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Water condition and identification of potential pathogenic bacteria from red tilapia reared in cage-cultured system in two different water bodies in Malaysia

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The need to conduct periodic surveillance on the presence, associated pathological alteration in tissues and the various environmental factors that could trigger some potential fish pathogens that result to disease outbreak in tilapia farming in Malaysia is paramount. This study was conducted to determine the presence of potential pathogenic bacteria in fish and water bodies that could trigger disease outbreak. Some potential pathogenic bacteria were isolated and identified from water, sediments and tissues of red tilapia reared in cage-cultured system in Kenyir Lake, Terengganu and Semantan River, Pahang, east of Peninsular Malaysia, also the water quality was assessed using standard techniques. The brain, eye and kidney were collected randomly from 30 tilapias from each of these water bodies. The bacteria were isolated and identified using standard methods. In Kenyir Lake, bacterial isolates that predominated in selected tissues of tilapia were *Micrococcus* spp., *Aeromonas hydrophila*, *Staphylococcus* spp., *Pseudomonas aeruginosa* and *Enterobacter cloacae* while in Semantan River, *A. hydrophila* and *Staphylococcus* spp. predominated. The water quality of Semantan River was found to be above the recommended limits of ammonia, sulphide, iron and nitrite-nitrogen levels. For the water sample, *Staphylococcus xylosus* was the most predominant bacteria isolates in Kenyir Lake, while *Staphylococcus lentus* was the most predominant of Semantan River. From the sediments, *Pseudomonas aeruginosa* and *Enterobacter cloacae* were isolated in Kenyir lake while, *A. hydrophila* was found in Semantan river. From this investigation, *A. hydrophila* and *Staphylococcus* spp. are the predominant bacteria in Red hybrid tilapia; water quality, animal and human activities may play a role in the susceptibility of red tilapia to these potentially pathogenic bacteria which have not being previously observed in Malaysia. There is need for periodic surveillance of water, sediment and tissues of fish to detect the pathogens of paramount importance to Malaysian aquaculture industry.

Key words: Bacteria, Red hybrid tilapia, analytical profile index (API) test, water quality, lake, river.

INTRODUCTION

Red hybrid tilapia (*Oreochromis* sp.) was first introduced into Malaysia in the mid 1980's. It was initially considered to be hardy and resistant to diseases (Siti-Zahrah et al., 2004; 2008). However, unlike higher vertebrates, fishes being less immuno- competent could be predisposed to innumerable disease outbreaks (Bowser et al., 1998).

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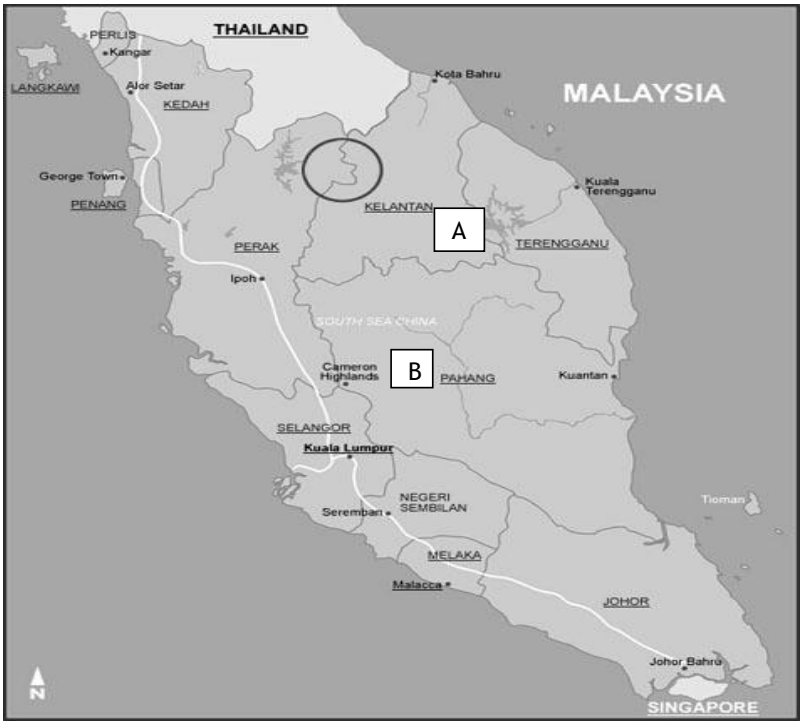

Figure 1. The study sites; A Kenyir Lake and B Semantan River.

