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Pathology and immunohistochemical evaluation of *Vibrio alginolyticus* infection in *Macrobrachium rosenbergii*

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Abstract

Vibriosis is one of the common bacterial diseases plaguing the prawn and shrimp industry. However, the sequential histopathological changes associated with *Vibrio alginolyticus*-induced vibriosis is scanty in literature. This study investigated the histological alterations in groups of *Macrobrachium rosenbergii* experimentally injected intramuscularly between the second and third abdominal segment with 50 µL of 107 CFU of *V. alginolyticus*. Twenty-four hours post inoculation, the organism was isolated and molecularly characterized while infected giant freshwater prawns were observed for histological alterations and immunoreactivity in different tissues due to the experimental challenge.

The lesions observed in the organs include incipient haemocytic infiltration into the interstitial space of the tubules of the hepatopancreas and loss of epithelial layer, muscular necrosis and disruption of muscular layer with haemocytic infiltration, hyperplasia of epithelial cells and degeneration of the epithelium, deformed and necrotizing lamellae, and enlargement of the lamellar sinus of the gill, and the heart is characterized by localized nodular haemocytic reaction and melanization. Organs such as the heart, gill, hepatopancreas and muscle following injection of the putative bacteria showed immunohistochemical activity. Hence, histology and immunohistochemistry still remain important tools for vibriosis disease diagnoses in giant freshwater prawns.

Keywords Pathology · Immunohistochemistry · *Macrobrachium rosenbergii* · *Vibrio alginolyticus*

Introduction

Diseases often encountered in shrimps are sequelae of a complex interaction of the shrimps, their environments and the pathogen themselves (Prakash and Karmagam 2013). Globally, disease outbreak in shrimps has caused severe economic loss of about \$US 3 billion (Vaseeharan and Ramasamy 2003). The major infectious diseases that cause a devastating

economic loss in shrimp and prawn industry are caused by bacterial and viral pathogens (Barman et al. 2011).

Vibriosis is one of the major bacterial diseases plaguing shrimp and prawn industry. The disease is caused by myriads of species, and at least 14 species have been identified (Longshaw 2011; Jayaprakash et al. 2006; Kennedy et al. 2006; Tyagi et al. 2007; Chen et al. 1992; Brock and Lightner 1990). *Vibrio* spp. are found to be normal members of the bacterial flora in the aquatic environments and may be considered as opportunistic pathogens and/or true pathogenic organisms (Mehdi 2011). The presence of any *Vibrio* sp. in the haemolymph or other tissues of shrimp or prawn is not exclusively an indication of *Vibrio* infection, as the bacteria may also be isolated from an apparently healthy aquatic crustacean without any form of clinical signs of the infection (Gomez-Gil et al. 1998). Hosts may be co-habiting with their potential pathogens under some conditions with little or no deleterious effect (Lightner and Redman 1998). This interaction often creates ambiguous interpretations of diagnosis when a sample of prawn is submitted for laboratory procedures. A correct diagnosis that is properly

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Table 1 Immunoreactivity quantification

	Mean	Min	Max
Fig.11.jpg-(Colour_2)	160.326	0	255
Fig.12.jpg-(Colour_2)	108.127	0	255
Fig.13.jpg-(Colour_2)	107.526	0	255
Fig.14.jpg-(Colour_2)	173.270	0	255

carried out is essential for an effective disease control and efficient treatment regimen. The diagnosis of *Vibrio* infection in shrimp can be based on the combination of clinical signs, histological examination and bacterial culture (Flegel 2012) since the palpable lesions of *Vibrio* infection are not pathognomonic. Other bacterial infections such as a chitinolytic bacterial disease that is caused by different bacteria spp. including *Aeromonas* spp., *Pseudomonas* spp., *Flavobacterium* spp. and *Spirillum* spp. share common clinical signs especially dark or brown discolouration of the prawn (Bower et al. 1994). Histological examination has reportedly been used as a good tool to monitor the extent of tissue changes due to stress and monitor the extent of tissue changes due to structural pathology associated with disease (Collins 2010) while immunohistochemistry is an important investigative and rapid diagnostic histological technique that enables identification of tissue constituents by means of antigen-antibody reactions via a colour change (Noraini et al. 2013). Both techniques are essential in prawn disease investigation. A complete diagnostic approach is expedient to arrive at a definitive and conclusive diagnosis

which is vital to the sustenance of prawn aquaculture in order to enhance better understanding of pattern, forestall the unnecessary spread of disease and enable timely and correct control or treatment plan. However, this study demonstrated the isolation and molecular identification of *Vibrio alginolyticus* from experimentally infected giant freshwater prawns (*Macrobrachium rosenbergii*) and is the first to investigate the histological alterations and immunoreactivity in different tissues such as the gill, muscle, heart, and hepatopancreas of the giant freshwater prawn primed with bacterial outer membrane proteins and experimentally challenged by *V. alginolyticus* (Table 1).

