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Short communication

A case of natural co-infection of Tilapia Lake Virus and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm experiencing high mortality

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ABSTRACT

This paper reports a case of natural co-infection of Tilapia Lake Virus (TiLV) and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm. In May 2017, a tilapia farm operator reported a mass mortality among cultured red hybrid tilapia juveniles, approximately 45 days after introduction into earthen ponds. Affected fish showed lethargy, loss of appetite, swim near the pond edge, pale and isolated from schooling group. There were skin redness and haemorrhages, particularly at the operculum area and at the base of dorsal, caudal and anal fins. Histopathological examinations revealed swollen hepatocytes, haemorrhagic spleens and perivascular cuffing consisted of mononuclear cells in the brains. PCR and sequence analyses confirmed the presence of TiLV and *A. veronii* in the diseased fish. Phylogenetic tree revealed that Malaysian's TiLV strain was more closely related with the virus isolated in Israel than in Egypt or Thailand, while Malaysian's *A. veronii* strain 5L was closely related with strains from China. TiLV infection is a significant threat to global tilapia industry. However, synergistic co-infection of TiLV and other bacterial might aggravate the problem.

1. Introduction

Tilapia is one of the most common fish species that is intensively produced all over the world. In 2015, the world tilapia production amounted to 6.4 million tonnes (MT) with an estimated value of USD 9.8 billion (FAO, 2017). Main producers of tilapia are China (1.8 MT), Indonesia (1.1 MT) and Egypt (875 thousand tonnes). The worldwide tilapia production is expected to increase in the future as they are quite resistance to diseases and hardy for intensive culture system making them an affordable protein source (FAO, 2017). In Malaysia, tilapia (*Oreochromis* sp.) is the second highest harvested freshwater fish, with a total annual production of 33,437 tonnes. The estimated wholesale value of this species was RM 259 million, which indicates the importance of tilapia farming in Malaysia (AFS, 2013).

Tilapia Lake Virus (TiLV), a novel RNA virus, is an orthomyxo-like virus. TiLV is an emerging disease that is responsible for causing massive mortalities of cultured tilapia in Israel, Ecuador, Colombia and Egypt (Eyngor et al., 2014; Ferguson et al., 2014; Bacharach et al., 2016; Del-Pozo et al., 2017; Fathi et al., 2017; Nicholson et al., 2017; Tsofack et al., 2017). Recently, outbreaks of TiLV infection have also been reported in Thailand, affecting cultured red tilapia (*Oreochromis* sp.) and Nile tilapia (*O. niloticus*) (Dong et al., 2017a, 2017b; Surachetpong et al., 2017).

Tilapia culture in Malaysia is commonly affected by *Streptococcus agalactiae* and *Aeromonas hydrophila*. However, *A. veronii* and viral infection have never been reported so far (Amal et al., 2008; Zamri-Saad et al., 2014; Ismail et al., 2016). This study describes a case of natural co-infection of TiLV and *A. veronii* in a Malaysian red hybrid tilapia (*O. niloticus* × *O. mossambicus*) farm.

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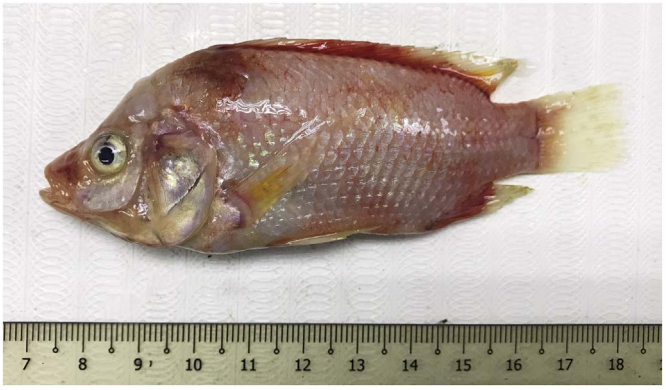

Fig. 1. Red hybrid tilapia juvenile that positive to TiLV and *Aeromonas veronii* showing skin redness and haemorrhages around the operculum area, body and base of dorsal, caudal and anal fins.

