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Short Communication

Antibiotic Sensitivity and Pathogenicity of *Aeromonas veronii* Isolated from Diseased Red Hybrid Tilapia in Malaysia

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ABSTRACT

This article reports the antibiotics sensitivity and pathogenicity of *Aeromonas veronii* 5L isolated from diseased red hybrid tilapia. The antibiotic sensitivity of *A. veronii* 5L was determined towards 13 antimicrobial agents. Then, the bacterial inoculums ranging between 0 and 10^6 CFU/mL were used for intraperitoneal challenge in red hybrid tilapia juveniles. *Aeromonas veronii* 5L showed resistance to ampicillin, streptomycin and sulfamethoxazole/trimethoprim. Following intraperitoneal exposure, mortality was observed as early as 24 h post infection leading to a total of 56.7% cumulative mortality at 10^2 CFU/mL, and 66.7% from 10^3 to 10^6 CFU/mL. Clinical signs and gross lesions including abdominal distension, detachment of

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scales and ulceration on the body surface, inflammation around the operculum and based of fins, hemorrhage of internal organs, and accumulation of fluid in abdominal cavity. Histopathological examinations revealed generalized congestion of the brain, necrotic hepatocytes, generalized tubular necrosis of kidney and multifocal necrosis of spleen with splenic infarction. The $LD_{50\text{-}336h}$ of *A. veronii* 5L was determined at $1.9 \times 10^{3.73}$ CFU/mL. This study revealed the capability of *A. veronii* as another important pathogen in tilapia culture in Malaysia.

Keywords: *Aeromonas veronii*, antibiotic sensitivity, fish disease, pathogenicity, tilapia

INTRODUCTION

Aeromonas veronii infection has been reported in several fish species around the world including carp (*Cyprinus carpio*), oscar (*Astronotus ocellatus*), Chinese longsnout catfish (*Leiocassis longirostris*), Nile tilapia (*Oreochromis niloticus*) and pond loach (*Misgurnus anguillicaudatus*) (Cai et al., 2012; Dong et al., 2015; Sreedharan et al., 2011; Yu et al., 2010; Zhu et al., 2016). Affected fish exhibit either abdominal distension, abnormal swimming behavior, loss of appetite, reddish foci on the skin, dark bodies, hemorrhages all over the body surface and ulceration with muscular necrosis, severe haemorrhage of internal organs, liver congestion, enlarged spleen and kidney, enteritis and gut displayed a significant amount of yellowish liquid (Eissa et al., 2015; Sreedharan et al., 2011; Yu et al., 2010; Zhu et al., 2016).

Tilapia (*Oreochromis* spp.) culture in Malaysia is commonly affected by *Streptococcus agalactiae*, *S. iniae* and *Aeromonas hydrophila* infections (Amal et al., 2010; Ismail et al., 2016; Nur-Hidayahanum et al., 2016; Rahmatullah et al., 2017). Recently, the isolation of *A. veronii* from diseased cultured red hybrid tilapia (Nile tilapia × Mozambique tilapia (*O. mossambicus*)) that concurrently infected with Tilapia Lake Virus (TiLV) has been reported in Malaysia (Amal et al., 2018). However, the pathogenicity of the isolated *A. veronii* was not conducted. Thus, we report the antibiotics sensitivity and pathogenicity of *A. veronii* isolated from diseased red hybrid tilapia in Malaysia. In this study, we revealed the virulent and capability of *A. veronii* as another important pathogen in tilapia culture in Malaysia in the future.

MATERIALS AND METHODS

Aeromonas veronii 5L was used in this study. It was isolated from diseased red hybrid tilapia juvenile that was concurrently infected with TiLV (Amal et al., 2018). The isolate was identified as *A. veronii* based on Gram staining, oxidase and catalase tests, API®20NE system (bioMérieux, Marcy l'Etoile, France), PCR and sequencing analyses. Stock of *A. veronii* 5L was cultured in *Aeromonas* medium base agar (AMBA) (Oxoid, Hampshire, United Kingdom) and incubated for 24 h at 30°C. Then, 10

colonies of the isolate were inoculated into 100 mL of tryptic soy broth (TSB) (Merck, Darmstadt, Germany), and incubated in an orbital incubator at 0.75 × *g* for 24 h at 30°C. The bacterial concentrations were calculated based on standard ten-fold serial dilutions and spread plating methods onto AMBA, prior to fish challenge experiment.