Materials and methods

Experimental animals

Apparently healthy adult freshwater prawns with the average weight of 26 ± 2 g were obtained from a farm in Sungai Dulang, Bagan Datoh, Perak, Malaysia. The prawns were acclimatised for 1 week prior to the commencement of the experiment, and haemolymph was randomly collected to screen the crustaceans for the presence of *Vibrio* spp. The prawns were divided into three groups A, B and C of ten prawns each with replicates in 6 (150 l) glass aquaria and positive control group of ten prawns only. Group A was fed with OMPs-mixed diet, group B with OMPs-FIA (Freund's

Fig. 1 Agarose gel (1.0%) electrophoresis of polymerase chain reaction (PCR) amplified DNA product of collagenase gene of *V. alginolyticus*. Lanes 1 and 5, 1 kb molecular weight marker; lane 2, negative control; lane 3, positive control (800 bp); lane 4, *V. alginolyticus* isolate (800 bp)

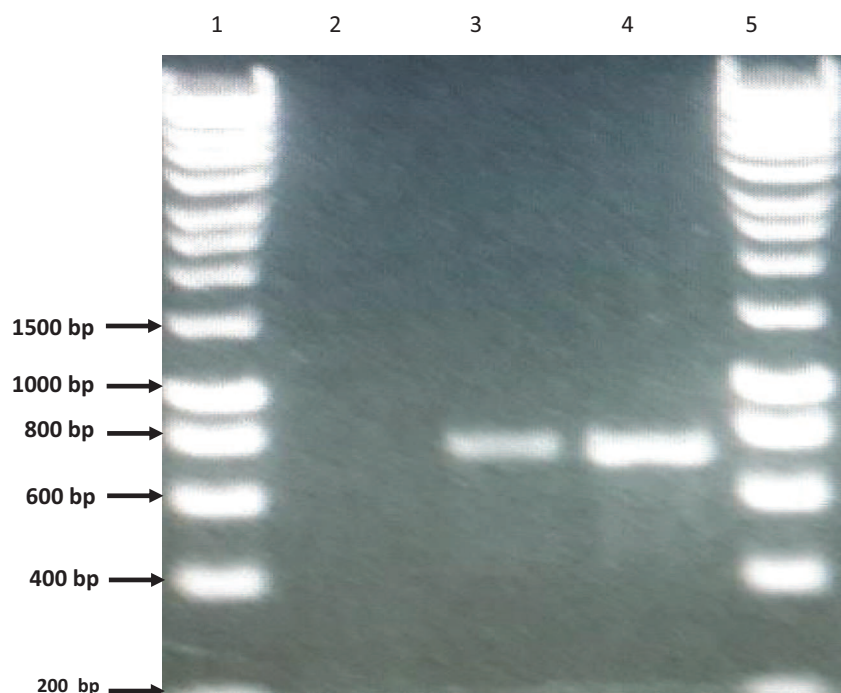
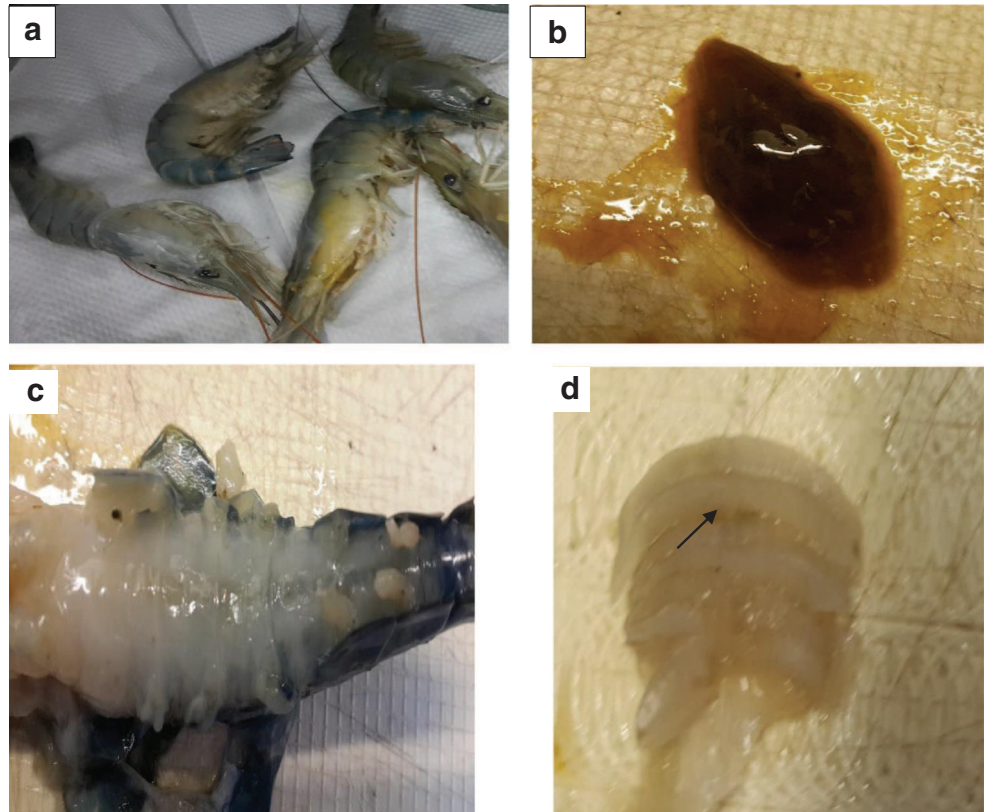


