Disease prevalence of tiger grouper, *Epinephelus fuscoguttatus* cultured in nursery cages in Sabah, Malaysia

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Abstract: A survey of fish diseases in tiger grouper *Epinephelus fuscoguttatus* at two sites of nursery cages in Sabah was conducted during June-December 2009. From the examination of 90 and 60 juvenile tiger groupers from Tanjung Badak and Pulau Layang-Layang nursery cages respectively, 5 taxa of parasites (*Trichodina*, *Benedenia*, *Caligus*, *Pseudorhabdosynochus* and unidentified metacercariae); 2 viruses (VNN and iridovirus) and 10 bacterial groups (*Staphylococcus*, *Vibrio*, *Photobacterium*, *Non-fermentor*, *Micrococcus*, *Pseudomonas*, *Gemella*, *Erwinia*, *Cedecia* and *Aerococcus*) were recorded. This study highlighted the prevalence of pathogen in nursery cages of tiger grouper cultured in Sabah waters.

Keywords: tiger grouper, *Epinephelus fuscoguttatus*, diesease, nursery cages, Sabah

Abstrak: Kajian mengenai penyakit ikan kerapu harimau *Epinephelus fuscoguttatus* di dua tapak nurseri sangkar, Sabah telah dijalankan pada Jun-Disember 2009. Pemeriksaan keatas 90 dan 60 juvenil ikan kerapu harimau dari sangkar nurseri Tanjung Badak dan Pulau Layang-Layang menunjukkan terdapat 5 taxa parasit (*Trichodina*, *Benedenia*, *Caligus*, *Pseudorhabdosynochus* dan metaserkariae); 2 virus (VNN dan iridovirus) dan 10 kumpulan bakteria (*Staphylococcus*, *Vibrio*, *Photobacterium*, *Non-fermentor*, *Micrococcus*, *Pseudomonas*, *Gemella*, *Erwinia*, *Cedecia* and *Aerococcus*). Kajian ini menekankan prevalen patogen pada ikan kerapu harimau yang diternak dalam nurseri sangkar di perairan Sabah.

Introduction

Grouper (*Epinephelus* sp.) farming is one of the most popular mariculture activities worldwide, especially in Asian countries. The main species farmed are *E. coioides*, *E. tauvina*, *E. fuscoguttatus*, *E. lanceolatus*, *Plectropomus leopardus* and *Cromleptes altivelis*. In Malaysia, the supply of grouper for local market comes mainly from aquaculture. Its export increased from only 2572 metric tons in 2005 to 4,400 metric tons in 2008 with estimated wholesale value of UDS$5 million (Department of Fisheries, Annual Statistics, 2005 and 2008). Tiger grouper (*E. fucsoguttatus*) is among the top three most cultured species in Malaysia and it is a species with great potential for aquaculture in Southeast Asia. In Malaysia, this high-value food fish is cultured in floating net cages placed in coastal pond or open sea cages. The local price for live tiger grouper ranges between US$10 - 13.00 per kilogram and fetched up to USD$16-20 per kg in export market in 2009.

Sadovy (2000) reported that the production of cultured groupers was influenced by factors such as cannibalism, poor water quality and disease outbreaks. Arthur and Ogawa (1996) identified the principal diseases in grouper (primarily *Epinephelus* spp.) cultured in Southeast Asia were mainly due to the pathogen virus, bacteria and parasite. Viral diseases such as viral nervous necrosis (VNN); golden eye disease; spinning grouper disease (picorna-like virus); sleepy grouper disease (iridovirus) and red grouper reovirus have been reported since 1995 (Chew-Lim *et al.*, 1992). A range of bacterial diseases of grouper has also been reported, including *Vibrio* sp., *Pseudomonas* sp., *Pasteurella piscida* and *Flexibacter* sp. (Melba *et al.*, 2000) Bacteriosis caused by *Pseudomonas* sp. among *E. tauvina* cultured in Malaysia was first reported by Nash *et al.* (1987), where all age groups were affected with mortalities ranged from 20-60% during an outbreak in 1982-1986. Affected fish showed extensive haemorrhagic erosions and ulcerations of the skin, fins and tail. Vibriosis was reported among *E. malabaricus* and *Epinephelus* sp. in Malaysia (Wong and Leong, 1990; Palanisamy *et al.*, 2000). Grouper also suffered parasitic diseases such as protozoan infection (*Amyloodinium* sp., *Brooklynella* sp., *Cryptocaryon irritans*, *Trichodina* sp.) and monogenea group such as *Benedenia* sp., *Megalocotyloides epinepheli* and *Pseudorhabdosynochus epinepheli* (Koesharyani *et al.*, 1999).