Table 1
Comparison of phenotypic characteristics of *A. veronii* from present and previous studies.

Characteristics	*A. veronii* red hybrid tilapia (present study)	*A. veronii* Nile tilapia (Dong et al., 2017c)	*A. veronii* Channel catfish (Liu et al., 2016)
Gram stain	− (rod)	− (rod)	− (rod)
Haemolysis (horse blood)	+	NA	+
Oxidase	+	NA	+
Catalase	+	NA	NA
Growth at 0.0% NaCl	+	NA	NA
Growth at 0.5% NaCl	+	NA	+
Growth at 1.0% NaCl	+	NA	+
Growth at 1.5% NaCl	+	NA	−
Growth at 2.0% NaCl	+	NA	−
Motility	+	+	+
Reduction of nitrates to nitrites	+	+	−
Indole production	+	+	+
Fermentation of glucose	+	+	+
Arginine dihydrolase	+	+	−
Urease	−	−	−
Hydrolysis of β-glucosidase	−	NA	NA
Hydrolysis of gelatin	+	+	+
B-galactosidase	+	NA	NA
Assimilation of glucose	+	+	+
Assimilation of arabinose	−	−	−
Assimilation of mannose	+	NA	+
Assimilation of mannitol	+	+	+
Assimilation of maltose	+	NA	NA
Assimilation of potassium gluconate	+	NA	NA
Assimilation of capric acid	+	NA	NA
Assimilation of adipic acid	−	NA	NA
Assimilation of malate	+	NA	NA
Assimilation of trisodium citrate	+	NA	NA
Assimilation of phenylacetic acid	−	NA	NA

*Note: + = positive, − = negative, NA = not available. All biochemical tests are included in the API 20NE identification kit.

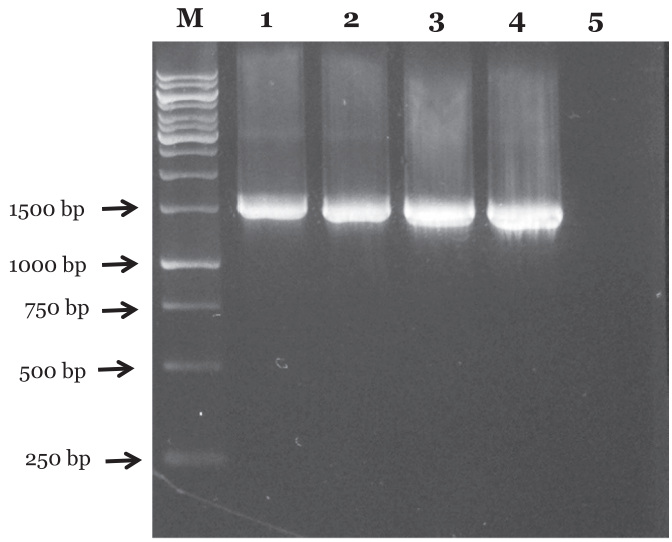

Fig. 2. PCR amplification of the 16S rRNA gene of *Aeromonas* spp. strains isolated from red hybrid tilapia showing 1500 bp. Lane M = DNA marker (1 kb DNA Ladder, Promega, USA); Lane 1–4: *Aeromonas* isolates isolated from infected tilapia; 5: Negative control.

2. Materials and methods

2.1. Case history

In May 2017, a tilapia farm operator in Selangor, Malaysia reported a mass mortality among juveniles of cultured red hybrid tilapia (*O. niloticus* × *O. mossambicus*) that were reared in earthen ponds. The batch of approximately 100,000 red hybrid tilapia fry was initially stocked in two ponds in late March 2017. First mortality was noticed in early May 2017, approximately 45 days after the fry were introduced into the ponds. Between 300 and 1800 of estimated death fish were recorded daily, with mortality rate peaked at around 5 to 9 days after first noticeable mass mortality that continued for the next three weeks. The fry were obtained from a local hatchery in Negeri Sembilan, Malaysia.