Fig. 2 Photographs of different organs of a positive control group of prawns 24-h post-injection. **a** The bodies of the dead prawns. **b** Dark-brown hepatopancreas. **c** Moderately cloudy muscle. **d** Gills with a brown spot (arrow)



incomplete adjuvants)-mixed diet, while group C was fed with OMPs or adjuvant-free diet (control diet). Groups A and B were fed for 7 days, alternated with control diet for 7 days and a booster dose for another 7 days, thus, a total of 28 days. Water parameters such as temperature, dissolved oxygen, pH and salinity were measured every other day using YSI (Yellow Spring Inc. OH, USA). All experimental prawns in group A, B and C were injected intramuscularly between the second and third abdominal segments with 50 μ L of 10^7 CFU of

V. alginolyticus and positive control with 50 μ L of PBS using a 1-mL sterile syringe (25 gauge). By 24-h post-infection, the prawns had died and samples were collected for further investigations.

Isolation and culture of bacteria from challenged prawns

The putative bacterium was aseptically isolated from the gills and hepatopancreas of the prawns using a sterile cotton swab.

Fig. 3 Photomicrograph of the hepatopancreas from positive control group showing infiltration of haemocytes (black arrow) and obliteration of tubular lumen and severe coagulative necrosis at 24-h post-infection (H&E, 100 μ m)

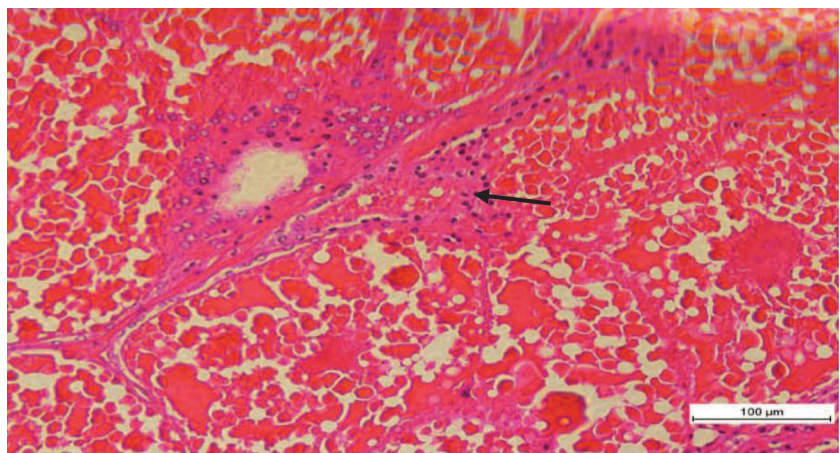
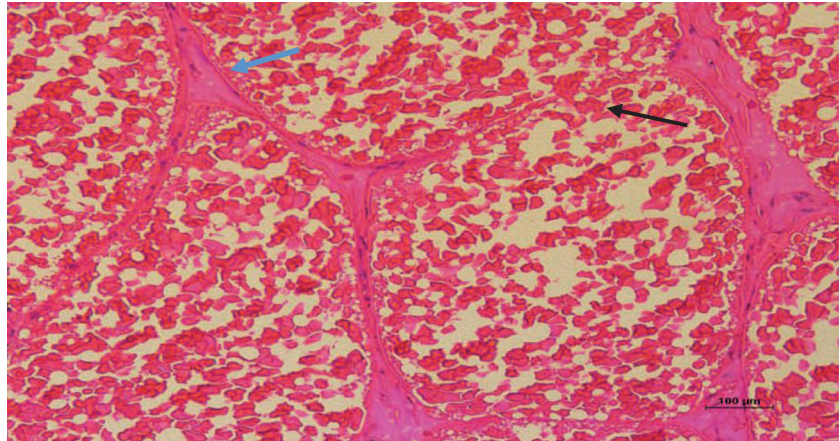


Fig. 4 Photomicrograph of the hepatopancreas from OMPs group showing degeneration of epithelium (black arrow) and thickened inter tubular septa (blue arrow) and loss of tubular lumen at 24-h post-infection (H&E, 100 μ m)



The swab was then streaked on TCBS agar and incubated for 24 h at 30 °C and Gram stain was performed.