The increasing disease problems and its impact to the fish farming industry have showcased the relevance of disease monitoring and surveillance program in the country, as they are considered to be of socioeconomic importance (Hastein, 1995;, Hastein *et al.*, 1999; 2001). The term "disease surveillance" is used to describe a more active system and implies that some form of directed action will be taken if the data indicate a disease level above a certain threshold (Martin *et al.*, 1987). Hence as we introduce culture of tiger grouper in submerged cages at Pulau Layang-Layang, Sabah, a disease surveillance program at the nursery level is needed to document the status of disease, to eradicate a disease or to keep a disease under control within certain conditions. For some viral diseases caused by Viral Nervous Necrosis (VNN) diseases or iridovirus, the ultimate aim will probably be prevention of their occurrence or keep the disease level at a minimum. During the culture period percentage weight gain was used as an indicator for fish health. This paper reports the findings of the study conducted on the disease surveillance particularly on the prevalence of pathogens at two sites of nursery cages in Sabah waters.

Materials and Methods

Source of fish

The tiger grouper fry were produced locally at Tanjung Badak Hatchery, Kota Kinabalu, Sabah in November 2008. The fry were cultured in hatchery until January 2009 before sent off to the nursery cages at Tanjung Badak and Pulau Layang-Layang, Sabah. At 100 g and above, the fish were transferred into submerged cages at Pulau Layang-Layang. The initial weight was obtained from the fish management group of Pulau Layang-Layang and Tanjung Badak in order to compare the weight after few months of culture at the site. Health monitoring program of the first batch of tiger grouper began in June 2009 and ended in October 2009. Standard diagnostic procedures and bimonthly samplings were carried out during the sampling period.

Gross observation

Approximately 30 fish were sampled from each site and a total of 90 and 60 juvenile tiger grouper from Tanjung Badak and Pulau Layang-Layang nursery cages examined respectively. We managed to obtain 3 sets of sampling data from nursery cages at Tanjung Badak (June, August and October 2009) and only 2 sets from Pulau Layang-Layang (August and October 2009). The fish were randomly scooped from the nursery cages. The fish from Pulau layang-Layang were caught on the same day of sampling at nursery cages at Tanjung Badak. The fish were air freighted to Tanjung Badak hatchery and upon arrival the tiger grouper were conditioned in the same plastic bag before undergoing necropsy procedures. The weight and length from each fish were recorded and water in the plastic bag was disinfected with 40 ppm chlorine. Clinical signs both external and internal were recorded in database by using Epi-Info software (Centers for Disease Control and Prevention, United State).

Parasitology

Fish ectoparasites were isolated from body and gill for examination under compound microscope. Methods used in preparing specimens for taxonomic studies and identification of parasite were based on Kabata (1985).

Bacteriology

Lesion and internal organs (liver and kidney) was sampled using transport media swab and brought back to NaFisH for bacterial growth and identification. Swabs were inoculated on TSA agar plates and sub-cultured several times to obtain pure bacterial isolates. Gram staining was done on pure isolates to divide them into Gram-negative and Gram-positive bacteria group. Pure cultures of Gram-negative bacteria were subjected to presumptive test using McConkey media plates (Difco™, France), TCBS (Merck, Germany), OF (Merck, Germany), vibriostat 0/129 (Oxoid Ltd., England) and motility (Oxoid Ltd., England). API 20E (bioMerieux, France) identification strip procedure soon followed and bacterial profile determined by APIWEB software (bioMerieux, France) and Bergey's Manual of Determinative Bacteriology, 9th Edition. Pure cultures of Gram-positive bacteria were subjected to catalase enzyme test using H_2O_2 as substrate to differentiate between *Staphylococcus* and *Streptococcus* group. API 20 STAPH (bioMerieux, France) and API 20 STREP (bioMerieux, France) identification strip procedure soon followed and bacterial profile determined by APIWEB software and Bergey's Manual of Determinative Bacteriology, 9th Edition.

Virology

Two viral strains, i.e viral nervous necrosis (VNN) and iridovirus (IRIDO) were selected for disease surveillance using commercial kits (IQ2000™ VNN and IQ2000™ Iridovirus (Detection kit, Farming IntelliGene Tech. Corp, Taiwan). Protocol for the viral RNA and DNA extraction and the polymerase chain reaction (PCR) were according to the kit's manual without any modification. Targeting organs for both viral diseases were spleen, eye, kidney and brain. First, organs were ground with mortar and pestle, homogenized and extract using RNA extraction solution and CTAB-DTAB solution for VNN and IRIDO respectively. The resultant pellet were dried and dissolved in diethylpyrocarbonate treated (DEPC) water. Two (2) µl of this extracted RNA/DNA were then used in the subsequent reverse transcription-polymerase chain reaction (RT-PCR) for VNN and direct PCR for IRIDO. The thermal cycler program for both viruses followed the kit's instruction. At the completion of the PCR reaction, the PCR products were read using 2% electrophoresis gel prepared in LB buffer (Faster Better Media LLC, USA) and stained with ethidium bromide (Sigma-Aldrich, Inc, USA) before viewed under gel documentation equipment (Syngene, UK).

