Journal of Sustainability Science and Management
Volume 12 *Number* 2, December 2017: 167-175
ISSN: 1823-8556
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HEALTH SURVEILLANCE OF FRESHWATER PRAWN, *Macrobrachium lanchesteri* IN SETIU WETLAND, TERENGGANU, MALAYSIA

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Abstract: Freshwater prawn *Macrobrachium lanchesteri* is commonly present in the rivers in Setiu Wetland and is regarded as a key health indicator of waterbody. The present study was undertaken to survey the health status of *M. lanchesteri* in Nyatoh and Chalok Rivers in Setiu Wetland. Prawns were sampled from each river by seining for bacterial, mycological and viral screenings, as well as histopathogical examination. Bacterial screening was done using tryptic soy agar (TSA), glutamate starch phenol red (GSP) agar, thiosulfate citrate bile salts sucrose (TCBS) agar, pseudomonas agar F, xylose lysine dextrose (XLD) agar, eosin methylene blue (EMB) agar and horse blood agar. Identifications were done by Gram staining, oxidase and indole tests and BBL Crystal Identification System. Selected isolate was also identified by BLAST® analysis of 16S rRNA gene sequence. Fungi were isolated using potato dextrose agar (PDA) with penicillin G (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) identified by macro and micromorphologies and BLAST analysis of internal transcribed spacer (ITS) sequence. Viral screening was done for white spot syndrome virus (WSSV), Monodon baculovirus (MBV) and infectious hematopoietic and hypodermal necrosis virus (IHHNV) by polymerase chain reaction. A total of 14 isolates were obtained from the prawn hepatopancreas and eggs including *Pseudomonas aeruginosa*, *Enterobacter* spp., *Klebsiella* spp, *Enterococcus* spp., *Aerococcus viridans* and *Kluyvera cryocrescens*. The *P. aeruginosa* isolate (N5) was beta hemolytic whereas all other isolates were alpha hemolytic. Mycological screening isolated mostly *Ascomycetes* sp. Other fungi isolated were *Fusarium* sp., *Galactomyces* sp., *Basidiomycete* sp. and *Trichosporon* sp. The prawns were tested negative for WSSV, MBV and IHHNV. Histopathology revealed degeneration of hepatopancreas tubule cells and loss of the typical star-shaped lumen in cross-section profile in the samples from the Chalok River. Samples from the Nyatoh River also exhibited tubular degeneration, loss of the star-shaped lumen profile and reduced lumen size. Isolate N5 from Nyatoh River could be potentially pathogenic and of public health concern. Further characterizations on antibiotic susceptibility and virulence are needed to determine its pathogenicity. The *Fusarium* sp. isolate from Chalok River may be pathogenic to penaeid shrimp, thus further study is needed to identify the isolate to the species level. The absence of WSSV, MBV and IHHNV in the present study does not rule out the possible presence of these viruses in *M. lanchesteri* in Setiu Wetland. The cause of degenerative changes in hepatopancreas tubules could not be determined. In the present study, *Pseudomonas aeruginsa* and *Fusarium* sp. isolate may be potentially pathogenic to penaeid shrimp compared to other species.

Keywords: *Macrobrachium lanchesteri*, health screening, histopathological examination, Setiu Wetland.

Introduction

Macrobrachium lanchesteri is a relatively small but hardy native prawn species found in many types of freshwater habitats such as rivers, streams, rice fields, lakes, ponds and reservoirs. This prawn is a good swimmer, thus less restricted to bottom dwelling than many palaemonids and usually occurs in large numbers in the habitats

