Mass Mortalities of Golden Pomfret (*Trichinotus blochii*) at Floating Cages Pulau Aman, Penang Associated with Oxygen Crisis, Multiple Infections of Parasites and *Vibriosis*

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Abstract: Mass mortalities (80%–100%) of golden pomfret aged between one to five months old have been reported by fish farmers at floating cages off the coast of Seberang Perai, Pulau Aman, Penang. Hence, the objective of this study is to determine the cause of fish mortalities. On-site investigation with sampling of fish (n=13) and waters were taken for laboratory analysis. Parasite was examined from skin and gills. Aseptic method for bacterial inoculation and isolation on tryptose soy agar (TSA+1.5% NaCl) were performed from the internal organs of kidney, spleen, liver and brain. Pooled organ tissues of each sample was fixed in 95% alcohol for detection of viruses causing Viral Nervous Necrosis and Red Sea Bream Iridoviral diseases using polymerase chain reaction. Genomic analysis using Next Generation Sequencing of bacterial and capsalid species *were* performed on NovaSEQ6000 Illumina. *In-situ analysis of water parameters* showed low dissolved oxygen (3.0–3.5 mg/L) while other parameters were within normal range for aquaculture. High prevalence of *Neobenedenia melleni* (92%) and marine leech *Zeylanicobdella arugamensis* (31%) were found in golden pomfret with mean density of 5 and 2, respectively. *Vibrio harveyi* were isolated with six virulent factors of Type III Secretion System (T3SS) found as a single or mixed isolates with *V. vulnificus*. High mortalities of golden pomfrets occuring on the site were caused by low dissolved oxygen and multiple infection from parasitic infestations of *N. melleni*, *Z. arugamensis* and bacterial infection.

Keywords: mass mortalities, low dissolved oxygen, parasite, bacteria

Abstrak: Kematian besar-besaran (80%–100%) ikan bawal emas berumur antara satu hingga lima bulan telah dilaporkan oleh penternak ikan di sangkar terapung di luar pantai Seberang Perai, Pulau Aman, Pulau Pinang. Justeru, objektif kajian ini adalah untuk mengenal pasti punca kematian ikan yang berlaku. Penyiasatan di tapak dengan pensampelan ikan (n=13) dan air laut telah dibuat untuk analisis di makmal. Parasit diperiksa di kulit dan insang. Kaedah aseptik dalam inokulasi bakteria dan pengasingan pada agar soya tryptose (TSA+1.5% NaCl) dilakukan daripada organ dalaman buah pinggang, limpa, hati dan otak. Tisu organ terkumpul setiap sampel telah dirawat dalam alkohol 95% untuk pengesanan virus yang menyebabkan Nekrosis Saraf Viral dan penyakit Iridoviral Ikan Siakap Merah menggunakan tindak balas rantai polimerase. Analisis genom menggunakan Penjujukan Generasi Seterusnya spesies bakteria dan capsalid telah dilakukan pada NovaSEQ6000 Illumina. Analisis *in-situ* parameter air menunjukkan kandungan oksigen terlarut yang rendah (3.0–3.5 mg/L) manakala parameter lain berada dalam julat normal untuk aktiviti akuakultur. Prevalen tinggi parasite *Neobenedenia melleni* (92%) dan lintah laut *Zeylanicobdella arugamensis* (31%) ditemui dalam ikan bawal emas dengan ketumpatan purata 5 dan 2, masing-masing. *Vibrio harveyi* berjaya diasingkan dengan enam faktor virulen Sistem Rembesan Jenis III (T3SS) ditemui sebagai pencilan tunggal atau

campuran dengan *V. vulnificus*. Kematian tinggi bawal emas yang berlaku di tapak adalah disebabkan oleh oksigen terlarut yang rendah, infestasi daripada serangan parasit *N. melleni*, *Z. arugamensis* dan jangkitan bakteria.