Results

Gross observation

The tiger grouper cultured at Tanjung Badak achieved 112% in weight gain against the 22% obtained in Pulau Layang-Layang (Fig.1). Approximately 47 to 80% with average of 31.11 ± 18.36 g of tiger grouper cultured at nursery cages at Pulau Layang-Layang showed external clinical signs as compared to 10-43% with average of 63.66 ± 23.57g in tiger grouper cultured at Tanjung Badak nursery cages (Table 1). The external signs such as ulcer, red boil, bloated stomach, tail and fins rot were seen throughout the culture period (photo a and b). Most of the ulcers were seen on the abdomen, fins, operculum and jaw. The red boil signs were recorded on the abdomen areas. The internal organs showed congested kidney, enlarged spleen and swim-bladder (Table 2, Photo 2c).

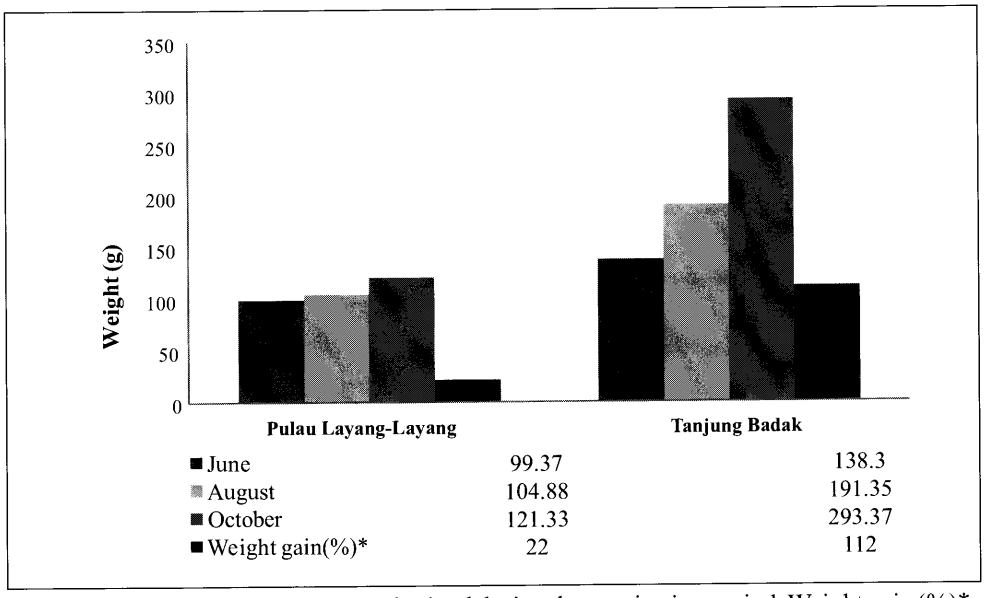


Figure 1: Body weight (g) of tiger grouper obtained during the monitoring period. Weight gain (%)* = (final weight -initial weight) x 100/initial weight

Table 1: Gross observation based on the external signs of the tiger grouper cultured in the two nursery sites

Clinical signs	Tanjung Badak			Pulau Layang-Layang	
	Jun 09	Aug 09	Oct 09	Aug 09	Oct 09
Ulcer	23.33	3.33	6.67	16.67	6.67
Tail and fin rots	20	0	0	0	36.67
Cloudy eye	0	3.33	0	0	0
Abnormal	0	3.33	0	0	0
Red boil	0	0	0	23.33	0
Blind eye	0	0	0	3.33	0
Hemorrhage	0	0	16.67	0	3.33
Bloated stomach	0	0	3.33	0	0
Blind eye and red boil	0	0	0	3.33	0
Rot and hemorrhage	0	0	6.67	0	0
Hemorrhage and ulcer	0	0	3.33	0	3.33
Hemorrhage and bloated stomach	0	0	3.33	0	3.33
Ulcer and rot	0	0	0	0	23.33
Hemorrhage and fot	0	0	0	0	3.33
Total fish with signs	43.33	9.99	40.00	46.66	79.99

Table 2: Gross observation based on the internal signs of the tiger grouper cultured in the two nursery sites