DNA extraction

The DNA of the cultured bacterial cell was extracted using Promega Wizard® Genomic DNA extraction kit (Promega, USA), and the manufacturer's instruction was followed. Overnight, broth culture (1 mL) was centrifuged for 2 min at 13,000 $\times$ g, and the supernatant was discarded. The pellet was re-suspended in 600 μ L nuclei lysis solution and gently mixed, after which the mixture was incubated at 80 °C for 5 min and cooled to room temperature. This was followed by addition of protein precipitation solution (200 μ L), vortexed and incubated on ice for 5 min. Upon incubation, the mixture was centrifuged at 13,000 $\times$ g for 3 min. The supernatant was transferred into a new clean tube containing 600 μ L of isopropanol, gently mixed by inversion and centrifuged as mentioned above. The supernatant was discarded, and 600 μ L of 70% alcohol was added, mixed gently and

centrifuged at 13,000 $\times$ g for 2 min. The ethanol was aspirated, and the pellet was air dried for 15 min. The DNA was rehydrated in 100 μ L rehydration solution and kept overnight at 4 °C. The DNA was stored at – 20 °C until used for analysis.

Polymerase chain reactions

The extracted DNA was subjected to polymerase chain reactions (PCR) using *V. alginolyticus* specific forward primer (5'-TGGTGAACAGCCAGTAAA-3') and reverse primer (5'-AAACCCATCATAAGTAG-3'). Briefly, the PCR was carried out with the following constituents according to the manufacturer's guideline (1st Base, Malaysia); extracted DNA (6 μ L), 2 $\times$ PCR BIO *Taq* mix (25 μ L), forward primer (2 μ L), reverse primer (2 μ L) and distilled water (15 μ L) give a total volume of 50 μ L in an Eppendorf tube. The tube containing PCR mixture was placed in Eppendorf master cycler. The cycler was programmed as follows: initial denaturation at 95 °C for 1 min followed by 34 cycles at 95 °C for 15 s, annealing at

Fig. 5 Photomicrograph of the hepatopancreas from negative control group showing normal cellular structure with mild vacuolations (H&E, 100 μ m)

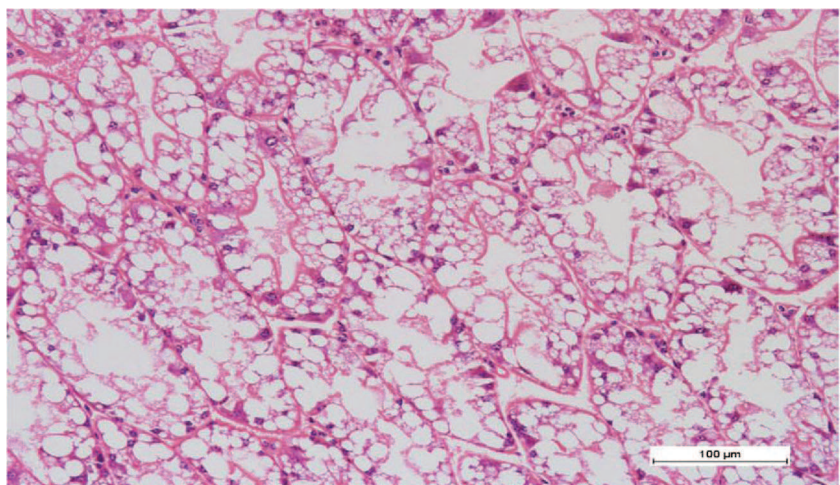
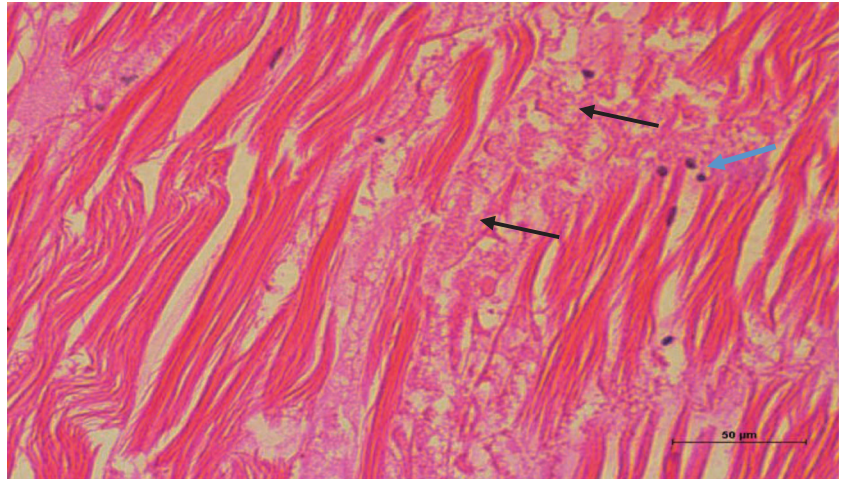


Fig. 6 Photomicrograph of the muscle from positive control group showing diffuse areas of necrosis (arrows) and infiltration of haemocytes (blue arrow) (H&E, 100 μ m)



47.6 °C for 15 s, extension at 72 °C for 30 s, final extension at 72 °C for 10 min and hold at 4 °C.