(FAO, 2016). It is an edible species favored by the locals, either consumed raw, cooked or fermented together with small fish and is also used as fishing bait. This species has been found in the temperature range of 25.5 to 36.0 °C and is able to live in shallow waters with rather high water temperature for several hours (FAO, 2016). Johnson (1964) has mapped the distribution of *M. lanchesteri* in Malaysia, followed by a number of new records later. It is known to inhabit the rice lands of central Terengganu and near Kuala Sedili Besar in eastern Johore. The Setiu Wetland, part of the Setiu River Basin and the larger Setiu-Chalok-Bari-Merang basin wetland complex, lie in Terengganu on the east coast of Peninsular Malaysia. It comprises of narrow strip of vegetation including mangroves and swamps growing next to riverbanks. *M. lanchesteri* is commonly present throughout the rice lands and numerous fish ponds and rivers in the wetland complex. It is considered a key indicator of waterbody health because the adult prawn has been reported to be sensitive to copper (Cu), cadmium (Cd), zinc (Zn) and lead (Pb) of which Cd was most toxic with a 96-h LC50 at 7.0 µg/L, followed by Pb, Cu and Zn at 35.0, 32.3 and 525.1 µg/L respectively (Shuhaimi-Othman *et al*., 2011). The present study was undertaken to establish baseline health status data of *M. lanchesteri* in Setiu Wetland.

Methodology

Sampling

Live prawns were sampled from the usual fishing grounds in Nyatoh and Chalok Rivers (5°37'0" N and 102°49'1") at Setiu Wetland by seining. The prawns were transported with aeration to the Fish Disease Laboratory at Universiti Malaysia Terengganu (UMT) for further works. Ten prawns each were randomly picked for body weight and size measurement and used for health surveillance.

Bacterial Screening

Isolation and Identification

The prawns were screened individually. The prawn hepatopancreas and eggs were aseptically prepared for bacterial isolation using tryptic soy agar (TSA), glutamate starch phenol red (GSP) agar, thiosulfate citrate bile salts sucrose (TCBS) agar, pseudomonas agar F, xylose lysine dextrose (XLD) agar, eosin methylene blue (EMB) agar and horse blood agar (Merck, Germany). Incubation was done at 25°C for 24-48 h. Colonies were subcultured on TSA to obtain pure cultures and stored for further uses. The isolates were identified by Gram staining, oxidase and indole tests, followed by BBL Crystal Identification System. Selected isolate was also identified by 16S rRNA gene sequence analysis. DNA was extracted by boiling technique (Sambrook & Russell, 2001) and amplified by 16S rRNA gene polymerase chain reaction (PCR) with primers fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3') according to Weisburg *et al.* (1991) in a Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Germany) using program: 1 cycle at 95°C for 2 min, 40 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min 30 s, final extension at 72°C for 7 min and 10°C hold until collected. PCR product was electrophoresed in 2% agarose gel preloaded with ethidium bromide in 1x TBE buffer and visualized under UV-transillumination. The 16S rRNA gene amplicon was purified and subjected to commercial Sanger sequencing (First BASE, Malaysia). The resulting DNA sequence was analyzed by Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Mycological Screening

Isolation and Identification

The mycological screening was part of surveillance for *Aphanomyces invadans*, the causative agent of epizootic ulcerative syndrome (EUS). The prawn samples above were also used for fungal isolation using potato dextrose agar (PDA) supplemented with penicillin G (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}). Incubation was done in CO_2 incubator at 25°C for 7 days. Fungal colonies were subcultured

on PDA until free from contamination. Pure isolates were stored on PDA slant for long-term preservation. Isolates were identified based on macro and micromorphologies. The macromorphological features included the culture appearance (visible to naked eye or under low magnification), growth rate, colony texture and colour. Micromorphological examinations were conducted by lactophenol cotton blue staining and light microscopy observation. The fungal isolates were also identified by internal transcribed spacer (ITS) sequence analysis. The mature fungal mycelia (40 mg) were collected and ground in liquid nitrogen using mortar and pestle, then subjected to DNA extraction using Wizard® Genomic DNA Purification Kit (Promega, USA). ITS fragment was amplified from the fungal DNA by PCR using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990) using program: 1 cycle at 95°C for 2 min, 35 cycles of 95°C for 15 s, 58°C for 30 s, 72°C for 45 s, followed by final extension at 72°C for 2 min and 10°C hold until collected. PCR products were analyzed and processed for DNA sequencing as above.