Clinical signs	Tanjung Badak			Pulau Layang-Layang	
	Jun 09	Aug 09	Oct 09	Aug 09	Oct 09
Enlarged spleen (SP)	20	13.33	6.67	3.33	13.33
Enlarged gall bladder (GB)	13.33	3.33	10	0	3.33
Enlarged Liver (L)	0	0	0	10	0
Enlarged kidney (KID)	0	0	0	6.67	0
Pale liver	0	0	0	3.33	0
Brown patches L	0	0	0	3.33	0
Enlarged L & SP	0	0	0	10	0
Enlarged SP & KID	0	0	16.67	0	6.67
Enlarged GB & SP	53.33	10	23.33	0	16.67
Enlarged GB & KID	3.33	3	3.33	0	3.33
Enlarged GB, SP & KID	3.33	33.33	13.33	3.33	33.33
Enlarged GB, SP & L	0	0	0	3.33	0
Enlarged GB & pale liver	0	10	0	0	0
Enlarged GB, KID & L	0	0	0	3.33	0
Enlarged GB, KID, L & SP	0	0	0	6.67	0
Enlarged KID & L	0	3.33	0	0	0
Enlarged KID & SP	0	6.67	0	6.67	0
Enlarged KID,SP & L		3.33	0	26.67	0
Enlarged KID & GB, pale L		3.3	0	0	0
Enlarged kid & SP, pale liver		10	0	6.67	0
Total fish with signs	93.32	96.62	73.33	93.33	76.66

Photo 2: Clinical signs of tiger grouper. (a) ulcer at the abdomen (arrow), (b). bloated stomach (arrow) and (c). enlarged spleen (arrow)

Parasitology

Similar ectoparasites were found at both nursery sites but with Tanjung Badak having more diversity (4 taxa: *Trichodina, Benedenia, Caligus* and *Pseudorhabdosynochus)* than Pulau Layang-Layang (2 taxa: *Pseudorhabdosynochus* and unidentified metacercariae). However, both sites had high prevalance of ectoparasites: 73.33 - 83.88% at Tanjung Badak and 86.66 to 96.66% at Pulau Layang-Layang nursery cages respectively (Fig. 3).

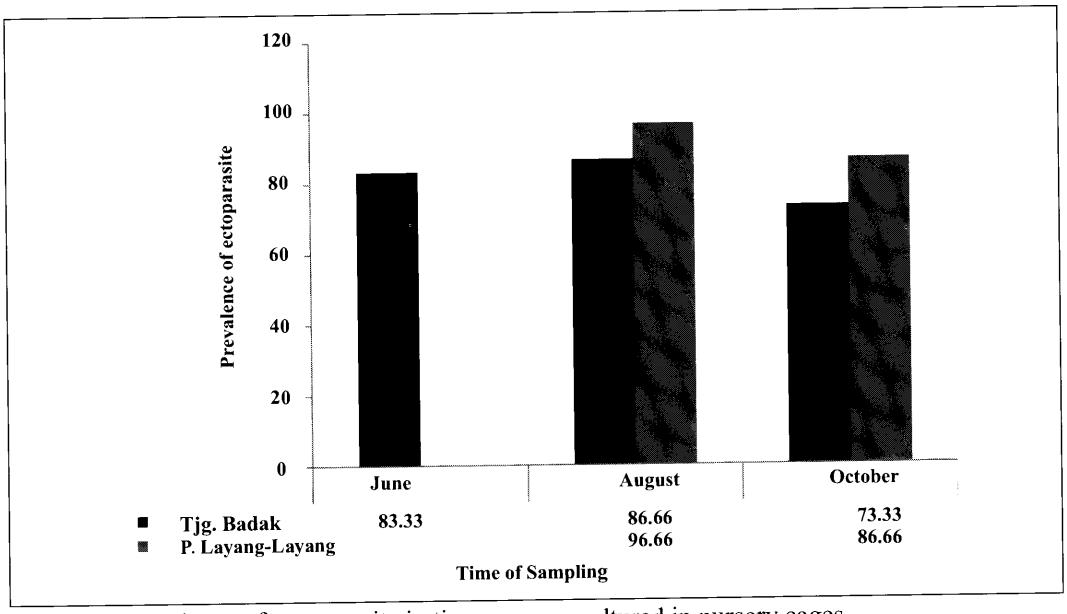


Figure 3: Prevalence of ectoparasite in tiger grouper cultured in nursery cages

Bacteriology

At Tanjung Badak hatchery, percentage of bacterial-infected fish increased from the month of June (7%) to August (97%) but decreased to 63% in October sampling (Fig. 4a). Ten bacterial groups were isolated from infected fish with the highest being *Staphylococcus* (36%) followed by *Vibrio* (13%), *Photobacterium* (10%), *Non-fermentor* (2%), *Micrococcus*, *Pseudomonas*, *Gemella*, *Erwinia*, *Cedecia* and *Aerococcus* (1% each) (Fig. 4b).

Fish sampled from Pulau Layang-Layang showed 67% bacterial infection in August and decreased to 40% in October 2009 (Photo 4c). Twelve bacterial groups were isolated from infected fish with the highest being *Staphylococcus* (28%) followed by *Vibrio* (9%), *Kocuria* (7%), *Pseudomonas* (3%), *Photobacterium* (2%), *Enterobacter, Erwinia, Leuconostoc, Micrococcus, Non-enterobacteria, Shigella* and *Chrommobacterium* (2% each) (Fig. 5).