Outbreak of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide (Zorrilla et al., 2003). The bacteria that had been associated with fatal outcomes in tilapia were *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., *Streptococcus* spp., *Enterococcus* spp., *Micrococcus* spp., *Staphylococcus* spp., *Plesiomonas* spp., *Moraxellaceae* and *Enterobacteriaceae* (Daskalov, 2006; Najiah et al., 2012). Of these, *Streptococcus* spp. had been the most important fish pathogen in Malaysia.

Since poor water condition including elevated nitrite and ammonia levels has been reported to be responsible for both morbidity and mortality in fish farming worldwide (Moraes and Martins, 2004), the need to conduct periodic surveillance on the presence, associated pathological alteration in tissues and the various environmental factors that could trigger some potential fish pathogens resulting to disease outbreak in Malaysian aquaculture industry is paramount. Thus, the study focuses on the isolation and identification of potential pathogenic bacteria from tissues of tilapia, water and sediments in two different water bodies. It also compared the water conditions from the cage-cultured of red tilapia in Kenyir Lake, Terengganu and Semantan River, Pahang in Malaysia.

MATERIALS AND METHODS

Location sampling and time

This study was conducted in cage-culture tilapia located in the east of Peninsular Malaysia. The most popular fish among the farmers were the Red hybrid tilapia. Sampling 1 was done at Kenyir Lake (5.00916°, 102.63310°), Hulu Terengganu, State of Terengganu and sampling 2 was located at Semantan River (3.48786°, 102.25317°), Temerloh, State of Pahang (Figure 1).

These locations were the commonly used for tilapia farming in Malaysia. Kenyir Lake is an artificial lake located in the state of Terengganu in northeast Malaysia, created in 1985 by the damming of the Kenyir river to create the Sultan Mahmud Power Station. The 260 km lake is reputed to be the largest artificial lake in Southeast Asia and it serves as a gateway to Taman Negara and Thailand.

Semantan River in State of Pahang is the longest river in in the Peninsular Malaysia with a length of about 435 km Pahang River. The climate is of equatorial type where the temperature is uniform throughout the year with temperature ranging from 21 to 32°C.

The humidity is consistently in the region of 75 to 80% throughout the year with an annual rainfall average of around 200 to 300 cm. The weather can be divided into dry (mid of year) and wet season (end to early year). Samples were collected during the wet season (rainfall season) for this investigation.

Fish, water and sediment

Thirty (30) fish were randomly selected per collection point. All the fish were measured and weighed. The external observations of the fish were also recorded. Also, ten samples of water and sediments were also collected in each site. Water samples were collected in 15 ml of sterile Bijou bottles under the water surface with the depth of 10 to 15 cm in different location within the cage-cultured farm from the lake and river.

All the samples were kept in the sterile containers and preserved in low temperature with icepack.