Introduction

Mass mortalities of fish culture are becoming more frequent over the years in Southeast Asia region and worldwide, particularly in countries where aquaculture industry is booming and intensified due to continuous demand of high value protein source. Pollutants, originating from both land and sea as well as increase in fish farms activities add more nutrients to waters, whereas rapid climate change, heavy rain after prolonged dry and warm weather are among many factors in tropical region known to cause lethal and sub-lethal effects on marine life. Fish kills create a concern to general public due to the worries of pollution and the effect to human health by consuming the contaminated fish or sharing common water bodies. Prolonged dry and warm weather followed by heavy rain is a fatal combination that can cause massive fish kills in lakes or open waters within few hours (Svennevig, 2020). When it happens, aquaculture farmers are often badly affected. Mass mortalities of fish culture in Malaysian waters, are often associated with low dissolved oxygen due to algal blooms following heavy rain pour which may last for few days (Lim, 2012; Lim et al., 2014). The runoff through ditches and drainage system that contains high contaminants including suspended solid or garbage materials, inorganic and organic pollutions, bacteria contaminants from industry, animal farms and domestic wastes are commonly been reported as the cause of fish kills (Duraisamy & Latha, 2011; Shah, 2019; Zanuri et al., 2020).

Prolonged dry spell and warm weather predispose fish to stress that weakened the immune system. It is common for the affected fish succumb to bacterial infections as their immune system is weakened when the underlying problem has not been resolved. Stressors, often inevitable in most culture systems, predispose fish to bacterial-borne diseases (Snieszko, 1974). Over time, bacterial or parasitic problems are found to be another problems which enhance the cumulative mortalities percentage in the affected farms. A gradual increase in mortalities caused by *Vibriosis* sp. may be reaching up to 50% (Liao & Leano, 2008; El-Galil & Mohamed, 2012). The actual role of these bacteria may vary from a primary pathogen to an opportunist invader associated with other pathogen involved in the disease process (Richards & Roberts, 1978). Many of these bacteria are usual component of the microflora in aquatic habitat.

The development of cage culture activities has been associated with the emergence of parasitic disease (Kent, 2000). The overlapping generations of fishes in the culture system provide a pool of pathogens for any newly placed fish (Leong, 1997). The common report of parasitic infestation in cage-cultured species in Malaysia are capsalid monogeneans (Kua et al., 2015; Ihwan et al., 2016), marine leech (Rajiv & Shariman, 2017), crustacean isopods (Kumar et al., 2015) and caligus (Maran et al., 2009). Multiple problems are commonly inter-related, associated with stress factors such as rapid climatic change, poor water quality and management practices which may trigger primary or secondary infections that lead to acute disease outbreaks in fish culture system. Hence, the objective of this study is to identify the cause of high fish mortalities occurring at the cage culture farms, off shore Pulau Aman, Penang.

Materials and Methods

Sampling of fish

A total of thirteen (13) specimens of golden pomfret (*Trachinotus blochii*) consisting of apparently healthy, sick and moribund fish from 1–2 months old of age (n=7) and 5 months of age (n=6) were collected for examination and laboratory analysis. Post-mortem examination of fish was performed on-site. *Bacterial inoculation and isolation from the k*idney, spleen, liver and brain samples were taken under aseptic condition, cultured onto tryptose soy agar (Oxoid, UK) with the addition of 1.5% NaCl. Pooled tissues of internal organs and brain of each sample (13) was fixed in 1.5 mL tube containing 95% alcohol for polymerase chain reaction (PCR) test of virus nervous necrosis (VNN) and iridovirus (RSIVD).

Water quality analysis

In-situ analysis of physical water parameters such as temperature (^{o}C), salinity (ppt), dissolved oxygen (mg/L), pH and total suspended solid (TTS, mg/L) were carried out at surface level and water depth of 7 meters using YSI 5908 probe (Yellow Springs, OH, USA) from 3 locations (inside cage, outside cage and outside premise/farm). Water was collected in 500 mL plastic bottles for basic chemical parameters such as total ammonia-N, nitrate, nitrite, sulphide and iron using HACH Kit (Loveland, CO, USA).

Parasitology

Five minutes fresh water dip of each fish sampled was performed in a small container after which the body of fish was gently stripped to remove any external parasites attached on the body for collection, identification and determination of mean density. The collected external parasites were fixed in 96% alcohol, placed on a glass slide followed by Giemsa staining, mounting with DPX and ready for microscopic examination and identification. A small piece of gill sections from the first and third segments of gills branch from both sides were cut off using scissors, placed on the glass slide and dried followed by fixation in methanol, Giemsa staining and microscopic examination.