Iridovirus and VNN are the most common viral diseases in cultured marine fish in Malaysia. In this study, iridovirus was detected in three of the samples from nursery cages at Tanjung Badak and two from the fish kept at nursery cages at Pulau Layang-Layang. Two positive iridovirus were detected in June 2009 while the other three were detected in August 2009. VNN was only detected from fish kept at Pulau Layang-Layang in August 2009 and one of these positive samples was found to be concurrently infected with iridovirus (Table 3). The result showed that there was not much difference in prevalence of VNN and iridovirus based on the detection and samples taken from both sites.

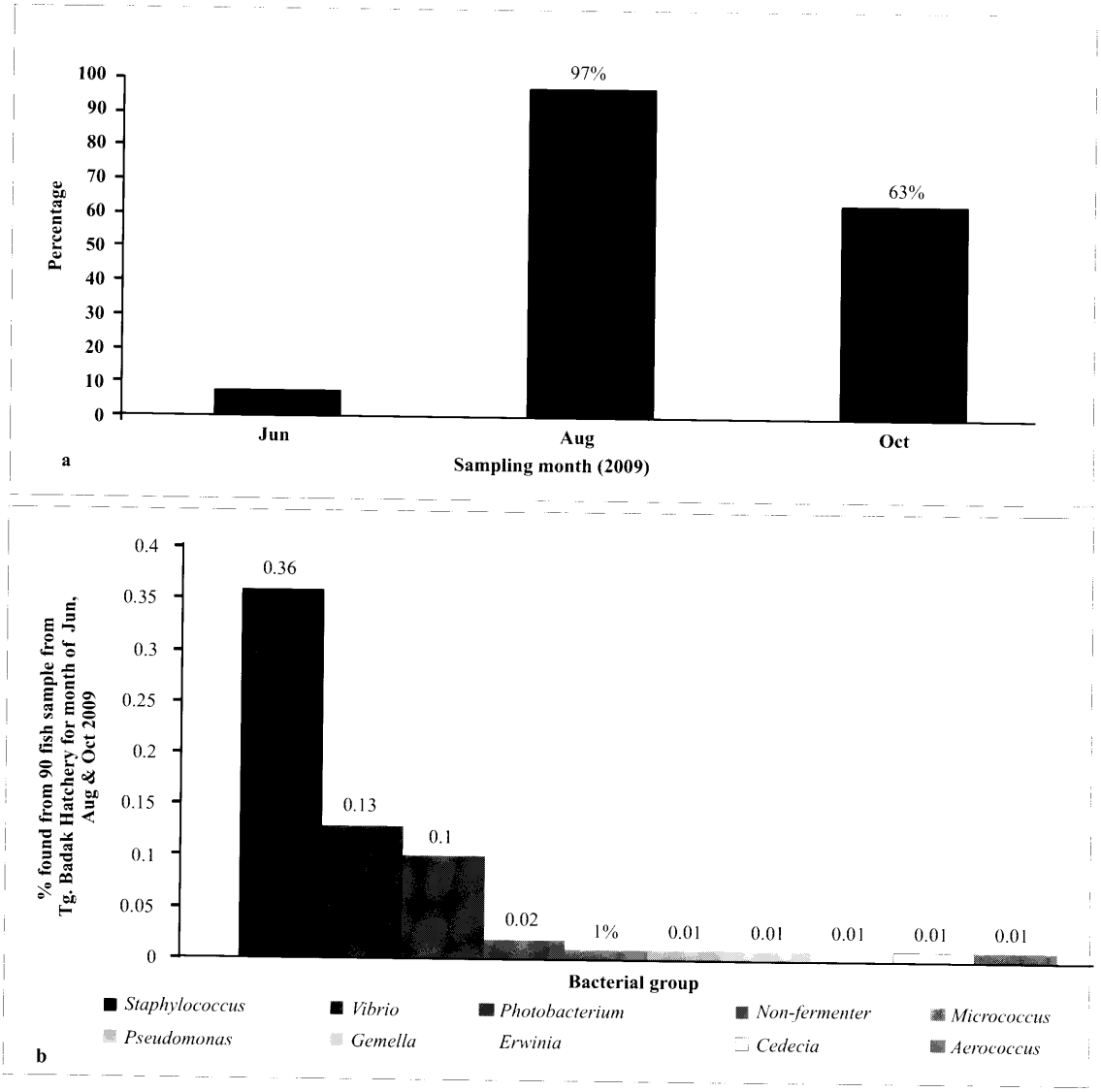


Figure 4: Percentage of bacteria in tiger grouper cultured at Tanjung Badak. (a). Fish infected with bacteria for each sampling month and (b) Bacteria found in June, August & October 2009