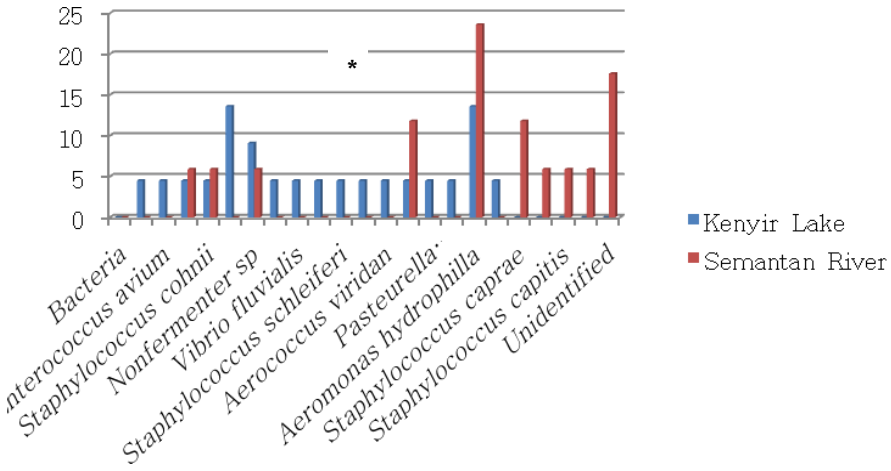

Figure 2. The predominant bacteria isolated from tilapia in two different water bodies. The asterisk shows that *A. hydrophila* was significantly higher ($P< 0.05$) in Semantan River as compared to Kenyir Lake.

Bacterial isolation and media

The fish were sacrificed and organs abnormalities were recorded. Brain, eyes and kidneys of each fish were collected for bacteriological analysis. Similar bacteriological analysis was done on the samples of water and sediments. Blood agar (BA) (Oxoid, UK) was used for the isolation before further characterization. All BA plates were labelled and sealed with adhesive tapes. The inoculated BA media were incubated at room temperature, ~22°C for 18 - 24 h.

These inoculated BA media were observed for the presence of bacterial growth after 24 h of incubation. After taking note of cultural growth characteristics, positive cultures were subjected to Gram's staining properties and cellular morphology observed with a light microscope (100x). Mixed colonies and Gram negative bacteria were subcultured on onto Trypticase Soya Agar (TSA) medium. Pure cultures of single colony type were transferred onto nutrient agar slants for a series of biochemical tests including catalase, oxidase and fermentative/ oxidative tests for identification following standard procedures (Austin and Austin, 1999). The bacterial colonies were then subcultured onto the BA media again to obtain the pure colonies of bacteria for analytical profile index test (API) (Morrison and Tillotson, 1988).

Biochemical test

Gram staining was done using the pure culture of bacterial colonies growth from TSA medium. For Gram-negative bacteria, API 20E was used including motility test, MacConkey medium bacterial inoculation, oxidase test, fermentation of glucose (OFF) and oxidation of glucose (OFO) to identify species of bacteria present. For Gram-positive bacteria, catalase test was done. Catalase positive by Gram-positive bacteria were subjected to API 20 Staph test. Catalase negative by Gram-positive bacteria will be further tested with API 20 Strep test. Catalase and oxidase tests were further used for the bacterial colonies from TSA medium. All the API test kit used was incubated in normal incubator at 30°C for 24 h. Identification of bacteria was done by using API test software.

Water quality measurement

The temperature, pH and dissolved oxygen and conductivity were measured using YSI 556 (YSI, USA). The ammonia, iron, sulphate and nitrate were determined using a DR 2800 Portable Spectrophotometer (Hach Company, Loveland, CO, USA).

Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 12.5.0.0 (MedCalc Software, Mariakerke, Belgium) and tested at 5% level of significance. The water conditions were analyzed using a one-way ANOVA and post-hoc test was administered using the Student-Newman-Keuls pairwise comparison test. The differences in other parameters were the figures.

RESULTS

Water quality

The water quality parameters (mean ± SD) were: dissolved oxygen, 4.55 ± 0.3 mg/L; temperature, 31.6 ± 0.8°C; pH, 7.40 ± 0.1; total ammonia, 2.40 mg L^{-1} and nitrate, 0.021 mg/L. All the parameters were within the normal range (El-Sayed, 2006)

Bacterial isolation

Figure 2 show the bacterial isolates from tilapia of Kenyir Lake which are mostly predominated by *Micrococcus* spp. and *Aeromonas hydrophila* with percentage of 13.64% of both bacteria and followed by non-fermenter sp. with 9.1%. In samples from Semantan River, the most predominant bacterial isolates are *A. hydrophila* with 23.53%, followed by *Staphylococcus xylosus* and *Staphylococcus caprae* both with 11.8%.