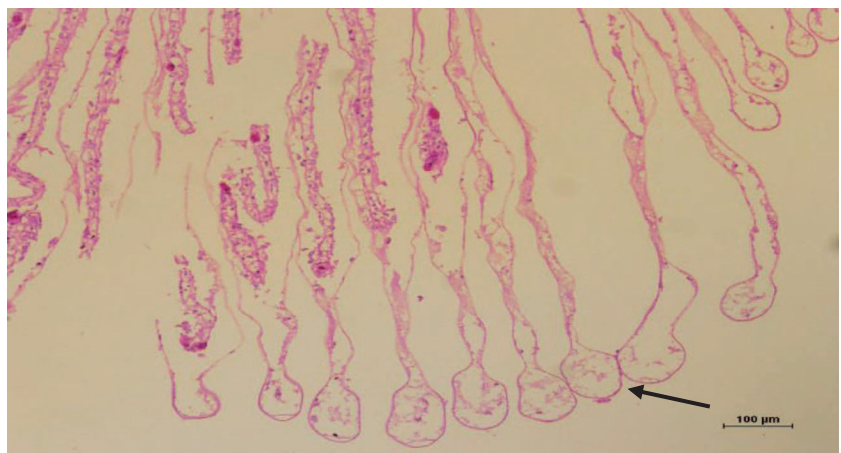
Detection of PCR product

Upon the completion of the 35 cycles, the amplified PCR products were separated by agarose gel electrophoresis (1.0% w/v agarose gel in 1× TBE). Five microliters of the PCR products were mixed with 3 μ L of loading dye and loaded onto the gel. Five microliters of Gene Ruler™ 1 kb DNA marker (Fermentas, USA) was used as a standard. The electrophoresis of the PCR product was done at 90 V (Power supply EPS 600, Pharmacia, Biotech) for 1 h. The gel was stained with GelRed™ nucleic acid gel stain before the bands were visualised under an ultraviolet light illuminator (Gene Snap program, UK).

Gross examinations

The prawns (whole body) were examined for any clinical sign or gross pathological changes 24 h post-*Vibrio* injection.

Fig. 7 Photomicrograph of the gill from OMPs group showing deformed lamellae with club tip (arrow) 24-h post-infection (H&E, 100 μ m)



Target organs such as muscles, gills, heart and hepatopancreas were also examined for gross lesions.

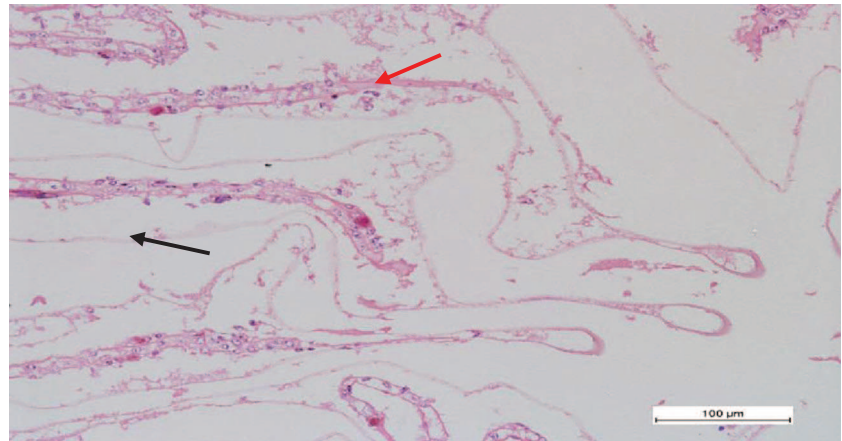
Histopathology

Tissues including muscles, gills, heart and hepatopancreas of both treatment and control groups were sampled. The samples were preserved in Davidson's AFA fixative for 24 h and later transferred to 70% ethyl alcohol. The standard histological procedure for shrimp and prawn as described by Bell and Lightner (1988) was followed. The fixed samples were then embedded in paraffin, and sections of 5 μ m thickness were prepared and placed on clean microscope slides. The slides were stained with haematoxylin and eosin (H&E) for light microscopy examination.

Immunohistochemistry

The method of Noraini et al. (2013) was followed with little modifications. The Davidson solution-fixed tissue samples of all the groups were embedded in paraffin,

Fig. 8 Photomicrograph of the gill from positive control group showing severe swelling and deformed architecture of the lamellae, separation of lamellar epithelium (black arrow) and gill necrosis (red arrow) 24-h post-infection (H&E, 100 μ m)



and serial 5 μ m thick were sectioned from the paraffin wax-embedded tissues onto saline-coated glass slides. The slides were dried for 15 min at 56 °C, deparaffinised in xylene for 5 min and rehydrated through a graded series of alcohol. The slides were rinsed with running tap water for 30 s and placed in PBS for 15 min. Heat-mediated antigen retrieval with citrate buffer solution in a microwave oven was employed to enhance immunoreactivity in the tissue. The slides were immersed in the citrate buffer and incubated in the microwave for 10 min at 50 W and rinsed with PBS. Freshly prepared 3% hydrogen peroxide was used to block the activity of endogenous peroxidase for 5 min at room temperature and gently rinsed and washed with PBS for 2 min. Tissue sections were blocked with blocking buffer (1% normal serum [Bovine serum albumin] in PBST), and then sections were incubated in rabbit anti-*V. alginolyticus* antibody at a dilution of 1:100 (antibody to 5% BSA in PBS ratio) for 1 h at 37 °C in an incubator. The sections were rinsed and washed in PBS for 5 min. Sections were incubated again with secondary antibody (goat anti-rabbit) at a dilution of