Affected fish appeared lethargic, loss of appetite, slow in movement, swim near the pond edge, pale and isolated from schooling group. They also showed skin redness and haemorrhages particularly at the operculum area and at the base of dorsal, caudal and anal fins before death (Fig. 1). The affected fish were between 7 and 20 g body weight and between 8 and 11 cm body lengths. A total of 20 affected morbid red hybrid tilapia juveniles were collected for further investigations.

2.2. Bacteria isolation and identification

Swab samples from the eye, brain, kidney, liver and spleen were streaked directly onto Tryptic Soy Agar (Merck, Darmstadt, Germany) with 5% horse blood. The agars were then incubated for 24 h at 30 °C. The grown bacteria were sub-cultured to get pure colonies before they were subjected to Gram staining, oxidase and catalase tests. A total of 18 isolates which were recovered either from eye, brain, kidney, liver and spleen of the diseased fish were proceed to identification using API®20NE system (bioMérieux, Marcy l'Etoile, France) and PCR.

Genomic DNA of the bacteria was extracted by Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA), according to the manufacturer's protocol. PCR assay targeting the 16S rRNA gene was carried out to confirm the bacterial isolates using universal primers 16S-F (5′-GAGTTTGATCCTGGCTCAG-3′) and 16S-R (5′-GACTACCAG GGTATCTAATC-3′) (Sfanos et al., 2005). The PCR amplifications were performed in reaction mixture containing 5 μL of Go Taq PCR buffer (Promega), 3 μL of 25 mM $MgCl_2$ (Promega), 2 μL of 25 mM $MgCI_2$ (Promega), 0.5 μL of 10 mM dNTPs (Promega), 1 μL of Taq polymerase

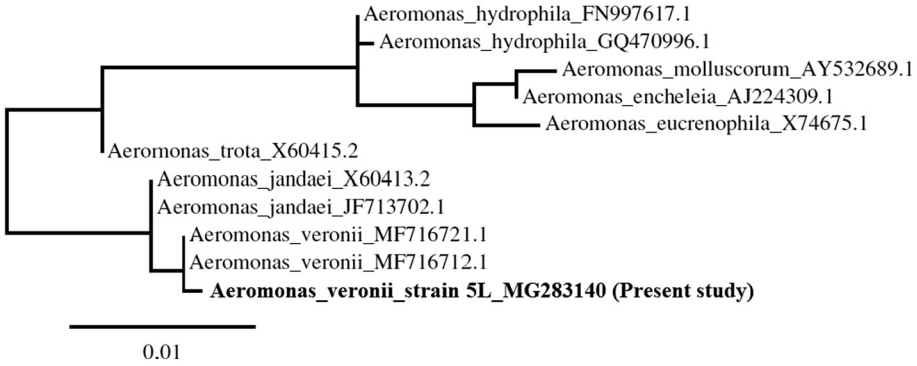

Fig. 3. Phylogenetic tree showing the relationships between the *Aeromonas veronii* strain 5L (MG283140) from Malaysia with other *Aeromonas* spp. *Aeromonas veronii* strain 5L was closely related with isolates of *A. veronii* from China (MF716712.1 and MF716721.1), compared to other species of *Aeromonas*. The tree is based on a 1500 bp gene sequence of *Aeromonas* spp.

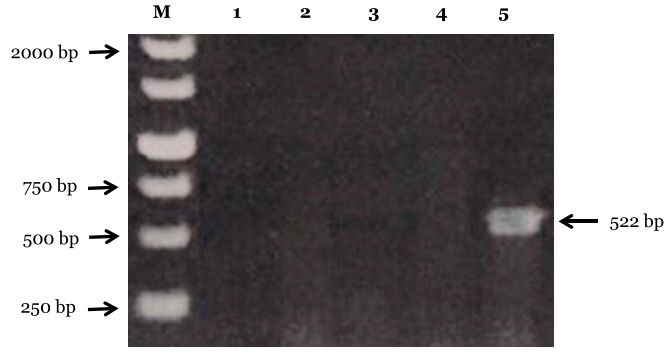

Fig. 4. The PCR product for TiLV from red hybrid tilapia showing the 522 bp band. Lane M = DNA marker (1 kb DNA Ladder, Promega, USA); Lane 1: negative control from non-infected farm; 2–4: non-infected tilapia; Lane 5: TiLV-infected tilapia.

