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Studies on Diseased Freshwater Prawn *Macrobrachium rosenbergii* Infected with *Vibrio vulnificus*

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Abstract: The present study was aimed at isolation and characterization of the pathogenic bacterium from diseased freshwater prawn. The effect of the bacterial pathogen on hepatopancreas, gills and exoskeleton was also investigated. Diseased freshwater prawn, *Macrobrachium rosenbergii* were collected from commercial hatchery in Behera Governorate, Egypt. The diseased prawn showed dark brown focal lesions and necrosis of appendage tips. The causative bacterial pathogen was isolated from haemolymph and hepatopancreas of the diseased prawn. Based on the morphological, biochemical and physiological characteristics, in addition EPI 20E test, the isolated pathogen was characterized as *Vibrio vulnificus*. Histopathology, hepatopancreas showed haemocytic infiltration in the interstitial sinuses, thickening and ruptures of the basal lamina and necrosis of its tubules. Similarly, the accumulation of haemocytes in the haemocoelic space, swelling, fusion of lamellae and abnormal gill tips. Also, the cuticular layers of the exoskeleton of diseased prawn had a rough or wrinkled surface and were disrupted and separated from the epidermis. The pathogen, *V. vulnificus* showed different degrees of sensitivity to different antimicrobial agents. It was highly sensitive to each of the antibiotics rifadin, virbamycin, oflaxcin, garamycin, flummox and trimethoprim/sulfamethoxzole) and resistant to nalidixic acid, unasyn, velosef, claforan, negram and amoxicillin. The minimal inhibitory concentration of trimethoprin/sulfamethoxzole for the studied pathogen, *V. vulnificus* was 0.31/5.93 μg.

Key words: *Macrobrachium rosenbergii*, *Vibrio vulnificus*, disease, haemdymph and hepatopancrease, antibiotics, pathogenicity and histopathology

INTRODUCTION

Freshwater prawn (*Macrobrachium rosenbergii*, de Man, 1879) is an important commercial species due to property as food supply as well as a valuable export product. In the last several years, prawn diseases have had a devasting effect on world prawn farming. Such diseases increase risk, deterring investment and commercial development. Out breaks of disease in prawns is often attributed to bacterial infection (Sung *et al*., 2000; Phatarpekar *et al*., 2002; Al-Harbi, 2003; Al-Harbi and Uddin, 2004). Vibrio species have been reported as the causative agents for numerous disease outbreaks (Alavandi *et al*., 2004; Jayaprakash *et al*., 2006; Kennedy *et al*., 2006). These species cause some diseases such as vibriosis and chitinolytic bacterial shell disease. Vibrios are a ubiquitous and predominant component of prawn culture environment and comprise a major part of the normal flora of crustaceans (Lightner, 1993; Gil *et al*., 1998; Ruangpan *et al*., 1999; Vandenberghe *et al*., 1999; Thmopson *et al*., 2003 ; Vaseeharan and Ramasamy, 2003; Vijayan *et al*., 2006).

Culture water is a major potential route for introducing pathogenic bacteria into shrimp hatcheries. However, absolute sterility of rearing water is very difficult to achieve in laboratory situations and impossible in commercial shrimp hatcheries. Wide range methods are employed to limit and reduce the number of potential pathogens occurring in hatchery water supplies, including antibiotic and other chemotherapeutic agents. Antibiotics have been applied for treatment of bacterial diseases (Brown, 1989; Coyne *et al*., 1994; Angka, 1997; Roque *et al*., 2001; Aja *et al*., 2002; Selvin *et al*., 2005; Abraham, 2005).

Because of the disease agent cause a distinct pathological effect of an infected population. Also, it cause significant economic losses, increased mortality, reduced growth rates, decreased product quality and increased management coasts. Thus the present work was designed to isolate and characterize pathogenic bacterium from the haemolymph and hepatopancreas of diseased freshwater prawn, *M. rosenbergii*. The histopathology of the hepatopancreas, gills and exoskeleton of diseased

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prawn was investigated. The sensitivity of the characterized bacterial pathogen to different antibiotics was also studied.

MATERIALS AND METHODS

Prawn samples: For histopathological and bacteriological analysis, diseased freshwater prawn, (*Macrobrachium rosenbergii*) measuring 6 to 8 cm in length were collected from commercial hatchery in Behera Governorate, Egypt, in June 2006 and July 2007. The collected samples were transferred alive to the laboratory then placed in glass jars containing water from the natural habitat. Jars were maintained at temperature of 26-28°C and aeration was supplied by compressed air with diffusers.