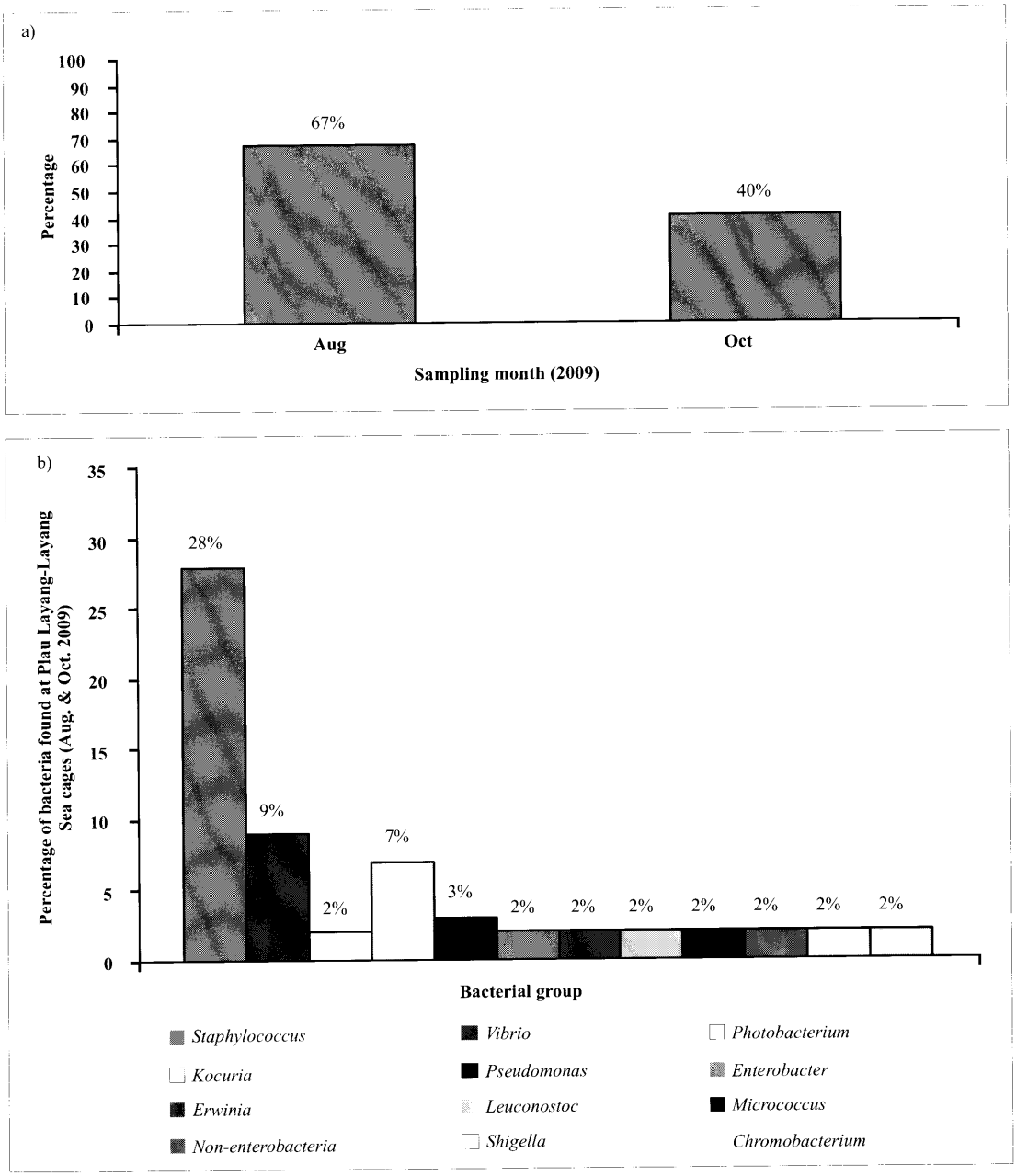


Figure 5: Percentage of bacteria found in tiger grouper cultured at Pulau Layang-Layang Sea Cages (a). Fish infected with bacteria and (b). bacteria found in August and October 2009

Table 3: Summary results of VNN and IRIDO detected in fish organs sampled in Jun, Aug and Oct 2009

Location	Results (positive/number of samples tested)	
	VNN	IRIDO
Tanjung Badak nursery cages	2/32 (6.25%)	3/38 (7.89%)
Pulau Layang-Layang nursery cages	0/24 (0%)	2/30 (6.66%)

VNN – Positive at 289 bp and/or 479 bp
IRIDO – Positive at 226bp and/or 450bp

Discussion

Gross observation

In the present study, tiger grouper showed a normal rate of weight gain in 150 days fed with normal pellet in both of the nursery cages. However, the weight gain (%) by the tiger grouper at Tanjung Badak was much higher than the fish reared in Pulau Layang-Layang. A lot of factors influenced the weight gain but the concern is usually for abnormal or unanticipated weight loss. A slower than normal rate of gain may be a more sensitive indicator of sub-optimal environment or health (Morton and Griffiths, 1985).