Water samples

In Kenyir Lake, the most predominant bacteria isolates

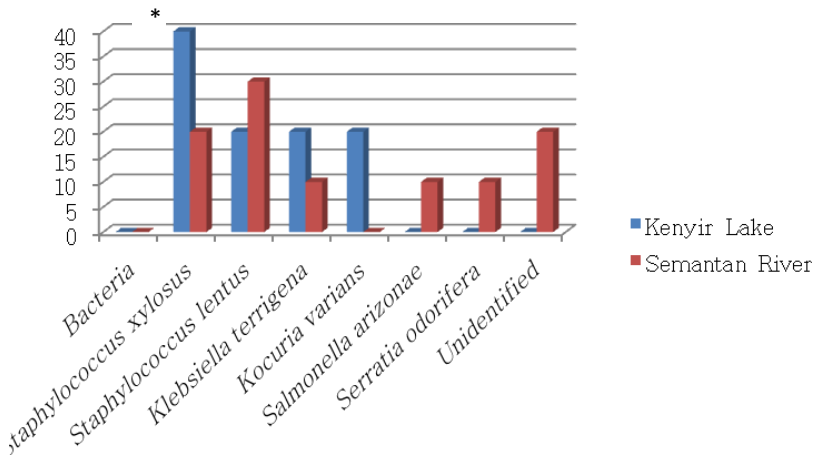

Figure 3. The predominant bacteria isolated from the water in two different water bodies. Asterisk shows the bacteria that were significantly higher (P< 0.05) in Kenyir Lake as compared to Semantan River.

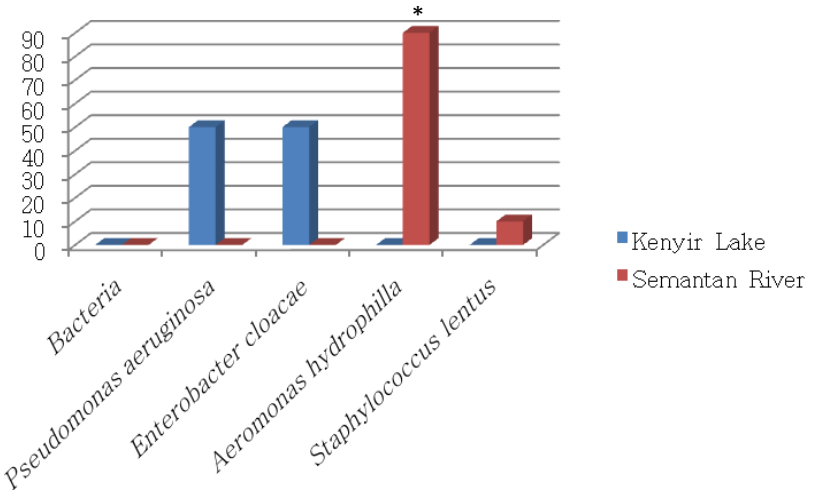

Figure 4. The predominant bacteria isolated from the sediment in two different water bodies. The asterisk shows the potential pathogenic bacteria which was significantly higher (P< 0.05) in Semantan River as compared to Kenyir Lake.

were *S. xylosus* (40%), followed by *S. lentus*, *Klebsiella terrigena* and *Kocuria varians* (20%), respectively. In Semantan river, *S. lentus* is the most predominant bacterial isolates (30%), and then followed by *S. xylosus* (20%). Meanwhile, the other bacterial isolates found are *K. terrigena, Salmonella arizonae* and *Serratia odorifera* which were 10%, respectively (Figure 3).

Sediment samples

There were two species of bacterial isolates isolated in Kenyir Lake, which are *Pseudomonas aeruginosa* and *Enterobacter cloacae* with 50%, respectively. About 90% of bacterial isolates in Semantan River are *A. hydrophila* and the remaining percentage are *S. lentus* (Figure 4).

Weight and length

The mean body weight of fish from Kenyir Lake, was significantly lower (P< 0.05) than that of Semantan River (Figure 5). However, both water bodies had about the same length of the fish (Figure 6).