Isolation of the bacterial pathogen from the diseased prawn: To isolate the bacterial pathogen, 0.1 g hepatopancreas of a diseased animal was ground in 1 mL sterile saline (0.85%) and also 100 μL of haemolymph was suspended in 400 μL saline and used as sources of the pathogen. Both hepatopancreas and haemolymph suspensions were diluted ten fold and 100 μL of each were spread separately on the surface of thiosulfate-citrate-bile salts sucrose (TCBS) plates. The plates were incubated at 30°C for 2 days and then the appeared colonies were maintained on slants of the same medium at 4°C.

Characterization of the isolated pathogen

Morphological studies: Diluted suspension of the isolated bacterium was spread on the surface of TCBS plates to study the color, shape, margin and elevation of the grown colonies. In addition, a smear of this bacterium was made and stained with Gram-stain to record the stain reaction and the cell form.

Physiological and biochemical study

Growth at different temperatures: The isolated bacterial pathogen was inoculated in tryptic soy broth (TSB) and incubated at different temperatures (Song *et al*., 1993).

Production of indole acetic acid: The characterized bacterium was grown at 30°C for 24 h in TSB. The culture was centrifuged and the supernatant was extracted with equal volume of ethyl acetate and evaporated under vacuum at 40°C (Wang *et al*., 1982). The residue was detected by paper chromatography using ethyl acetate in chloroform (3:2) as eluent and $FeCl_3$ (0.05% in sulphuric acid) as a color reagent (Langenbeck-Schwich and Grambow, 1984).

Degradation activities: The degradation of casein was determined by the method described by Song *et al*. (1993), using tryptic soy agar (TSA) plates supplemented with 7.5% skim milk. Starch degradation (1%) was detected in TSA plates. The inoculated plates were incubated at 30°C for 2 days and then flooded with iodine solution (Cowan and Steel, 1974). The urease activity was determined by using urea agar base medium (Song *et al*., 1993).

Acid production from sugars: Filter sterilized solutions of arabinos and mannitol, were added separately to a final concentration of 1% to peptone (Ono *et al*., 1984) water medium (Peptone, 10 g; NaCl, 5 g; 4 mL of 0.2% bromocresol purple; 1 L dist. H_2O). Change of bromocresol purple color into yellow-brown indicated positive result.

Oxidation fermentation test (O/F): Medium composed of glucose, l0 g; peptone, 2 g; NaCl, 5 g; K_2HPO_4 0.3 g; 0.002% bromocresol purple, 5 mL and 1 L dist water was used for O/F test. Glucose was sterilized separately by filtration and added after the adjustment of the medium to pH 7.2. The yellow-brown colour indicated positive result.

Citrate utilization: Citrate utilization was tested by using Simmon's citrate (Sod. citrate, 2 g; NaCl, 5 g; $MgSO_4$ 0.2 g, ammonium dihydrogen phosphate, 0.2 g; bromothymol blue 0.08 g; agar, 15 g; pH 7 and 1 L) agar medium (Edwards and Ewing, 1962). The characterized organism was inoculated onto the medium and incubated at 30°C for 24 h. The positive growth produces an alkaline reaction which changes the color of the medium from green to bright blue.

The characterization of the isolated bacterial pathogen was confirmed by API 20E strip system.

Histopathology: Hepatopancreas, gills and exoskeleton of healthy and diseased prawn were fixed in 10% formalin and processed for paraffin sectioning, then the sections were stained using haematoxylin and eosin and Azan stains.

Antibacterial sensitivity test: The sensitivity of isolated bacterium to some commercially available antibiotic was determined by the method described by Costa *et al*. (1998). Bacterial suspension (100 μL of 10^6 cfu mL^{-1}) was spread on to TSA and discs impregnated with the tested antibiotic were used. After incubation period of 24 h at 30°C, the diameters of inhibition zones were measured.

Determination of minimal inhibitory concentrations (MICs): The MICs of trimethoprin/sulyamethoxzole for the isolated pathogen was determined by spreading 10^6 cfu and then sterilized filter paper discs impregnated with serial concentration of the selected antibiotic were placed over the agar surface. The plates were kept 2 h at 4°C and then incubated at

30°C for 24 h. The lowest concentration which inhibited the tested bacterium was the MIC.