1:300 (antibody to 5% BSA in PBS ratio) at 37 °C for 30 min. The slides were rinsed and washed again in PBS for 5 min before DAB was applied at 1 mL diluents to a 1 drop DAB (3,3'-diaminobenzidine) (DAKO, USA) for the development of the colour change. The slides were immediately rinsed with distilled water once the sections turned brown and the slides were counterstained using Mayer's haematoxylin solution for the background colour. All slides were analysed and captured using image analyser NIS-Elements D 3.2 (Nikon, Japan). The images were quantified for staining intensity using the open Fiji (ImageJ) software (Ferreira and Rasband 2012).

Results

Bacteriological examination and molecular identification

The isolated organisms turned green TCBS into yellow thus, confirming sucrose fermentation of the organism. Gram stain

Fig. 9 Photomicrograph of the heart from positive control group showing moderate haemocytic infiltration and mild nodular haemocytic reaction (arrows) 24-h post-infection (H&E, 100 μ m)

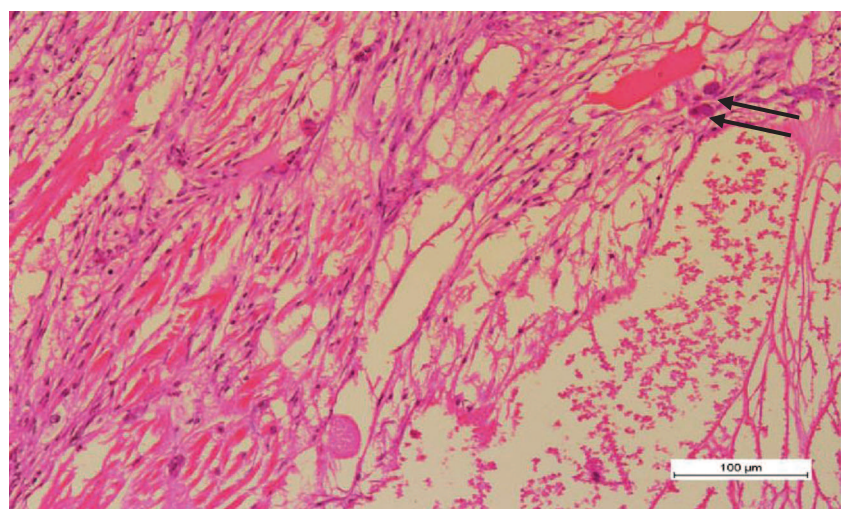
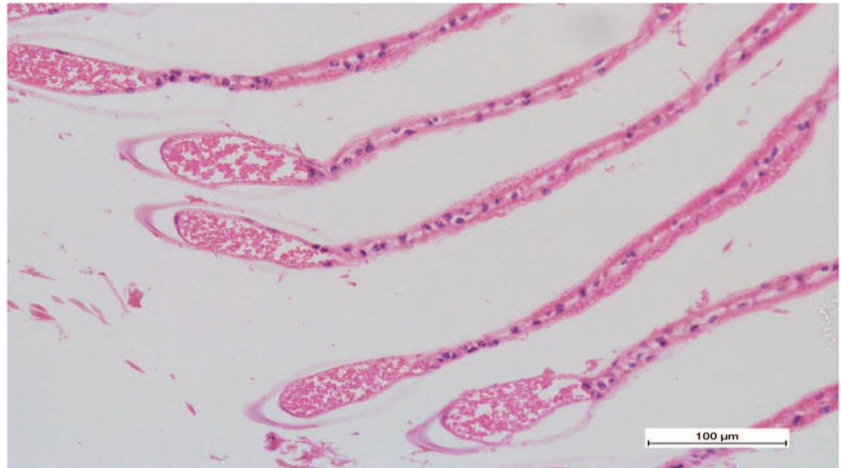


Fig. 10 Photomicrograph of the gill from negative control group showing the normal architecture of the gill (H&E, 100 μ m)



revealed a Gram-negative (pink colour) bacilli and PCR showed positive bands (using *V. alginolyticus* specific primers) at 800 bp with the positive control (Fig. 1).

Gross signs

The mortality rate was 100%. There were no apparent differences in the gross appearance between the treatment and control groups. However, some of the control group of prawns without OMPs or OMPs-FIA feed administration revealed mild cloudy muscles, loss of appendages and soft and friable dark-brown hepatopancreas (Fig. 2). The gills with focal necrosis and heart appeared apparently normal.