Water quality

There was no significant difference (P> 0.05) in the water

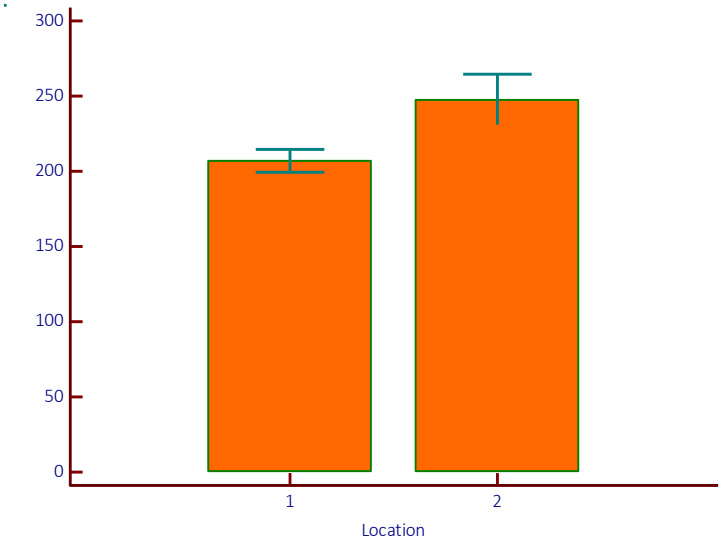

Figure 5. The body weight of tilapia from two different types of water bodies. Semantan River shows tilapia had similar body weight with that of Kenyir Lake.

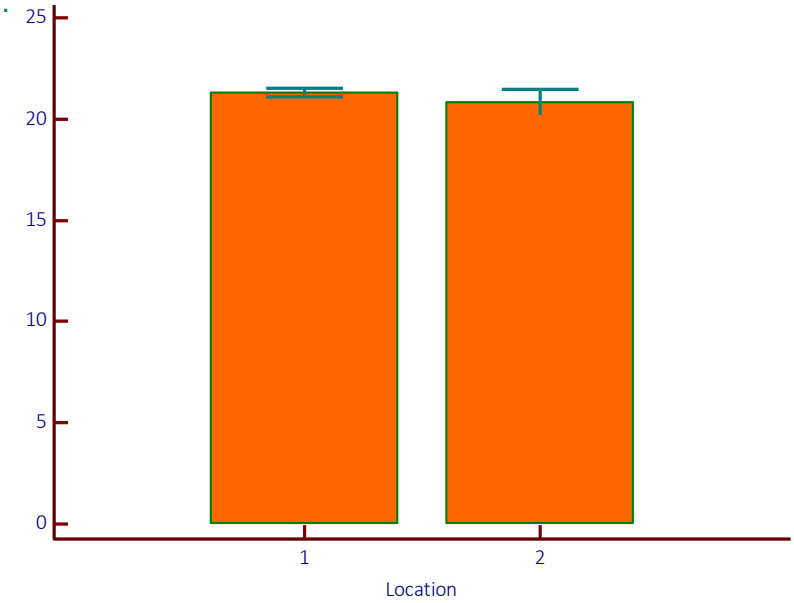

Figure 6. Tilapia from different types of water bodies showing similar length.

quality (pH and conductivity) between Kenyir Lake and Semantan River. Of the parameters measured, sulphide, Fe and NH_3 in Semantan River were significantly higher ($P< 0.05$) than that of Kenyir lake while temperature, DO and NO_2:N (Figure 7) were similar.

DISCUSSION

This investigation describes the isolation and identification of potential fish pathogens in water, sediments and tissues of Red hybrid tilapia reared in cage-cultured system in two different water bodies in Malaysia.