RESULTS

Clinical signs: Diseased prawns showed opaque and whitish muscles and subsequent melanization body and appendages. Gross signs of vibriosis are dark brown focal lesions and necrosis of appendage tips (Fig. 1). Moribund prawns congregate at the edges of ponds and swim slowly near the surface.

Characterization of the pathogenic

Morphological characteristics: The colony of the characterized bacterium appeared green in colour on TCBS plates, circular and low convex with entire margin. This organism exhibited negative reaction to Gram stain and the cells were short rods in shape.

Physiological and biochemical characteristics: As represented in Table 1 the characterized pathogen was able to grow well at 28 and 37°C but had no growth at 5°C in TSB. The organism was indole negative and could not utilize citrate. This bacterium recorded positive result for oxidation-fermentation test and had the ability to produce urease. The organism hydrolyzed starch and did not casein. The tested bacterium was able to produce acid from mannitol but not from arabinose.

According to the above mentioned morphological, physiological and biochemical results in addition to the confirmation by API 20E test, the isolated bacterium was characterized as *Vibrio vulnificus*.

Histopathology: The hepatopancreas of healthy prawn is a large compact ducts and blind ending tubules. Each tubule consists of single layer of epithelial cells enclosed a lumen. Haemocyte and muscle cells are found in the connective tissue around the tubules (Fig. 2).

Table 1: Morphological, physiological and biochemical characteristics of bacterium isolated from diseased prawn, *M. rosenbergii*

Test	Result
Colony morphology	Circular, low convex with entire margin
Color in TCBS	Green
Cell form	Short rods
Gram reaction	Negative
Growth at	
5°C	-
28°C	+
37°C	+
Acid from	
Arabinose	-
Mannitol	+
O/F test	+/+
Indole formation	-
Citrate utilization	-
Urease production	+
Casein hydrolysis	-
Starch hydrolysis	+

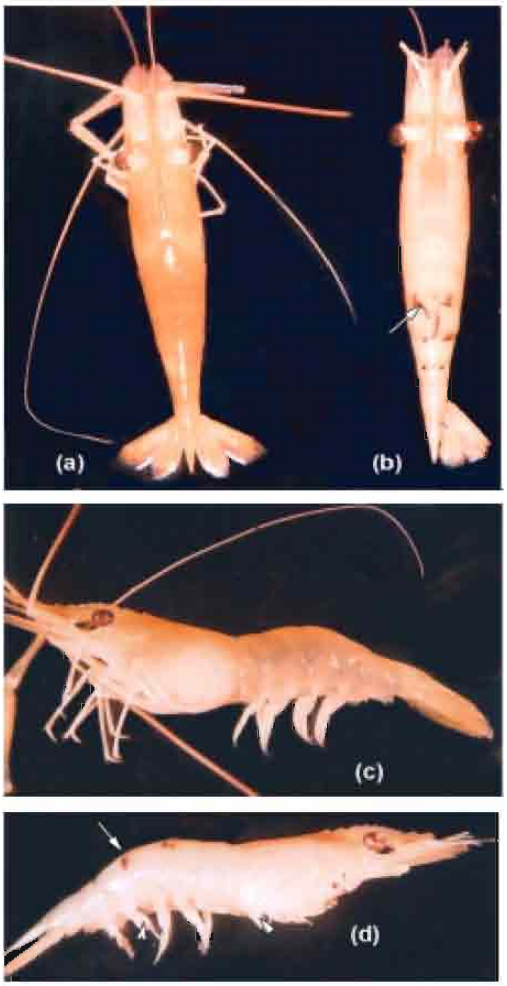

Fig. 1: Dorsal and lateral views of healthy (a and c) and diseased (b and d) *Macrobrachium rosenbergii*, respectively

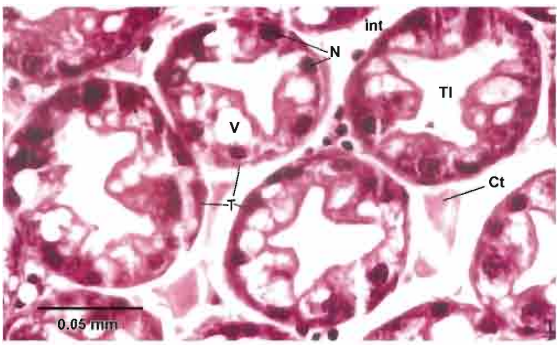

Fig. 2: Light micrograph of hepatopancreas of healthy *M. rosenbergii*, showing: ct., connective tissue; int: Intertubular space between tubules; N: Nucleus; T: Tubules; Tl: Tubule Lumen and V: Vacuole stained with Haematoxylin and Eosin. Bar = 0.05 mm