Histopathology

Different organs including muscles, heart, gills and hepatopancreas were examined for histopathological changes. The observed lesions, however, were similar in all groups challenged with *V. alginolyticus* but more severe in the positive control group. Negative control group revealed no distinct

histopathological changes. Histologically, the hepatopancreas is characterised by incipient haemocytic infiltration into the interstitial space of the tubule, putative bacteria infiltration, loss of epithelial layer, hyperplasia of the epithelial cells and degeneration of the epithelium (Figs. 3 and 4; for the normal cell structure, see Fig. 5). The muscular layer is disrupted with infiltration of haemocytes and bacterial cells and multi-focal necrosis (Fig. 6). There were also hyperplasia and haemocytic infiltration of the branchial arches of the gill with deformed and necrotising lamellar (Figs. 7 and 8). The heart is characterised by localised nodular haemocytic reaction, mild melanisation and bacterial infiltration (Fig. 9). However, in the negative control group, the heart, gills, muscle, and hepatopancreas were apparently normal with normal cell structure without alterations of the cellular integrity (Figs. 5, 10, 11, 12, 13, and 14).

Discussion

This study describes the gross and histopathological changes associated with *V. alginolyticus* infection in giant freshwater

Fig. 11 Photomicrograph of immunostaining of the muscle from positive control 24-h post-infection showing *V. alginolyticus* with moderate immunoreactivity to polyclonal antibody (arrow). Haematoxylin counter stain, 100 μ m

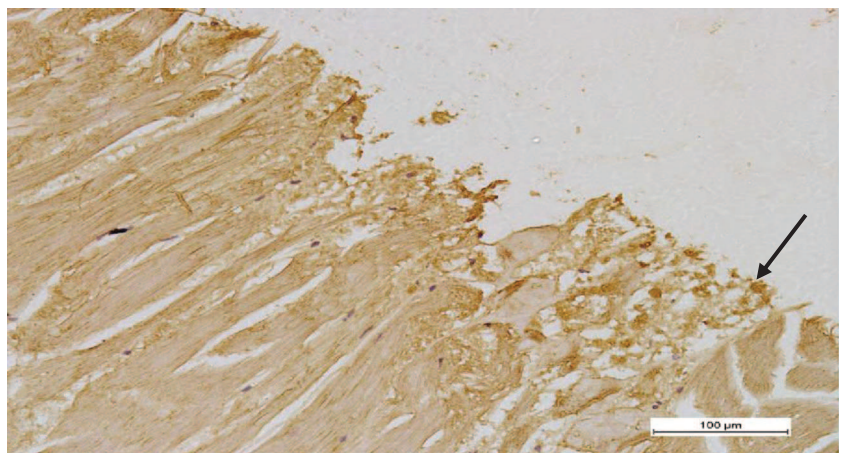


Fig. 12 Photomicrograph of the hepatopancreas from positive control 24-h post-infection showing *V. alginolyticus* with moderate immunoreactivity to polyclonal antibody (arrows). Haematoxylin counter stain, 100 μ m

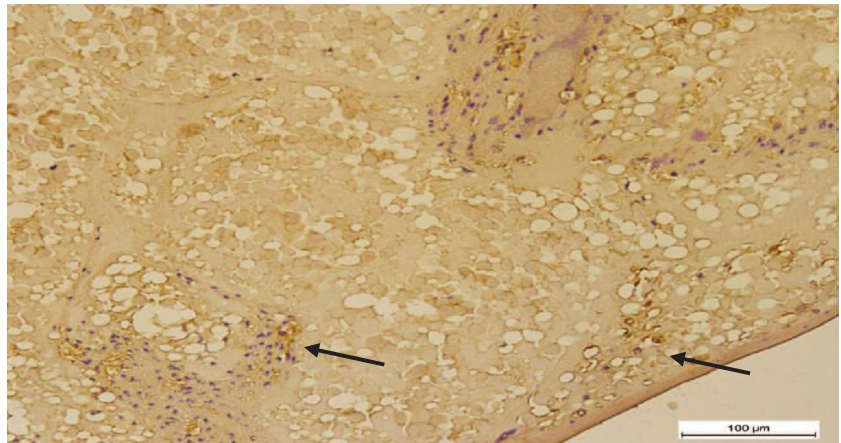
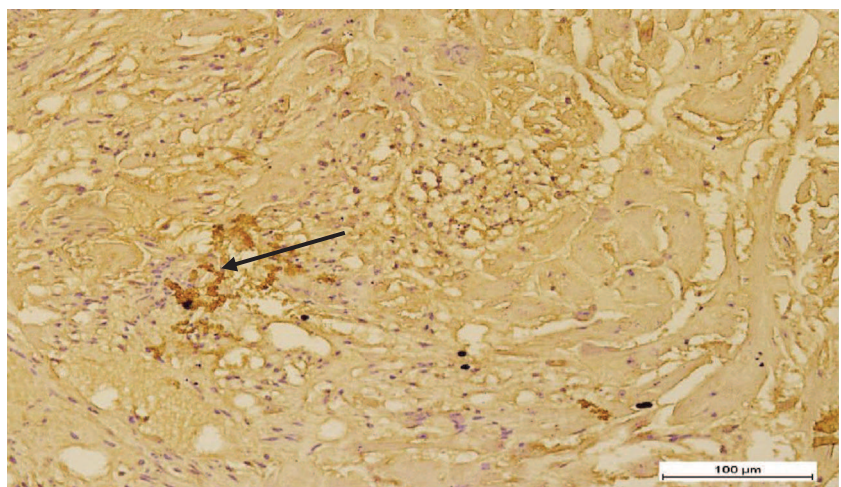