Viral Screening

The prawn samples were pooled into pools of two and screened for white spot syndrome virus (WSSV), Monodon baculovirus (MBV) and infectious hematopoietic and hypodermal necrosis virus (IHHNV) by polymerase chain reaction (PCR). Gills were used for WSSV and IHHNV PCR whereas the hepatopancreas and midgut were used for MBV PCR. WSSV PCR was done using primers GS (5`-ATTCCTGGCACTGACCATTTTCAT-3`) and GA (5`-AAAGGGATATTTTCTTGGCT GCA-3`), followed by inner primer TS (5`-GCCACTTGGCTCCGGCTGGAGA-3') and TA (5`-CCACGCGGCACCTGGCGTAGTT -3`) (Peng *et al*., 1998). MBV PCR was carried out using primers MBV1.4F (5'-CGATTCCATATCGGCCGAATA-3') and MBV1.4R (5'-TTGGCATGCACTCCCTGA GAT-3'), followed by nested primers MBV1.4NF (5'-TCCAATCGCGTCTGC GATACT-3') and MBV1.4NR(5'-CGCTAATGGGGCACAAGT CTC-3') (Belcher & Young, 1998). IHHNV PCR was carried out using primers 389F (5'-CGGAACACAACCCGACTTTA-3') and 389R (5'-GGCCAAGACCAAAATACGAA-3') (Gangnonngiwa *et al.,* 2010).

Histopathological Examination

Hepatopancreas was fixed in Davidson fixative solution and subjected to standard tissue processing procedure for histopathological examination with hematoxylin and eosin (H&E) staining.

Results

The prawns from Chalok River had an average body weight of 4.29 g and average body length of 5.1 cm whereas those from Nyatoh River were averagely 5.30 g and 5.6 cm respectively (Table 1).

Bacterial Screening

A total of 13 isolates (N1, N1-E, N2, N2-1, N2-E, N3-E, N4, N5, N7, N9, C2, C2-E and C5) were successfully obtained from the hepatopancreas and eggs, whereas only 1 isolate (C) was obtained from the water (Table 2). Isolate N5 was beta hemolytic whereas the rest showed alpha hemolysis. BLAST analysis showed 94% homology of N5 to *Pseudomonas aeruginosa* strain GIM 32 (accession no. HM067869), indicating the isolate as *P. aeruginosa*, thus

Table 1: The average body weight, total length and body length of prawns from Chalok and Nyatoh Rivers

Origin	Average Body Weight (g)	Average Total Length (cm)	Average Body Length (cm)
Chalok River	4.29 ± 0.20	6.1 ± 0.21	5.1 ± 0.26
Nyatoh River	5.30 ± 0.12	6.8 ± 0.21	5.6 ± 0.2