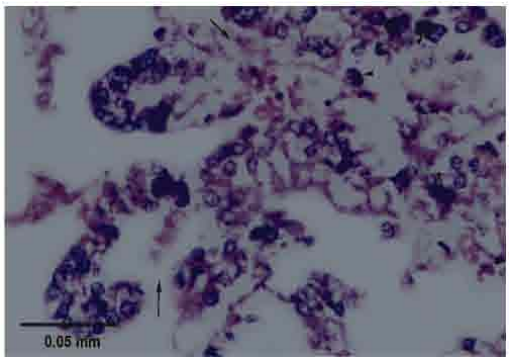

Fig. 3: Light micrograph of hepatopancreas of diseased prawn showing: necrotic and collapsed tubules (arrow) and necrotizing foci (arrowhead). Stained with H. and E. Bar = 0.05 mm

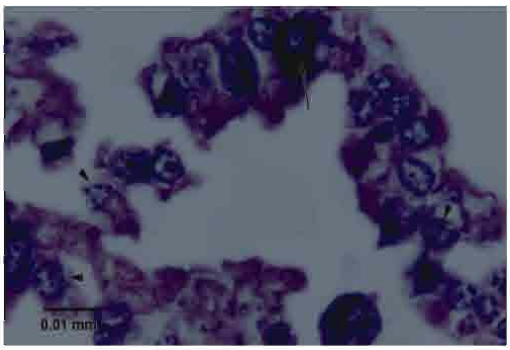

Fig. 4: Light micrograph of hepatopancreatic tubules from diseased prawn showing: microcolonies (arrowhead) in the hemal sinus of hepatopancreas, hypertrophied nuclei with marginated chromatin (arrow). Stained with H. and E. Bar = 0.01 mm

On the other hand, histological sections of the diseased sample showed lesions characteristic of prawn with bacterial infection. Many tubules were collapsed and contained intraluminal haemocytes. Light photographs (Fig. 3, 4) revealed extensive hepatopancreatic tubular necrosis and tubules were replaced by bacterial haemocytic nodules, often melanized and marked hemolytic enteritis.

Gills of healthy *M. rosenbergii* composed of elongated axial structure with a series of lateral lamellae. They showed uniform arrangement of lamellae with uniform inter lamellar space and normal haemocoelic space with optimum number of haemocytes (Fig. 5).

There are structural changes that occurred in the gills of the diseased prawn as a result of *Vibrio* infection. These changes include the accumulation of haemocytes

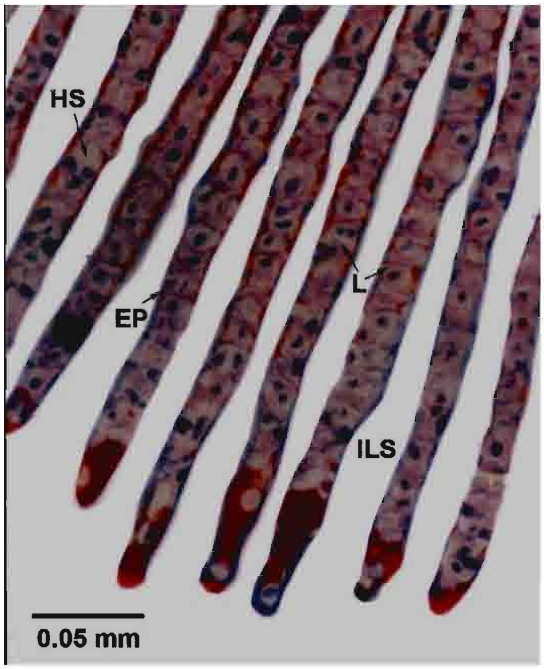

Fig. 5: Light micrograph showing longitudinal section of gill lamellae of healthy *M. rosenbergii*; EP: Epithelial cells; HS: Haemocoelic Space; Ils: Interlamellar Space; L: Lamellae. Stained with Azan. Bar = 0.05 mm