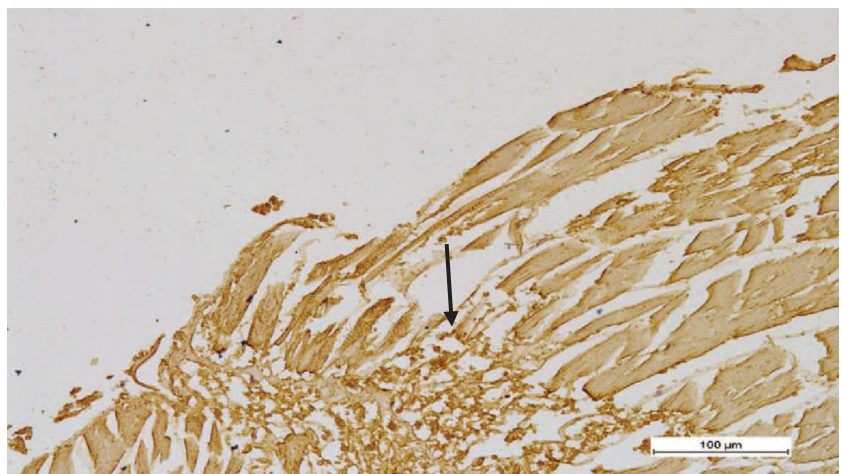
Fig. 13 Photomicrograph of immunostaining of the heart from positive control group 24-h post-infection showing *V. alginolyticus* with moderate immunoreactivity to polyclonal antibody (arrows). Haematoxylin counter stain, 100 μ m



prawn. Since vibriosis is an important bacterial disease of crustacean that is characterised by high morbidity and mortality (Khuntia et al. 2008), the need for effective and efficient methods of rapid diagnoses is an important approach towards the control of the disease. As observed in this investigation,

V. alginolyticus infection is characterised by necrosis and septicaemia as observed by various workers (Sayuthi 1993; Jayaprakash et al. 2006) although the disease may also be recognised as a primary infection and sometimes secondary (Saulnier et al. 2000).

Fig. 14 Photomicrograph of the muscle from positive control group 24-h post-infection showing *V. alginolyticus* with severe immunoreactivity to polyclonal antibody (arrow), 100 μ m



The apparently non-substantial gross lesions observed in this study could be as a result of acute nature of the disease course as observed by Khuntia et al. (2008) in *Vibrio parahaemolyticus*-infected prawns and in juvenile shrimps (*Penaeus monodon*) (Lavilla-Pitogo et al. 1998). The lesions observed with this infection are similar to those described by other workers which include cloudy musculature, loss of appendages and focal necrosis of hepatopancreas (Zorriehzahra 2014; Khuntia et al. 2008).

The cause of death could not be primarily ascertained as the sequelae of the bacteria challenge. Although, Adams (1991) also reported that injection of shrimps with heat-killed bacteria resulted in high mortality.

Systemic vibriosis as observed in this study results in disruption and sloughing of tubule epithelium of the hepatopancreas and significant proximal haemocytic infiltration (Lightner 2012). The musculature of the prawns was also characterised by haemocytic infiltration and necrosis. This could be associated with the site of injection and mobilisation of haemocytes to areas of injury or bacterial invasion. Similar earlier findings by Liu and Chen (2004) reported that necrosis of the muscle cells infiltrated with accumulating haemocytes and presence of a large amount of *Vibrio* organisms were observed in *Vibrio* infection of *Litopenaeus vannamei*.

Immunohistochemistry is an important investigative and diagnostic tool that enables the identification of tissue constituents through antigen-antibody reaction (Evensen et al. 1996). This present study demonstrated that specific antigen of *V. alginolyticus* could be identified in Davidson's fixative paraffin-embedded tissue samples of giant freshwater prawns challenged with the putative organism using histochemical technique. This finding was in consistence with the report of Alday-Sanz et al. (2002) whose study used immunoperoxidase technique to detect the antigens of *Vibrio vulnificus* in the tissues of black tiger shrimp (*Penaeus monodon*).

In crustacean pathology, diagnosticians and researchers alike depend greatly on case history, gross lesions and behavioural signs and morphological pathology with less focus on immunohistochemistry (Lightner and Redman 1998). A holistic approach with the use of immunohistochemistry would be beneficial to arriving at an understanding of the disease course, accurate diagnosis and effectual proper treatment or control plans in poor resource settings. It is also important in tissue localization of organisms.

In conclusion, the lesions observed in the organs including the hepatopancreas, heart, gill and muscles following intramuscular injection of pathogenic *V. alginolyticus* were described. The study also showed that histology and immunohistochemistry are vital tools in the diagnosis of vibriosis in giant freshwater prawns for effective control and treatment regimen.

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