Table 2: Characteristics of bacterial isolates

No	Isolate ID	Origin	Organ	Gram	Medium Agar	Colony Colour	Bacterial Species	Confidence Level (%)	Blood Hemolysis
1	N1	Nyatoh River	Hepato-pancreas	-	GSP	Pink	*Klebsiella pneumoniae spp. ozaenae*	98.72	Alpha
2	N2			-	TSA	Whitish cream	*Kluyvera cryocrescens*	91.54	Alpha
3	N2-1			-	TCBS	Yellow	*Klebsiella pneumoniae spp rhinoscleromatis*	97.54	Alpha
4	N4			-	EMB	Pink with black at the center	*Enterobacter cloacae*	94.01	Alpha
5	N5			-	PSEUDO F	Fluorescent	*Pseudomonas aeruginosa*	99.71	Beta
6	N7			-	XLD	Yellow	*Klebsiella pneumoniae spp pneumoniae*	99.79	Alpha
7	N9			+	TSA	Yellow	*Aerococcus viridans*	99.98	Alpha
8	N1-E		Egg	+	TSA	Whitish cream	*Enterococcus durans*	99.11	Alpha
9	N3-E			+	TSA	Whitish cream	*Enterococcus faecalis*	98.91	Alpha
10	N2-E			-	TSA	Whitish cream	*Enterobacter aerogenes*	99.87	Alpha
11	C2	Chalok River	Hepato-pancreas	-	EMB	Pink with black at the center	*Enterobacter aerogenes*	96.67	Alpha
12	C5			-	TSA	Whitish cream	*Klebsiella pneumoniae spp rhinoscleromatis*	99.27	Alpha
13	C2-E		Egg	-	TSA	Whitish cream	*Klebsiella pneumoniae spp rhinoscleromatis*	97.54	Alpha
14	C		Water	+	TSA	Whitish cream	*Enterococcus faecalis*	99.89	Alpha

in agreement with the BBL result of 99.71% confidence.

Mycological Screening

A total of eight distinct isolates were obtained, five from Chalok River (3-C6, 6-C2, 7-C3, 8-C3 and 10-C3) and three from Nyatoh River (5N, 15-N3 and 16-N2). However, none of the isolates was *A. invadans*. Isolates 7-C3, 8-C3, 10-C3 and 16-N2 were identified as *Ascomycetes* spp. based on ITS sequence analysis. Other isolates were identified as *Galactomyces* sp. (5-N), *Trichosporon* sp. (15-N3), *Basidiomycete* sp. (3-C6) and *Fusarium* sp. (6-C2) (Table 3).

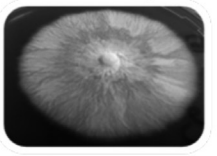
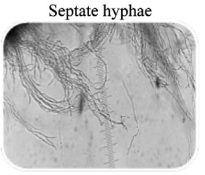
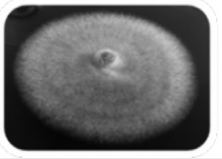
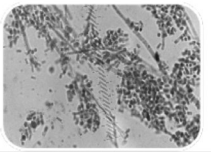
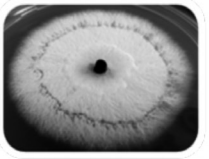
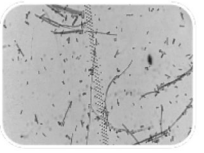
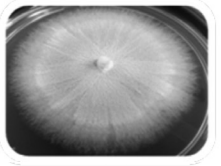
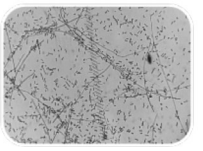
Viral Screening

The prawns were tested negative for WSSV, IHHNV and MBV (data not shown).

Histopathological Examination

Histopathology revealed degeneration of hepatopancreas tubule cells and loss of the typical star-shaped lumen in cross-section profile in the samples from the Chalok River. Samples from the Nyatoh River also exhibited degeneration of the tubule and loss of the star-shaped lumen profile, as well as reduced lumen size (Figure 1).

Table 3: Fungal isolates from Chalok and Nyatoh rivers

Origin	Isolation organ	Label	Morphology		Identity
			Macro	Micro	
Chalok River	Hepatopancreas	3-C6	White and thick mycelia 	Septate hyphae 	*Basidiomycete* sp.
	Egg	6 -C2	White mycelia with red and yellow basement 	Septate hyphae 	*Fusarium* sp.
		7 -C3	White and thick mycelia 	Aseptate hyphae with cylindrical spores 	*Ascomycetes* sp.
		8-C3	White and thick mycelia with black color at the center 	Aseptate hyphae with cylindrical spores 	*Ascomycetes* sp.
		10 -C3	White and thick mycelia 	Aseptate hyphae with cylindrical spores 	*Ascomycetes* sp.