Both internal and external clinical signs occurred in tiger grouper reared at Pulau Layang-Layang compared to those cultured at Tanjung Badak. Some of the clinical signs were asystematic symptoms for some diseases. Woo *et al.* (1999) reported that vibriosis normally showed skin lesions such as red boil that become ulcer and in an acute situation a blood exudates known as hemorrhage. Enlarged spleen and kidney could indicate gastroenteritis vibriosis and infection of viral diseases in some cultured groupers (Yii *et al.*, 1997; Lee *et al.*, 2002). Vibriosis could also cause vasculitis and eye lesions in fish (Austin and Zhang, 2006). In our study, there were also corneal lesions followed by blindness. In acute and severe epizootics, the course of the infection will be rapid, and most of the infected fish die without showing any clinical signs. In our study, no high mortalities were recorded and we believed that it was not acute or severe epizootics.

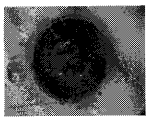
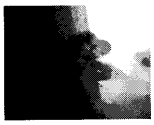
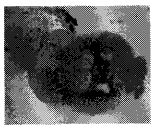
Parasitic infection

Ectoparasites such as ciliates (*Trichodina* sp.) and gill monogenean (*Pseudorhabdosynochus* spp.) are normally harmless ectocommensals, particularly in fingerlings. However, these ectoparasites often become pathogenic to fingerlings newly introduced into cages (Table 4). They become more susceptible to parasitic infections due to handling, transportation and environmental stresses. Once infected, the control of these ectoparasites becomes difficult. Infestation of body monogenean (*Benedenia* sp.) and crustacean copepod (*Caligus* sp.) are often seen in intensive mariculture. The intensity of parasitism may increase rapidly over a short period of time. The main infection sites are the skin. According to Leong (1992), marine finfishes cultured in Southeast Asia have been affected by monogenean epizootics. Usually, vibriosis occurs concurrent to monogenean infections in grouper, seabass and snapper. As for copepod infestation, it may not cause mortalities, however, infections will cause reduction in growth, anemia and subsequent consumer rejection.

Bacterial diseases

Staphylococcus bacteria were constantly found at each sampling month from the Tanjung Badak hatchery and Pulau Layang-Layang cages, suggesting its involvement in fish mortality at both sites. The staphylococcal infections comprised of infectious systemic diseases characterised by septicaemia and also lesion (Varvarigos). The disease spreads rapidly among fish population as well as to neighbouring cages holding the same or other fish species. Usually, there is good response subsequent to the administration of the appropriate therapy. However, the condition often persists and mortality gradually reappears. Field evidence suggests that it is not easy to eradicate a staphylococcal infection when established in a fish population. Apparently, it is not possible to eliminate the pathogen from the fish or the environment (Varvarigos). Gram-negative bacteria that were isolated from these two sampling sites appeared in low percentage as secondary infection; thus it was not considered as the main cause of fish mortality in the two sampling sites. The presence of Gram negative bacteria in secondary infection usually reflects poor water quality (mostly caused by high concentration of organic matter in culture area).

Table 4: Ectoparasites in tiger grouper during the surveillance period

Parasite	Picture	Disease symptoms	Possible treatments
Ciliates *Trichodina* spp.	Trichodina sp in gill (stained with giemsa) 	High infestation • Erratic swimming • Scraping against walls • Erosion of fins, skin ulcers • Gill hyperplasia	Formalin bath
Gill Monogenenan *Pseudorhabdosynochus* spp.	Adult *Pseudorhabdosynochus* spp on gill wet mount 	High infestation • Excessive mucus on the gill	Formalin bath
Body Monogenenan *Benedenia* sp.	Adult *Benedenia* sp from the skin 	High infestation • Skin darkening • Excessive mucus on the body • Associated with tail and fin rot diseases	Freshwater bath
Digenena Metacercariae	Metacercariae from wet mount from the gill 	High infestation • Mortality if mass penetration of the parasite	Break the life cycle: prevent snail or birds access to the site
Crustacean copepod *Caligus chiastos*	Adult female *Caligus chiastos* 	High infestation • Associated with ulcer and lesion or secondary skin bacterial infection	Freshwater bath

Viral diseases

VNN and iridovirus are the most common viral diseases in cultured marine fish in Asean generally and in Malaysia specifically. VNN disease was first described by Chuah and Kua (2004), when they found the first outbreak of VNN in *L. erythropterus* in Langkawi waters. The fish was imported from another Asean country and the detection was confirmed by histopathological changes of the brain and eye and RT-PCR. The disease is being transmitted either horizontally or vertically and showed clinical signs of dark body colouration and abnormal swimming behavior. It occurs mostly in the juvenile fish at hatchery at the age between 1 to 40 days old. In our study, we found that VNN could also be detected in bigger size fish. Further epidemiological studies at different sampling sites in Malaysia (2006-2009) revealed that VNN had been detected in healthy fish of seabass (*L. calcarifer*) and groupers (*Epinephelus* sp.) whether at hatchery or grow-out stages (Azila *et al.*, 2010). As the vaccine for VNN is still underway, a good management practice by screening of broodstocks and fish fry is essential to prevent its occurrence in the culture system. In addition, treatment of eggs with iodine has proven effective against it in the hatchery.