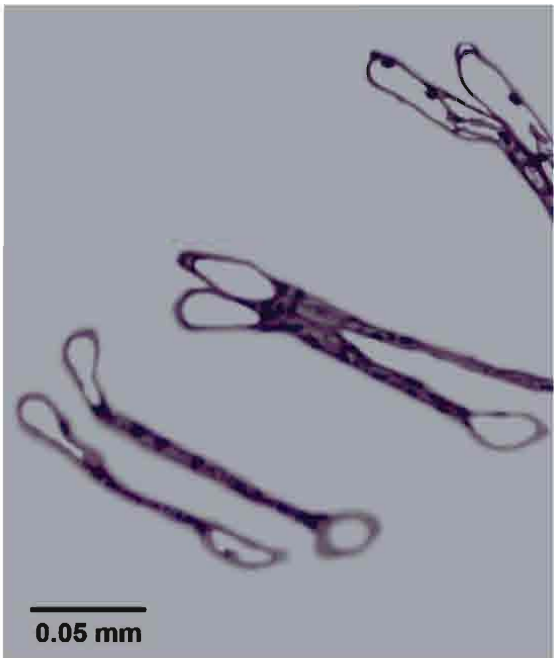

Fig. 6: Light micrograph gill of diseased prawn showing malformations of gill lamellae tips (arrow). Stained with H. and E. Bar = 0.05 mm

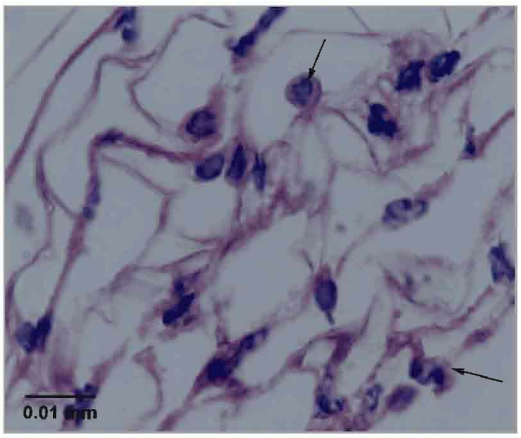

Fig. 7: Diffuse necrosis of gill lamellae in diseased prawn with pyknotic and karyorrhectic nuclei (arrow). Stained with H. and E. Bar = 0.01 mm

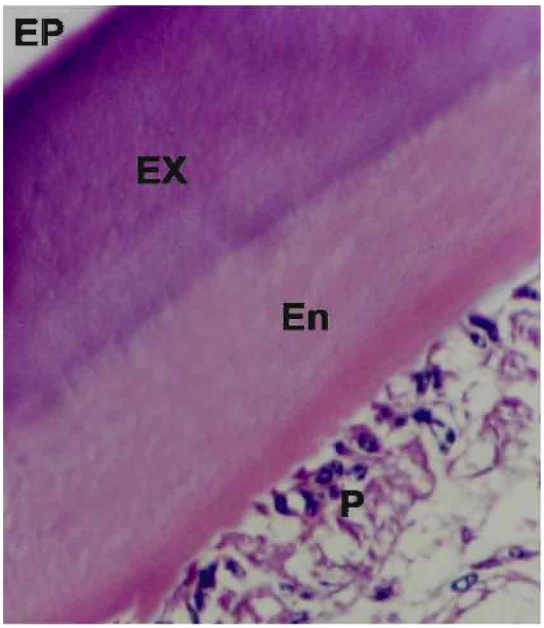

Fig. 8: Exoskeleton of healthy prawn showing, EP: Epicuticle; Ex: Exocuticle; En: Endocuticle; P: epidermis. Stained with H. and E. Bar = 0.01 mm

in the haemocoelic space, fusion of the lamellae and abnormal gill tips (Fig. 6). Light micrograph (Fig. 7) showed diffuse necrosis of gill lamellae pyknotic and karyorrhectic nuclei.

Light microscopic observations showed that the cuticular layers of the exoskeleton of healthy *M. rosenbergii* are intact and attached to the epidermis. The cuticle of prawn is composed of outer thin epicuticle, a thick exocuticle which appears to be lamellated