Discussion

The present study was undertaken to survey the health status of *M. lanchesteri* as a key health indicator of waterbody. In this study, 14 bacterial isolates were identified including *Klebsiella pneumoniae, Kluyvera cryocrescens, Enterobacter cloacae, E. aerogenes, Pseudomonas aeruginosa, Aerococcus viridans, Enterococcus faecalis and E. durans. Enterobacter* and *Pseudomonas* have been reported as the most common bacterial genera encountered in freshwater prawn *M. rosenbergii* (Muto *et al.,* 2003). The present study showed some similarities in bacterial flora in *M. lanchesteri.* The beta hemolytic *P. aeruginosa* (N5) from the prawn in Nyatoh River could be of public health concern because the ability to induce hemolysis is a strong indication of bacterial pathogenicity (Cappuccino & Sherman, 2001). *Pseudomonas aeruginosa* has been increasingly recognized as an emerging opportunistic pathogen of considerable medical importance. One of the most worrying characteristics of *P. aeruginosa* is its low antibiotic susceptibility attributed to multidrug efflux pumps with chromosomally-encoded

Nyatoh River	Eggs	5 –N	White and thick mycelium	Septate hyphae	*Galactomyces* sp
		15 -N3	Milky white mycelium and soft structure	Septate hyphae	*Trichosporon* sp.
		16 -N2	White mycelium with yellow basement	Sub-fusiform spore	*Ascomycetes* sp.

genes and the low permeability of its cellular envelope (Muto *et al.*, 2003). Though *Klebsiella* sp., *Enterobacter* sp. and *Enterococcus* sp. are fecal coliforms, *Klebsiella* are not necessarily fecal in origin. In comparison, *Enterococcus* sp. are more fecal and human-specific and are recommended by the United States Environmental Protection Agency (USEPA) as a better indicator of health risk from water contact besides *Escherichia coli* (USEPA, 2012). Though *Enterococcus* were not isolated from the water of Nyatoh River as in Chalok River, the presences of *E. faecalis* and *E. durans* in the eggs of the prawn from Nyatoh River indicated prior exposure to fecal pollution. *Enterococcus* infect almost exclusively hospitalized patients with significantly compromised body defenses (Ryan & Ray, 2004). This implicates potential risk of consuming raw or undercooked prawn from the rivers. *Enterobacter aerogenes* and *E. cloacae* have been largely associated with several outbreaks of hospital-acquired infections in Europe, especially in France. They are the important multi-resistant opportunistic human pathogens in hospital wards during the last three decades (Davin-Regli & Pagès, 2015). On the other hand, *E. cloacae* has been reported earlier in the oysters from Setiu Wetland (Najiah *et al.*, 2008).

Aphanomyces invadans is known to infect approximately 94 species of farmed and wild fish worldwide (OIE, 2016). If this fungus-like oomycete does present in the river system, it posts potential threat to the susceptible fresh and brackish water fish populations in Setiu Wetland. Though it does not appear to infect crustaceans, other species, *Aphanomyces astaci,* known to be carried by freshwater swamp crustacean *Procambarus clarkii* can cause an OIE-notifiable disease called crayfish plague in *M. rosenbergii* and *A. astaci* is therefore under the import risk analysis for *M. rosenbergii* in certain country, for example New Zealand (BNZ, 2006). Although *Ascomycetes* sp. was most frequently isolated (four out of eight) in the present study, *Fusarium* sp. is the fungus of concern because secondary *Fusarium* infection has been associated with black spot cuticular disease in *M. rosenbergii* , though it appeared to be opportunistic rather than invasive (Burns *et al.*, 1979). Besides, *Fusarium* is also associated with black gill disease and cuticular necrosis in penaeid shrimp. Fungal infection during grow-out stage is a clear indication for pond improvement (Lightner, 1996). *Fusarium*,

Aspergillus and *Penicillium* are the most clinically relevant fungi in farmed shrimp and are also potentially pathogenic to humans and other animals due to their abilities to produce Aflatoxin B and protease (Silva *et al.,* 2011).