On the other hand, iridovirus was first reported in cultured tiger grouper, *E. fuscoguttatus* in Malaysia (Oseko *et al.*, 2002). However, the detection confirmed that the disease was caused by a genus of iridovirus which was lymphocystis virus. This disease was known to cause a whitish nodules or growth structures on the body, fins or mouth of the fish. In our study, the positive samples did not show such clinical signs except enlarged spleen and kidney or haemorrhage of the swim bladder. These clinical signs are associated with other genus of iridovirus especially the ranavirus (Azila, unpublished data). However, genus determination was not done in the current studies.

Concurrent infection of VNN and iridovirus are quite common. At certain period, the disease might cause other unspecific clinical signs due to the lower immune status caused by both viruses (Azila *et al.*, 2006). Furthermore, these 2 viruses can also be found in the trash fish (Kim *et al.*, 2007). Thorough studies on the presence of these 2 viruses in trash or wild fish have been done in Malaysia but few samples did show positive results (Azila, unpublished data). Hence, the use of trash fish for feeding and the wild broodstocks should be screened from time to time to prevent horizontal transmission of both viruses.

In conclusion, five different taxa of parasites (*Trichodina, Benedenia, Caligus, Pseudorhabdosynochus* and unidentified metacercariae); two types of viral diseases (VNN and iridovirus) and ten bacterial groups (*Staphylococcus, Vibrio, Photobacterium, Non-fermentor, Micrococcus, Pseudomonas, Gemella, Erwinia, Cedecia* and *Aerococcus*) were recorded at the tiger grouper cultured in nursery cages in Sabah. There is no difference between the prevalence of parasitic infection and viral diseases (VNN and IRIDO) at nursery cages at Tanjung Badak and Pulau Layang-Layang throughout the 3 sampling periods (Fig. 6). However, there are differences between the findings of bacteria where the percentage was lower in August as compared to the other months for tiger grouper reared at Tanjung Badak nursery cages. As the causes for this observation remains unknown surveillance needs to be continued with regular sampling in another batch of fish to more information as Pulau Layang-Layang has been identified as a new culture site in Malaysia.

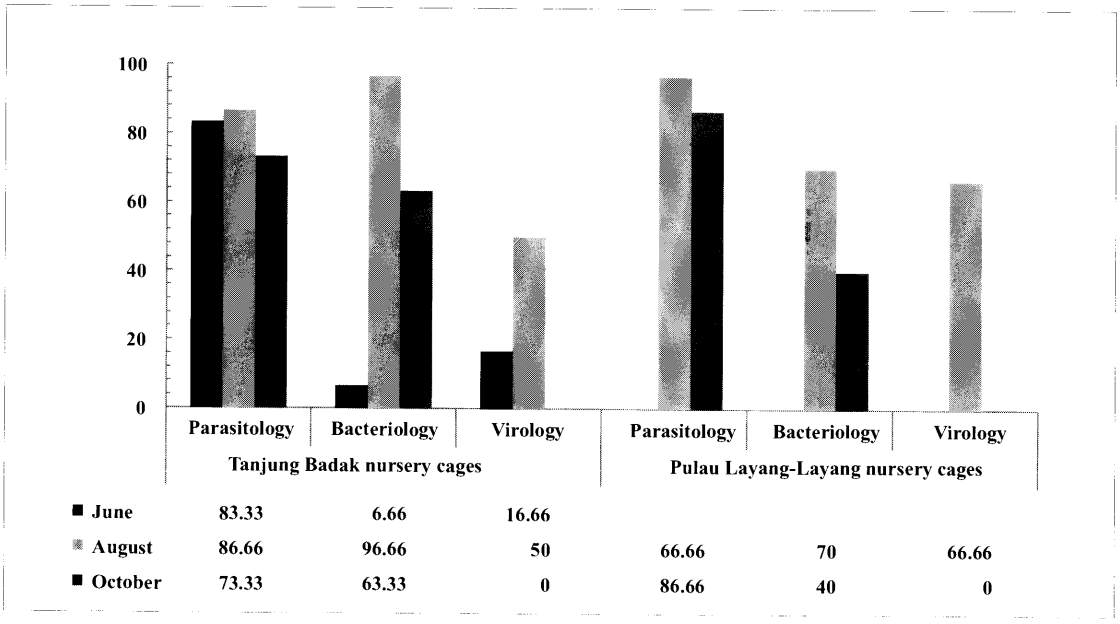


Figure 6: Summary of diseases pathogen at nursery cages of Tanjung Badak and Pulau Layang-Layang

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