Bacteriology

Pure culture of the isolate was further tested for characteristic growth on thiosulfate-citrate-bile salt-sucrose agar (TCBS), O/129 vibriostat sensitivity, Gram stain, oxidase, catalase, motility and API 20 NE (Biomerieux, France). Analytical profile index 20 NE Kit was used for biochemical identification of bacterial species with *i*dentification of more than 92.5% similarity to the referred database. Detection of virulent factors haemolysin (vvh) from eight isolates of V. vulnificus and 3 isolates of *V. alginolyticus* (collagenase gene) were tested using PCR method (Brauns et al., 1991; Di Pinto et al. 2005).

Three bacterial isolates (BE8B, BE9K and BE78K) and parasite *N. melleni* was sent to a private laboratory for sequencing and genomic identification. Sequencing was performed on a NovaSEQ6000 (Illumina, San Diego, CA) generating approximately 1 gb of paired-end data (2×150 bp) for each sample. Contigs smaller than 500 bp representing mostly sequencing artifact were removed and the filtered assembly was used for subsequent analysis. Genome assembly statistics were generated using QUAST (Gurevich et al., 2013). ABRicate (https://github.com/tseemann/abricate) for

mass screening of contigs that represent a consensus region of DNA for VFs was employed to perform a BLAST, based on nucleotide similarity search of the assembled genome in the National Center for Biotechnology Information (NCBI) and the Virulence Factor Database (VFDB). The Reference Gene Catalogue facilitates the examination of the genomic links among bacteria and virulent genes (Chen et al., 2005). Determination of VFs gene is based on identity threshold of more than 80% against VFDB.

Virology

DNA/RNA extraction procedures were carried out using taco™ DNA/RNA extraction kit (GeneReach Biotechnology Corp., Taiwan) following protocols outlined by the manufacturer. The PCR program for the detection of RSIVD was modified from the suggested MyTaq Red Mix protocol (Meridian Bioscience, Inc., Cincinnati, Ohio, USA) as follows; initial denaturation at 95°C for one min and 35 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and extension at 72°C for 10 sec with final extension at 72°C for 3 min and final hold at 4°C. The size for the amplicon is expected at 570 bp using primer 1F/1R (Kurita et al., 1998). Reverse-transcriptase polymerase chain reaction (RT-PCR) profile for VNN was: reverse transcription 45°C 20 min, initial denaturation 95 °C 1 min, denaturation 95°C 10 sec, annealing 60°C 10 sec, extension 72°C 30 sec at the end of 40 cycles followed by final hold at 4°C. The PCR products were prepared for gel electrophoresis (1.5% agarose gel at 100 V for 30 min) and analyzed under gel documentation system for detection of VNN with expected size base pair of 258 bp (Nishizawa et al., 1995).

Results

Gross observation

Infestation of capsalid *Neobenedenia* sp. and marine leech (*Z. arugamensis*) were identified following the fresh water bath treatment and microscopic examination. The infested fish was badly affected with high mortalities estimated about 80% (**Figure 1**), scale drops and white patches on body, skin wound ranging from inflammation/redness on body in mild cases (46%) to serious skin ulceration (46%) were observed in golden pomfret size between 7–13 cm of total length (1–2 months old) (**Figure 2**). Internal changes observed were congestion of kidney (31%), pale liver (54%) and enlarged spleen (8%) with eodematous fluid in the abdomen.

Figure 1: Accumulative mass mortalities of more than 80% in golden pomfret (*Trachinotus blochii*) aged 1–2 months old of culture.

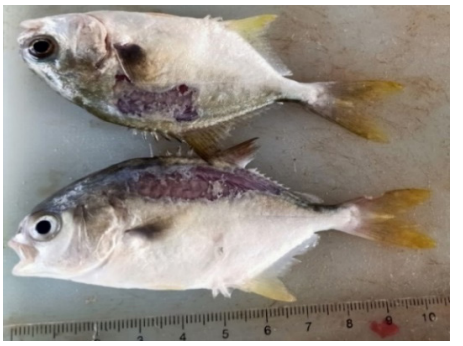

Figure 2: Skin ulceration on body in 46% of the fish (*Trachinotus blochii*).

Parasitology

Capsalid species at 92% prevalence, mean density of 5 and marine leech with prevalence of 31%, mean density of 2 were found on the body. Microscopic examination of gills tissues showed no other parasites detected from the normal gills. The prevalence of parasites was relatively low as farmers have treated the fish with fresh water bath a day earlier. Capsalid problems was heavy from the previous freshwater treatment carried out by a farmer. Mortalities was reported to continue and increased overnight as fish appeared very weak after treatment especially in golden pomfret aged 1–2 months.