The predominant bacteria isolated from tissues of Red hybrid tilapia from Kenyir Lake includes *Micrococcus* spp. and *Aeromonas hydrophila* while that of Semantan River, are *Aeromonas hydrophila, Staphylococcus xylosus* and *Staphylococcus caprae*. This showed that the bacteria isolated from fish vary with the water source and pollution level. The predominant bacteria from the two study sites are *A. hydrophila*, which has been reported by other workers as part of the normal flora of freshwater fish

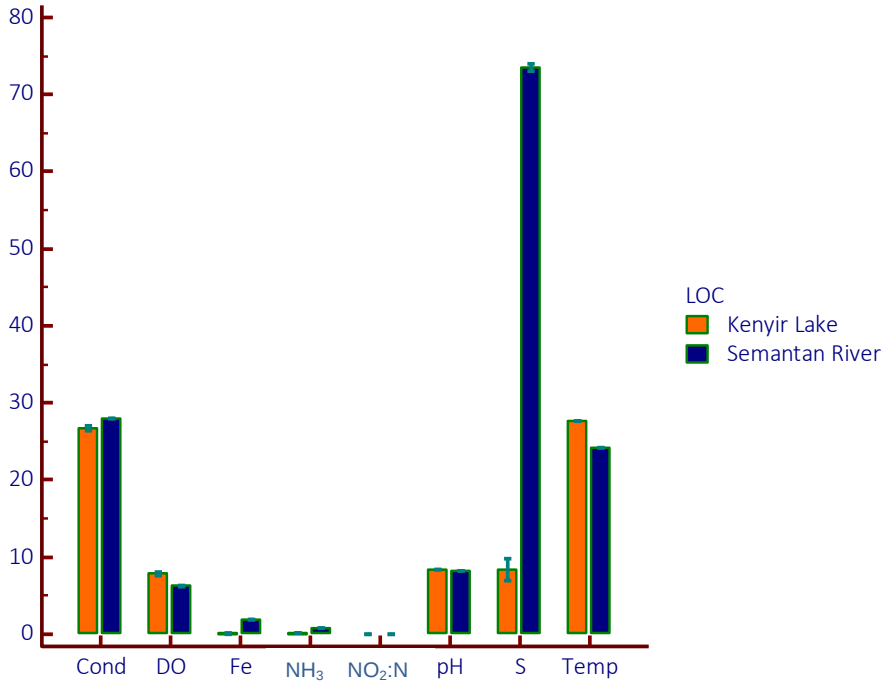

Figure 7. Water conditions in Semantan River and Kenyir Lake.

(Sugita et al., 1982; Santos et al., 1998; Thayumanavan et al., 2003). In this study, the tilapia that *A. hydrophila* were isolated showed no clinical signs, however at necropsy, there were evidence of renal hypertrophy and congestion, encephalomalacia and hepatomegaly. These findings are in agreement with the reports of other workers (Ghosh and Homechaudhuri, 2012). Since *Aeromonas* spp. had been reported to be an opportunistic pathogens that do invade heavily stressed fish (Inglis et al., 1993; Saha and Pal, 2000), the high mortality observed in Semantan River during sampling and isolation of *A. hydrophila* from the fish suggested *A.hydrophila* as a possible pathogenic bacterium that could lead to clinical diseases of stressed Red tilapia in Malaysia as earlier reported elsewhere (Lio-Po et al., 1983). It should be noted that in this present study, *Streptococcus agalactiae* was not isolated from the samples despite the reports of heavy mortality associated with outbreak of the disease in Malaysia (Amal et al., 2008). This could be associated with the reports of Siti-Zahrah et al. (2004, 2008) that *Micrococcus* and *Staphylococcus* spp. are the predominant bacteria often isolated in Kenyir Lake.

Micrococcus spp. infected fish showed no clinical sign but gross lesions include swollen or congested kidney and pale liver. The occurrence of lesions without any clinical signs further strengthens the need for periodic surveillance of this nature that will help in the detection of subclinical infections.

