.vulgaris* as was described by Austin and Austin (2007).

The Results of the current study indicated that the percentage of infection of *P. vulgaris* in the examined cultured fishes was (12 %). The percentage agree with the percentage of 15.8% that was reported in sharptooth catfish (Ahmed and Elkamel., 2006), and 10% that was reported in Cyprinus carpio L. (Al-Imarah, 2008). Meanwhile disagree with Salihuelal., (2012) who could isolate *P.vulgaris* from freshwater fishes caught from Sokotp river ,Sokoto,Nigeria at percentage of 3.15%. The incidence of *P.vulgaris* infection In *P.clarkii* was 24% and this result nearly agrees with Saad El-deen (2009).

Results of experimental challenge in the present study proved that *P. vulgaris* isolated from naturally infected Nile tilapia is pathogenic to both healthy tilapia (autologous species) and to red swamp crayfish (heterologous species). On the same moment, P. vulgaris isolated from freshwater crayfish is pathogenic to Nile tilapia and freshwater crayfish. An increase in the intensity of infection was observed in Nile tilapia experimentally infected with the *P.vulgaris* isolated from fish as compared to those infected with the same strain isolated from red swamp crayfish. Equivalent experiments demonstrated that *P.vulgaris* derived from the Nile tilapia was virulence to red swamp crayfish than that isolated from crayfish. Interestingly, the pathogenesis and the extension of lesions to Nile tilapia and red swamp crayfish were more intense by strain isolated from Nile tilapia; these results raise questions about the original host, most susceptible host, and source of infections, inter-species transmission, and whether Nile tilapia is main freshwater reservoir species.

Clinical examination of experimentally infected fishes showed that skin lesions and general septicemia. Septicemia may be due to generalized infection where liver and most internal organ become congested. Badran *et al.* (1994) concluded that clinical signs of fish infected with human Enterobacteriaceae, which are not classical fish pathogens bacteria, are not quite different than those of classical fish pathogens. Okaeme (1989) stated that signs of infected with *P.vulgaris* and other bacteria are ulceration and necrotic lesions of skin, and haemorrhagic septicemia.

P.vulgaris isolated from *P.clarkii* could also produce a low-level infection in *O.niloticus* during their experiments. The low of response might be attributed to the variations in virulence of the bacterial strains, as reported by Figucrdo and Plumb (1977) Moreover, Snieszko (1958) stated that the disease in fishes is dependant upon 3 factors, host susceptibility, pathogen virulence and environmental conditions (Omprakasami and Manohar1991). Postmortem examination of experimentally infected red swamp crayfish showed brown coloration on the hepatopancrease**.** This observation may be attributed to immune system of crayfish (Phenoloxidase is a copper –containing protein and a key enzyme in melanin synthesis (Shiao *et al.,* 2001 and Soderhall and Ajaxon, 1982).

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مدى قابلية اسماك البلطي النيلي واستاكوزا المستنقعات الحمراء للعدوى بميكروب البروتياس فولجارز

آية جلال سعد الدين

إن الهدف من هذا البحث هو دراسة إصابة أسماك البلطي النيلي واستاكوزا المستنقعات الحمراء بميكروب البروتياس فولجارز، وكذلك دراسة حدوث العدوى التصالبية لميكروب البروتياس فولجارز بين اسماك البلطي النيلي واستاكوزا المستنقعات الحمراء في محافظة أسيوط. وقد تم عزل ٨ من عترات ميكروبات البروتياس فولجارز من الأعضاء الداخلية لـ٦ سمكات من أصل٥٠ سمكة بلطي نيلي كما تم عزل عدد ١٢ عترة من ١٢ استاكوزا المستنقعات الحمراء من عدد ٥٠ استاكوزا التي تم جمعها من نهر النيل وترعة الإبراهيمية بمحافظة أسيوط. وقد تم تصنيف العترات المعزولة علي أنها ميكروب البروتياس فولجارز. تم دراسة مدى قابلية اسماك البلطي النيلي واستاكوزا المستنقعات الحمراء للعدوى بميكروب البروتياس فولجارز عن طريق عمل العدوى الصناعيه فوجد إن اسماك البلطي أكثر حساسية لميكروب البروتياس فولجارز المعزول من سمك البلطي عن الميكروب المعزول من الاستاكوزا. وكان معدلات النفوق الإجمالية ٥٣.٣٣ و٣٣.٣٣% على الترتيب بنهاية التجربة. كما أظهرت النتائج زيادة حساسية استاكوزا المستنقعات الحمراء لميكروب البروتياس فولجارز المعزول من اسماك البلطي عن الميكروب المعزول من الاستاكوزا.وكان معدلات النفوق ٦٦.٦٧ و ٣٣.٣ %على الترتيب بنهاية التجربة.