(Promega), 100 ng of DNA template and DNase-free water up to the total volume of 50 µL. A thermocycler (Eppendorf, Hamburg, Germany) was used to perform the following PCR reactions: initial denaturation at 94 °C for 10 min, denaturation at 94 °C for 1 min each for 34 cycles, annealing at 50 °C for 1 min, extension at 72 °C for 1 min and 30 s and post extension at 72 °C for 10 min. The amplicons were subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide.

2.3. Virus RNA extraction and detection

The organs of each affected red hybrid tilapia juveniles, including the brain, kidney, liver and spleen were pooled and preserved in RNAlater reagent (Qiagen, Kuala Lumpur, Malaysia). In brief, total RNA was extracted from pooled organs using TRIzol™ reagent (Invitrogen, California, USA) according to the manufacturer's instructions. Tissue samples from healthy red hybrid tilapia from a non-affected farm were processed as controls. For cDNA synthesis, reverse transcription (RT) was carried out using Quantinova™ Reverse Transcriptase kit (Qiagen) according to the manufacturer's protocol.

For molecular identification, a diagnostic RT-PCR specific for TiLV amplifying a 522-bp fragment of segment 9; TiLV-Seg9-F (5′-ACGTCC TTAAAGTCATACTT-3′) and TiLV-Seg9-R (5′-ACAAGTCCGATTACTTT TTC-3′) was performed (Dong et al., 2017a). Each mastermix contained 25 µL of 2 µL of cDNA template, 1 µM of each forward and reverse primer, 2.5 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTPs (Invitrogen) and 1U Taq DNA Polymerase (Invitrogen). A thermocycler (Eppendorf) was used to perform the following PCR reactions: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s each for 35 cycles, annealing at 50 °C for 30 s, extension at 72 °C for 1.5 min and a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1% agarose gels and stained with ethidium bromide.

2.4. Bacteria and virus sequencing

A total of four and one purified PCR products of *A. veronii* and TiLV, respectively were used for sequencing (First Base Laboratories, Malaysia). The nucleotide sequences of bacteria and virus were compared with the known sequences in the GenBank database using Nucleotide Basic Local Alignment Search Tool (BLAST) program. Phylogenetic tree for *A. veronii* and TiLV was generated by Neighbor-joining of the MEGA 6.06 software (Tamura et al., 2013).

2.5. Histopathological assessment

The brain, kidney, liver and spleen of the affected fish were fixed in 10% buffered formalin for 24 h. The samples were then embedded in paraffin, sectioned at 4 µm (Leica Jung Multicut 2045, Germany) and stained with Harris haematoxylin and eosin (HE). The slides were examined using Nikon microscope Eclipse 50i Japan and the photomicrographs were recorded using Nikon NIS-Element D 3.2 Image Analyser (Nikon Instruments Inc., USA).

3. Results

3.1. Disease characterization and post mortem analysis

The estimated rate of mortality of the red hybrid tilapia juveniles in this outbreak was approximately 25%. Only red hybrid tilapias of the same batch and source in both earthen ponds were affected. Red hybrid tilapia was the only cultured fish species in this farm, while other fish batches and ponds were not affected during this outbreak. The affected fish in this study also showed severe haemorrhages of the liver, spleen and kidney with enlarged gall bladder.