Bacterialogy and PCR for detection of virulent factor

Bacteria isolation from the internal organs of kidney, liver and spleen showed the presence of *V. vulnificus* (69%), *V. alginolyticus* (31%) and *Pasteurella multocida* type 1 (31%) and *P. multocida* type 2 (15%) as mixed of two isolates or a single pathogen in organs of fish. PCR analysis of virulent factor haemolysin (*vvh*) from eight isolates of *V. vulnificus* and collagenase gene from three isolates of *V. alginolyticus* were found to be negative.

Using *in-silico* genome-genome hybridization approach, three isolates BE8B, BE9K and BE78K (identified as *V. alginolyticus* using API 20NE) were all assigned to the species *V. harveyi* with an average ANI value of 98.7%, a value that is much higher than the pairwise ANI against *V. alginolyticus* (86%). Virulent factors Type III Secretion System (T3SS) *vcrH, vcrD, vcrN, vcrI, vcrF* and *VPA0450* were identified from *V. harveyi* bacteria of diseased fish. Results are shown in Table 1.

Table 1: Identification of bacteria from golden pomfret using *in-silico* genome-genome hybridization and virulent factors (VFs) gene with identity threshold of >80% against VFDB.

Sample ID	*Bacteria*	ANI (%)	VFs gene	VFDB accession
1. BE8B	*V. harveyi*	98.7	*vcrH*	NP_798037
2. BE9K			*vcrD*	NP_798041
3. BE78K			*vscN*	NP_798047
			vscI	NP_798070
			vscF	NP_798073
			VPA0450	NP_779960

Virology

VNN and RSIVD were not detected from all sample.

Water quality analysis

pH, salinity, surface temperature, nitrate and phosphate (**Table 2**) were within the permissible limit according to Malaysia marine water quality Class 2 for aquaculture (Department of Environment, Malaysia, 2005). Dissolved oxygen in surface water was low (3.30–3.45 mg/L) at three locations within cages and at 7 metre depth (2.65–3.39 mg/L). Total ammonia level was slightly high (0.03 mg/L) due to an overnight accumulation of dead fish at the bottom of cages. Microscopic examination of water samples taken from three locations (inside and outside cage, outside farm area) showed

undetected to low count of microalgae cells (<10^3 cells/L). The water quality results are shown in **Table 2**.

Table 2. Water parameters at floating cages, Pulau Aman, Penang on 22 September, 2020.

Water parameters	**Cage culture site (3 locations)**		**Class 2 (Aquaculture)**
	Surface (mean±SD)	**7 meter depth** (mean±SD)	Acceptable range
1. Temperature (°C)	30.20±0.00	29.90±0.10	-
2. DO (mg/L)	3.40±0.09	2.99±0.37	5.0–10
3. pH	7.98±0.32	8.14±0.08	6.5–8.5
4. Salinity (ppt)	29.71±0.18	30.31±0.04	-
5. Total Ammonia-N (mg L)	0.022±0.011	-	0.07
6. Nitrite (mg/L)	0.035±0.001	-	0.055
7. Iron (mg/L)	0.02±0.00	-	<5
8. Sulphide (µg/L)	6.00±2.00	-	1–10

Discussion

Mass mortalities of more than 80% of golden pomfret and other fish species at floating cages off shore Pulau Aman, Penang on 22nd September, 2020 were caused by low dissolved oxygen (3.0–3.4 mg/L). Multiple infestations of capsalid *N. melleni*, marine leech *Z. arugamensis* and *V. harveyi* infections led to exacerbation of the existing problems in association with oxygen depletion and hypoxia in fish. Skin injury ranging from scale drop to laceration (46%) and ulceration on the body (46%) were observed in the golden pomfret aged 1–2 months old. Generalised inflammation and haemorrhages on body, fins and tail, congestion of kidney (31%), enlarged spleen (8%) and pale liver (54%) were suggestive of septicaemia.