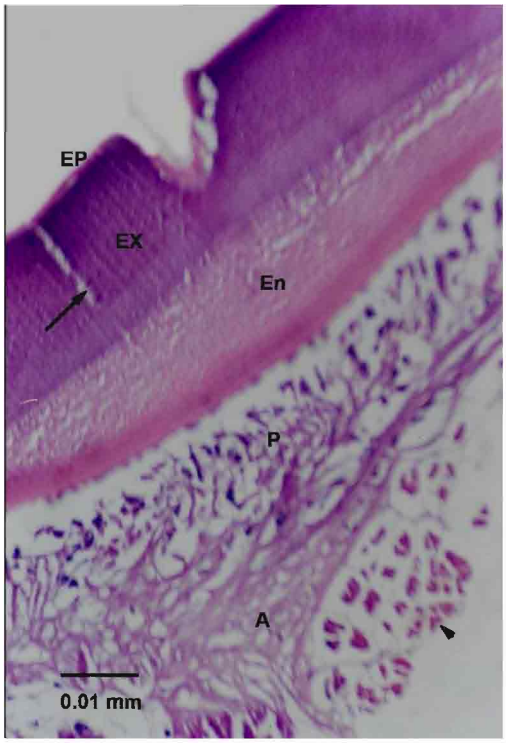

Fig. 9: Exoskeleton of diseased prawn showing erosion, degeneration and melanization of epicuticle and exocuticle, EP: Epicuticle; Ex: Exocuticle; En: Endocuticle; P: epidermis; M: Muscle. Stained with H. and E. Bar = 0.01 mm

Table 2: Sensitivity of *Vibrio vulnificus* to different antibiotics

Antibiotics	Concentration (μg)	Sensitivity
Rifadin	30	+++
Trimethoprim/sulfamethoxzole	1.25/23.75	+++
Vibramycin	30	+++
Oflaxcin	30	+++
Amikicia	30	+++
Garamycin	30	++
Flummox	30	+++
Nalidixic acid	30	R
Unasyn	30	R
Velosef	30	R
Claforan	30	R
Negram	30	R
Amoxicillin	30	R

+++: Highly sensitive; ++: Moderately sensitive; R: Resistant

and a relatively thinner endocuticle. The connective tissues next to the epidermal layer are directly in contact with the muscle tissues on the other sides (Fig. 8).

Histopathology of the diseased prawn showed erosion through the epicuticle which was characterized by necrosis, abrasions, scratches, cracking and melanization (Fig. 9). Erosion extended into the exocuticle with necrosis and loss of cuticular matrix, resulting in the formation of shallow carters in the carapace. Besides, cracking and moderate to severe melanization were observed (Fig. 10).

Fig. 10: Light micrograph of exoskeleton of diseased prawn showing cracking in epicuticle and exocuticle (arrow) and edematous. Fluid (A) accumulated between the cuticle and underlying muscle (arrowhead). Stained with H. and E. Bar = 0.05 mm

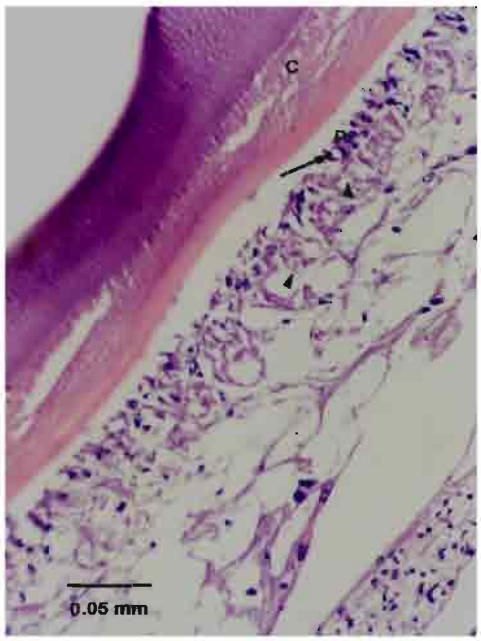

Fig. 11: Dissolution of columnar cell membrane of the epidermis, P: Under the cuticle; c: nuclear pyknosis (arrow), vacuolization and loss of cytoplasmic density (arrowhead). Stained with H. and E. Bar = 0.05 mm

Histopathological observations revealed that the diseased prawn had dissolution of columnar cell membranes of the epithelium under the cuticle, nuclear pycnosis, vacuolization and loss of cytoplasmic density (Fig. 10, 11). Also, diseased prawns showed marked edematous fluid accumulation between the cuticle and underlying muscle bundles with liquefaction necrosis (Fig. 10).