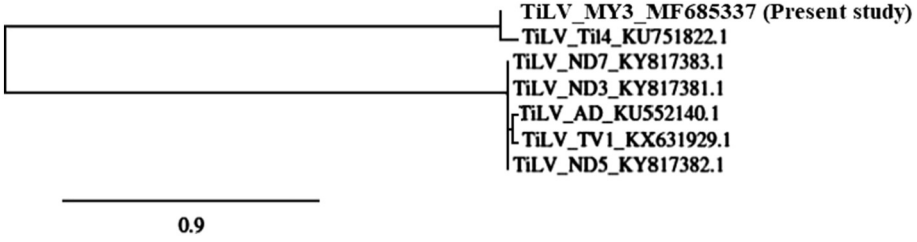

Fig. 5. Phylogenetic tree showing the relationships between the TiLV strains from Malaysia (MY3 MF685337), Israel (Til4 KU751822.1 and AD KU5552140.1), Thailand (TV1 KX631929.1) and Egypt (ND3 KY817381.1, ND5 KY817382.1 and ND7 KY817383.1). The tree is based on a 522 bp gene sequence of segment 9.

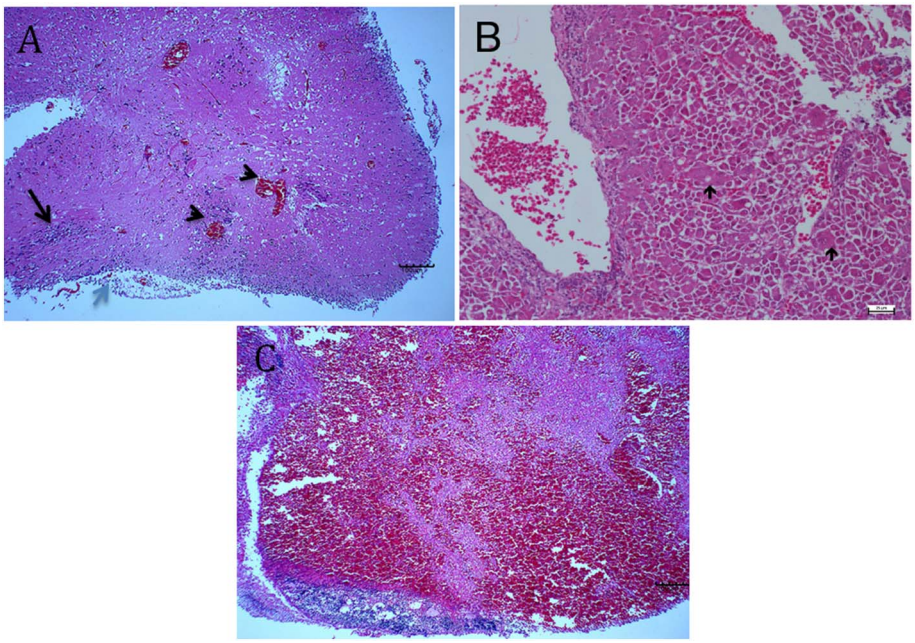

Fig. 6. A) Histopathological section of the brain showing focal inflammation in the leptomeninges (arrows) and congestion of blood vessels (arrowheads) HE × 100; B) Focal area of hepatocellular swelling and presence of syncytial hepatitis cells (arrows) HE × 200; C) The spleen shows focal haemorrhages with proliferating lymphocytes HE × 100.

3.2. Bacteria isolation and identification

Aeromonas veronii strain 5L was isolated and identified from the affected fish using API® 20NE (bioMérieux). Comparison of phenotypic characteristics of the isolated *A. veronii* with previous studies is described in Table 1. PCR successfully amplified the 1500 bp band, the 16S rRNA gene of *Aeromonas* spp. (Fig. 2). The BLAST analysis showed that the nucleotide sequence of 16S rRNA gene of *A. veronii* in this study was 98% similar with the published sequence of *A. veronii* strain JX1-2-2 isolated from China (GenBank accession no. MF716707.1). The nucleotide sequences for 16S rRNA gene of *A. veronii* strain 5L was deposited in the GenBank database with accession no. of MG283140.

Based on the phylogenetic tree, *A. veronii* strain 5L was grouped together with *A. veronii* from China (MF716712.1 and MF716721.1), compared with other species such as *A. jandaei* (JF713702.1 and X60413.2) and *A. hydrophila* (FN997617.1 and GQ470996.1) (Fig. 3).

Aeromonas veronii was successfully isolated from 50% (10/20) of the sampled red hybrid tilapia juveniles, and from all of the fish that were positive to TiLV.