Poor water quality have been reported by a farmer following the high tide water starting in the afternoon at about 2 pm on 20th September. High tides of 5.6 to 5.7 metre have been forecasted for September 19 and 20 by National Hydrographic Centre in Port Klang (Rajendra, 2020) with an alert of flood in the Selangor coastal areas. As expected, high tide water was also reported by farmers at Pulau Aman, Penang on 20th September, 2020. The water was reported to be fouled smell, milky brownish colour with high debris content. High organic content in waters was believed to have caused algae bloom leading to a decline in dissolved oxygen (DO). The DO level drop further in the early morning or late night leading to high mortalities of fish overnight as observed on a day before of occurance whereby the DO level of surface water (3.3–3.5 mg/L; mean 3.40±0.09) and water at 7 metre depth (2.7–3.4 mg/L; mean 2.99±0.37) were below the acceptable/safe level for aquaculture. Following the incident, fish health started to deteriorate when the appetite was reduced and fish started dying within two days.

Vibriosis affect all stages of fish growth and lead to as much as 50% mortalities in fish culture (Liao & Leano, 2008; El-Galil & Mohamed, 2012). *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi* are the most common bacteria associated with various health problems in marine farm fishes, particularly in many tropical countries (Khouadja et al., 2013; Dong et al., 2017; Nurliyana et al., 2019). *Vibriosis*-infected fish exhibit sluggish movement, fin rots, skin darkness, haemorrhagic patches all over the body particularly at base of fins, detached scales with abscess like lesion and haemorrhagic prolapsed vent (Abdelaziz et al., 2017).

Numerous bacterial products such as extracellular products, proteases, lipase, chitinase, collagenase, haemolysin, siderophore, biofilm, lipopolysaccharide, type I, II and VI secretion systems and quorum-sensing regulating system were described as typical virulence determinants mediating pathogenesis in marine vertebrates (Austin & Zhang, 2006; Ruwandeepika et al., 2012). *Vibriosis* caused by *V. alginolyticus and V. vulnificus in marine fish culture* has been *accounted for less than 45% mortalities (Lopez* et al., *2002;* Yan et al., 2007). *V. alginolyticus* infection causes haemorrhage, skin ulcers, skin darkening and fluid accumulation in the peritoneal cavity and consequently, most fish die within 7 days (Rajan et al., 2001). Haemolysin is a common virulence factor reported in *V. vulnificus* associated with both fish and human diseases (Fouz et al., 2002). *However, our study showed that the virulent factor haemolysin (vvh)* was not detected in eight *V. vulnificus* isolated or collagenase gene from three isolates of *V. alginolyticus* (BE8B, BE9K and BE78K). Instead, whole genome sequence and *in-silico* genome-genome hybridization approach showed that these isolates were *V. harveyi* with multiple VFs of T3SS consisting of *VPA0450, vcrH, vcrD, vcrN, vcrI* and *vcrF.*

More specific and accurate identification of *P. multocida* are based on differences in capsular polysaccaride designated as A, B, D, E and F (Boot et al., 2004; Carter, 1955). Thus, species identification of Pasteurellaceae needs more comprehensive phenotypic, genetic methods such as 16S rRNA gene sequencing and serology test. More importantly, study on pathogenesis of *P. multocida* in fish culture needs to be determined from the isolate obtained before any molecular or serology work. *P. multocida* is a gram negative coccobacillus bacteria normally found in the respiratory tract of a healthy animal. It can act as a primary pathogen causing haemorrhagic septicaemia in cattle, buffalo, fowl cholerae in avian, atrophic rhinitis in pigs and secondary infection of pneumonic pasteurellosis in stressed animals (Khoo et al., 2019). Thus, the isolation of this bacteria from the internal organ of fish showed high possibility of faecal waste contamination from animal farms. However, pathological lesion and post-mortem changes observed were more likely to suggest *Vibriosis* rather than *Pasteurelllosis*.

Conclusion

Low dissolved oxygen (<4 mg/L) was found as the main contributing factors that caused high mortalities in golden pomfret at Pulau Aman cage culture. Mortalities occurred mainly at night or in the early morning whereby the dead fish was found floating in cages the next day. P*arasitic infestations of N. melleni* (92% prevalence) and marine leech *Z. arugamensis* (31%) were observed with secondary infections of *V. harveyi* identified from whole genome sequence analysis of bacteria from the infected fish. These were supported from necropsy examination that showed various lesions on the body such as scale drops, skin wound from mild to severe skin ulceration and haemorrhages on body, based of fins, tail and septicaemia. We believed multiple stress factors such as oxygen crisis and parasitic infestation triggered the secondary bacterial infections of *V. harveyi* in golden pomfret thus causing more than 80% mortalities in juvenile fish and up to 50% mortalities in grow-out stages..

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