The presence of *Staphylococcus caprae* from Semantan River fish further showed that the farmer uses goat faeces as fish feed. This act should be with caution as there had been reports of bacteria isolated primarily from goats being pathogenic to fish (Carretto et al. 2005)

S. xylosus found in this study had also been previously reported (Siti-Zahrah et al., 2008). However, *S. xylosus* and other bacteria such as *Staphylococcus chromogens*, *Staphylococcus warneri*, *Staphylococcus capitis* and *Staphylococcus cohnii* had been reported to infect sea bream and sea bass in Greece causing dark body, fin and skin necrosis to shallow ulcers, hence isolating this bacteria from fish are of great concern, more importantly when infected fish showed no clinical sign except for a few with pale liver and congested kidney. The isolation of *Pasteurella multocida* from fish with no clinical sign and lesion showed that the infection is subclinical but worthy of note is the outbreak of pasteurellosis in tilapia hybrids from a fish farm on the shore of lake Kinneret, Israel (Nizan and Hammerschlag, 1993) and the source of infection was suggested to be the chicken manure used as organic fertilizer in the ponds. The possible transmission and pathogenicity of this bacterium in fish need to be investigated.

The presence of *Enterococcus avium* in fish may originate from contaminated water through the excretion of animals and humans waste. *Enterococcus* sp. had been reported to cause considerable economic losses in cultured turbot (*Scophthalmus maximus*) (Toranzo et al., 1995) and tilapia (Plumb, 1999). However, the infected fish also showed no apparent clinical signs or lesions.

Infected fish with *Vibrio fluvialis* also did not show clinical signs but only a pale liver and hyper-pigmentation of the eye. The detection of this bacterium in fish from the study sites is also of great concern as *V. fluvialis* have been reported to be pathogenic for tilapia hybrids (Xu et al., 1993) in Japan and Israel with haemorrhages around the base of the fins, prostration in the swimming movement and stiffness of the muscles (Bisharat et al., 1999).

S. epidermidis has been previously isolated from tilapia in Malaysia (Siti-Zahrah et al., 2008) but infected fish also did not show any clinical signs except a pale liver and a congested kidney. *Aerococcus* sp. and *S. aureus* had also been isolated from freshwater fish by other workers (Siti-Zahrah et al., 2008). The infected fish showed a congestion of the kidney and pale liver without the external lesion (Najiah et al., 2012). However, various reports abound on their pathogenicity in silver carp in India causing corneal damage (Shah and Tyagi, 1986). *Plesiomonas shigelloides* is a Gram-negative bacterium, though not specific for aquatic species but is widely distributed in water and soil in temperate and tropical regions hence it is considered as a potential fish pathogen (Cruz et al., 1986). The presence of *Salmonella arizonae* and *Enterobacter cloacae* in the fish tissue and in the water suggests pollution (Burras, 1993) possibly from contamination with animal waste from chicken as observed by Abd El-Aziz and Ehab (2003). *Pseudomonas aeruginosa* isolated from the debris of Kenyir lake, is a common bacterial pathogen of fish in most countries with remarkable septicaemia and haemorrhages in the skin of the mouth region, opercula and ventral side of the body (Buller, 2004).

Environmental stress has influenced the pattern of isolation and disease. The temperature of water in Kenyir lake was observed to be higher than Semantan River probably because lake tends to retain heat for a longer period, resulting to lower cooling, that could account for higher numbers of type of species of bacteria isolated since dissolved oxygen (DO) in the water column is one of the most important factor for maintenance of life of cultured organisms (Harada, 1978). Ammonia level in Kenyir Lake and Semantan river exceeded the acceptable limit (0.02 ppm) for fish. The high level of ammonia may be due to the high level of ammonia in the feed, where the goat faeces and visceral organs of chickens were used as a source of feed. The mortality of fish observed in Semantan River at the time of this study may be associated with the high level of ammonia. In Semantan River, the weight of fish was more than Kenyir Lake fish, and because the small-sized Nile tilapia had been found to be more tolerant to nitrite than larger fish (Atwood et al., 2001), it could explain the unusual high mortality observed with fish from Semantan River (Branson and Southgate, 1992).

In this investigation, more than one species of bacteria were isolated from the fish, which may be associated with the nature of feed, sediments and poor water quality observed. This passive surveillance of this nature is essential for the prevention of possible disease outbreak associated with the potential pathogenic bacteria observed especially during the critical period of environmental stress. The understanding of the virulence mechanisms of the pathogenic bacteria observed will aid the development of strategies for preventing or managing diseases caused by the potential fish pathogens in future.

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