3.3. Virus detection and characterization

TiLV was successfully detected from 20% (4/20) of the sampled red hybrid tilapia juveniles. The amplified product was observed at 522 bp (Fig. 4). The nucleotide sequence revealed 97% homology to the published TiLV sequence from Israel (KU751822.1), when compared to sequences from segment 9. The Malaysian's strain is closer to the Israeli's strain (Til4 KU751822.1), than to the Thailand's strain (TV1 KX631929.1) and Egyptian's strains (ND3 KY817381.1, ND5 KY817382.1 and ND7 KY817383.1) and another Orthomyxoviridae tilapia virus from Israel (AD KU552140.1) (Fig. 5). The nucleotide sequence for segment 9 of Malaysian's TiLV MY3 strain was deposited into the GenBank database with accession no. MF685337.

3.4. Histopathological findings

Histopathological examinations revealed oedema and haemorrhages in the leptomeninges, congested blood vessels and foci of gliosis and perivascular cuffings consisted mainly of lymphocytes. There were foci of hepatocellular swelling and presence of syncytial hepatitis cells suggestive of TiLV infection. The spleens showed focal haemorrhages with proliferating lymphocytes (Fig. 6).

4. Discussion

TiLV is an emerging pathogen causing syncytial hepatitis of tilapia (Ferguson et al., 2014). This disease was also linked for 'summer mortality syndrome' in tilapia, with potential economic impact of around USD 100 million (Fathi et al., 2017). TiLV was very contagious (Tattiyapong et al., 2017) and only tilapia cichlids are highly susceptible to this disease (Eyngor et al., 2014). In tilapia culture, apart from *A. hydrophila*, several aeromonads have been associated with diseases in tilapia including *A. veronii*, *A. sobria* and *A. jandaei* (Li and Cai, 2011; Dong et al., 2017c), while infectious disease caused by motile aeromonads is one of the most common problems in freshwater aquaculture (Austin and Austin, 2012).

In this study, the TiLV strain from Malaysia was closely related with the strain from Israel compared to Egypt and Thailand. Similarly, Thailand's TiLV strain also shares high sequence similarity with TiLV from Israel, indicating possibility of cross continent spread of the disease (Dong et al., 2017a; Surachetpong et al., 2017). *Aeromonas veronii* from Malaysia showed similar phenotypic characteristics with the strain of *A. veronii* recovered from Nile tilapia in Thailand (Dong et al., 2017c), but different from the strain isolated from channel catfish (*Ictalurus punctatus*) in China (Liu et al., 2016). However, the genotype of our *A. veronii* isolate was closely related with *A. veronii* isolated from the water of loach (*Paramisgurnus dabryanus*) culture in China.

The PCR and sequencing analyses confirmed the presence of TiLV and *A. veronii* in the cultured red hybrid tilapia in this study. The symptoms, clinical signs, mortality patterns and histopathological findings of the affected fish suggested the combination of TiLV and *A. veronii* infection (Dong et al., 2017a, 2017c; Surachetpong et al., 2017).

Co-infection occurs when hosts are infected by two or more different pathogens either simultaneous or secondary infections, so that two or more infectious agents are active together in the same host (Kotob et al., 2016). In this study, *A. veronii* was concurrently isolated with TiLV from the diseased fish as reported earlier by Nicholson et al. (2017). Similarly, multiple bacteria including *Flavobacterium*, *Streptococcus* and *Aeromonas* were also found in TiLV-infected fish (Surachetpong et al., 2017). In this study, the interactions of *A. veronii* and TiLV might be synergistic, which resulted in the enhancement of both pathogens and increasing the severity of the disease, as observed

in the affected fish. However, future *in vivo* study should be conducted to understand the relationship of both pathogens.

TiLV infection is a significant threat to global tilapia industry. However, synergistic co-infection of TiLV and other bacteria might aggravate the problem. In this study, we report the first case of natural co-infection of TiLV and *A. veronii* in a Malaysian red hybrid tilapia farm.

Acknowledgements

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