White spot syndrome virus (WSSV) is known to infect a wide range of crustaceans, however some of which will not die of the infection but act as carriers (Flegel, 1996; Rajendran *et al*., 1999). To certain extent *M. rosenbergii* is susceptible to WSSV (Peng *et al.,* 1998) and Rajendran *et al*. (1999) has shown with experimental proof that the virus does not cause serious mortality in the adult prawn. On the other hand, clearance of WSSV in experimentally infected *M. rosenbergii* has been reported in which the prawns recovered without any further gross signs of disease or any mortality over a period of 100 days post infection (Sarathi *et al.,* 2008). Nevertheless, prior to clearance of the virus, the carrier prawns may potentially spread the virus to a more susceptible crustacean species such as Pacific white shrimp *Litopenaeus vannamei* which is commercially cultured in Setiu. *Macrobrachium lanchesteri* could be potentially susceptible to WSSV, therefore was screened for WSSV in the present study.

On the other hand, MBV has been reported in *M. rosenbergii* postlarvae (Gangnonngiwa *et al.,* 2010) whereas IHHNV has been reported in *M. rosenbergii* postlarvae and sub-adults (Hsieh *et al.*, 2006). On these bases, *M. lanchesteri* was also screened for MBV and IHHNV in the present study. The negative results of WSSV, MBV and IHHNV screenings in the present surveillance do not imply the absence of the target viruses in the entire population of prawns in the ecosystem.

Histological analysis is a sensitive method for determining the cellular changes due to contaminants in target organs (Traversi *et al*., 2014). Histological alterations have been characterized in *M. malcolmsoni* and various species of *Macrobrachium*. For example, *M. lanchesteri* showed cellular alterations and changes in the hepatopancreas. Hepatopancreas is one of the most sensitive indicators of physiological disturbances in crustaceans as it acts as a center of intermediary metabolism (Kharat *et al.*, 2014). Hepatopancreas is composed of branched tubules lined with different types of epithelial cells (E-, R-, F- and B-cells). It is likely that exposure to harmful chemical such as copper sulfate would be reflected by structural alterations of tubules and epithelial cells (Kharat *et al.*, 2014). The hepatopancreas of healthy shrimp is composed of large compact ducts and blind-ending tubules. Each tubule consists of a single layer of epithelial cells enclosing the lumen. In this study, the prawn hepatopancreas from Chalok River showed degeneration of hepatopancreatic cells in the tubule and altered luminal morphology (loss of the typical star-shaped cross-section). Similar pathological changes as well as reduced lumen size were observed in the hepatopancreas of prawn from Nyatoh River. The cause of such histopathological changes, however could not be identified in the present study.

Conclusion

The isolation of *Enterococcus* spp. in the present study suggested possible human fecal pollution in the fishing grounds of both rivers. The presence of potentially pathogenic bacteria as indicated by the beta hemolytic property reflects the potential risk to the prawns and humans. The beta hemolytic *P. aeruginosa* (isolate N5) from the prawn in Nyatoh River could be potentially pathogenic and of public health concern. Further antibiotic susceptibility and virulence characterizations are necessary to determine its pathogenicity. The *Fusarium* sp. isolated from the prawn in Chalok River could be one of those species that infect penaeid shrimp, thus further study is necessary to identify the isolate to the species level. Though no WSSV, MBV and IHHNV were detected in the prawns in the present study, the possible presence of the viruses in *M. lanchesteri* in Setiu Wetland could not be ruled out. The cause of the degenerative changes in hepatopancreas could not be determined in this study.

Acknowledgements

This research is supported by Niche Research Grant Scheme (NRGS/2014/53131/9), MOHE, Malaysia and Sciencefund (E-SCIENCE 02-01-12-SF0120), MOSTI, Malaysia.

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