Susceptibility of *Vibrio vulnificus* to antibiotics: The tested pathogen, *V. vulnificus* was highly sensitive to each of the antibiotics rifadin, virbamycin, oflaxcin, garamycin, flummox (30 µg of each) and trimethoprim/sulfamethoxzole (1.25/23.75 µg) and moderately sensitive to amikicin. On the other hand, this pathogen exhibited resistance to nalidixic acid, unasyn, velosef, claforan, negram and amoxicillin (30 µg of each) (Table 2). The MIC of trimethoprin/sulfamethoxzole for the studied pathogen, *V. vulnificus* was 0.31/5.93 µg.

DISCUSSION

Vibrio species are part of the natural microflora of wild and cultured prawns and become opportunistic pathogens when natural defense mechanisms of the prawn are suppressed (Brock and Lightner, 1990). They are usually associated with multiple etiological agents. However, some *Vibrio* species, or strains of certain species, have been identified as primary pathogens. *Vibrio* species exist in the water used in prawn culture facilities. Bacteria enter prawns via wounds or cracks in the cuticle and are ingested with food (Lavilla-Pitogo *et al*., 1990; de La Pena *et al*., 1995).

Vibrio vulnificus did not successfully attach or invade wounded body surface, since water borne infection did not produce disease. It is possible that the transmission of *Vibrio vulnificus* occurs through the gastrointestinal tract (Song *et al*., 1993).

The mechanism of infection may be a combination of invasive and toxic pathways. Over certain concentration levels of the pathogen in the gut, vibrios lysed by digestive enzymes could release large amount of toxins (ciliostatic toxin, proteases, endotoxins) which would stop the digestive transit and it starts to degrade tissues. Subsequent growth of vibriosis would strengthen this attack until all tissues have been invaded (Nicolas *et al*., 1996; Verschuere *et al*., 2000).

In the current study, marked inflammatory response was detected in several organs of diseased prawn such as hepatopancreas, gill and exoskeleton

The diseased prawns had notable structural alterations of the gill lamellae including accumulation of haemocyte, fusion of lamellae and the formation of disorganized mass of disrupted gill lamellae. These observed inflammatory changes might be viewed simply as a protective mechanism, since the vulnerable surface of the gill is decreased in order to maintain the osmoregulatory functions. Similar observations were reported by Edgerton *et al.* (2000) in crayfish *Cherax quadricarinatus*. While Anderson *et al.* (1987) and Owens *et al.* (1992) showed the same disease in other crustaceans such as *Macrobrachium* sp.

Because of hepatopancreas is a sensitive organ and liable to injury by pesticides and other water pollutants (Bhavan and Geraldine, 2000). Thus, infected hepatopancreas appeared poorly vacuolated indicating low lipid and glycogen reserves (Anderson *et al.*, 1988). Systemic vibriosis typically results in the formation of septic haemocytic nodules in hepatopancreas (Jiravanichpaisal *et al.*, 1994). Previous studies showed that crustacean haemocytes exhibited degranulation in vitro when exposed to bacteria (Soderhall *et al.*, 1986; Kalia *et al.*, 2001; Jussila *et al.*, 2004; Sharshar, 2004). While, granules in haemocytes from a variety of healthy decapod crustaceans are known to contain lysosomal enzymes, prophenoloxidase and antibacterial compounds (Khoo *et al.*, 1999; Destoumieux *et al.*, 2000; Bartlett *et al.*, 2002). The haemocytes associated with the basal lamina of diseased shrimp *Sicyonia ingentis* were placed to light pathogens passing into the body through the mid gut (Martin *et al.*, 2004). The lesion observed in the hepatopancreas and gills of diseased prawns are likely to be structural manifestation of disruptions in the absorptive, storage and secretory functions of the hepatopancreas and in the osmoregulatory and respiratory physiological mechanisms of the gills.

In many areas, water for prawn culture comes from a multi user resource used to receive effluents from industry, commercial and domestic. Thus, good prevent selection, pond design and pond preparation are important to avoid bacterial disease (Nash *et al.,* 1992).

Also, vibriosis is controlled by rigorous water management and sanitation to prevent the entry of vibrios in the culture water and to reduce stress on the prawns (Lightner, 1993). In the present research several antibiotics showed high antimicrobial activities against the bacterial pathogen, *V. vulnificus* and could be used as an effective bacterial treatment in prawn cultures. The antimicrobial compounds used in such prawn culture should be changed periodically to avoid drug resistance development by prawn pathogens. Therefore, it is recommended that continue investigation to determine the annual changes of MICs for particular chemotherapeutants as a criterion of drug resistance and the persistence and degradability of antibacterial compounds used in aquaculture environments.

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