The antibiotic sensitivity of *A. veronii* 5L was evaluated using the Kirby-Bauer disc diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2016). Direct colony suspension of *A. veronii* with adjusted turbidity equivalent to 0.5 McFarland standard was used. A total of 13 representative antimicrobial agents (Oxoid, London, UK), including ampicillin (10 µg), cefotaxime (30 µg), cefepime (30 µg), cephalothin (30 µg), gentamycin (10 µg), kanamycin (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), ciprofloxacin (5 µg) and tetracycline (30 µg) were tested in duplicate. The resistance profiles (resistant, intermediate or susceptible) were interpreted using recommended criteria (CLSI, 2013, 2016). The multiple antibiotic resistance (MAR) index was determined according to Krumperman (1985). A MAR index higher than 0.2 indicated high-risk exposure to these antibiotics.

A total of 180 red hybrid tilapia juveniles weighing 20.5 ± 0.8 g were used for challenge experiment. The fish were divided into six groups and each group was in triplicate with 10 fish/replicate, including the control group. At the start of the experiment, each group was exposed intraperitoneal (IP) with different bacterial concentration that ranged between 10^2 and 10^6 CFU/mL of live *A. veronii* 5L, while the non-infected control group was similarly exposed to sterile phosphate-buffered saline. Each fish was IP injected with final volume of 100 µL of the inoculum.

Following infection, continuous aeration was provided to all groups, while feed was given twice daily. The experiment was conducted for 336 h, during which the clinical signs, gross lesions and mortality pattern were recorded. Freshly dead fish were collected for bacterial isolation and identification. The LD_{50} of *A. veronii* 5L infection in the fish was calculated at 336 h post infection (hpi), according to formula described by Ramakrishnan (2016). The mean ± SD of water quality parameters during the study period were as follows: dissolved oxygen at 5.60 ± 0.50 mg/L, pH at 7.2 ± 0.5, water temperature at 27.10 ± 0.50°C, ammonia at 0.02 ± 0.01 mg/L, and nitrite at 0.10 ± 0.50 mg/L.

Quantitatively, the brain, kidney, liver and spleen of dead fish were collected for histopathological changes observation (Amal et al., 2018). Following fixation in 10% buffered formalin for 24 - 48 h, the samples were processed in tissue processor, embedded in paraffin, sectioned at 4 µm thick and stained routinely with Harris haematoxylin and eosin (HE) for histological study. The sections were

examined and photographed using Nikon Eclipse 50i Japan microscope and The Nikon NIS-Element D 3.2 Image Analyser (Nikon Instruments Inc., USA).

RESULTS

Aeromonas veronii 5L displayed strong resistance to ampicillin (range: 0 mm), streptomycin (0 mm) and sulfamethoxazole/trimethoprim (0 mm), while intermediately sensitive to kanamycin (16 - 17 mm). The isolate was sensitive to cefotaxime (31 - 33 mm), cefepime (28 – 29 mm), cephalothin (23 - 24 mm), gentamycin (18 mm), tetracycline (28 mm), ciprofloxacin (33 mm), nalidixic acid (30 - 31 mm), nitrofurantoin (24 mm) and chloramphenicol (30 mm). The MAR index was 0.23.

Fish mortality was observed as early as 24 hpi in fish infected with 10^2, and 10^4 to 10^6 CFU/mL of *A. veronii* 5L (Figure 1). In general, the fish mortality showed increasing pattern until the end of the experimental period. Mortality among fish infected with 10^4 and 10^6 CFU/mL reached peak at 66.7% as early as 264 hpi, followed by those infected with 10^5 CFU/mL at 312 hpi, with 10^3 CFU/mL at 336 hpi. However, infection with 10^2 CFU/mL peaked with 56.7% cumulative mortality at 336 hpi. *Aeromonas veronii* 5L was successfully re-isolated from all dead fish. No fish mortality was observed in negative control group. The $LD_{50\text{-}336h}$ of *A. veronii* 5L in red hybrid tilapia juveniles was determined at $1.9 \times 10^{3.73}$ CFU/mL.

Infected fish tended to isolate from the schooling group, appeared lethargy, less responsive to stimuli, loss of appetite,

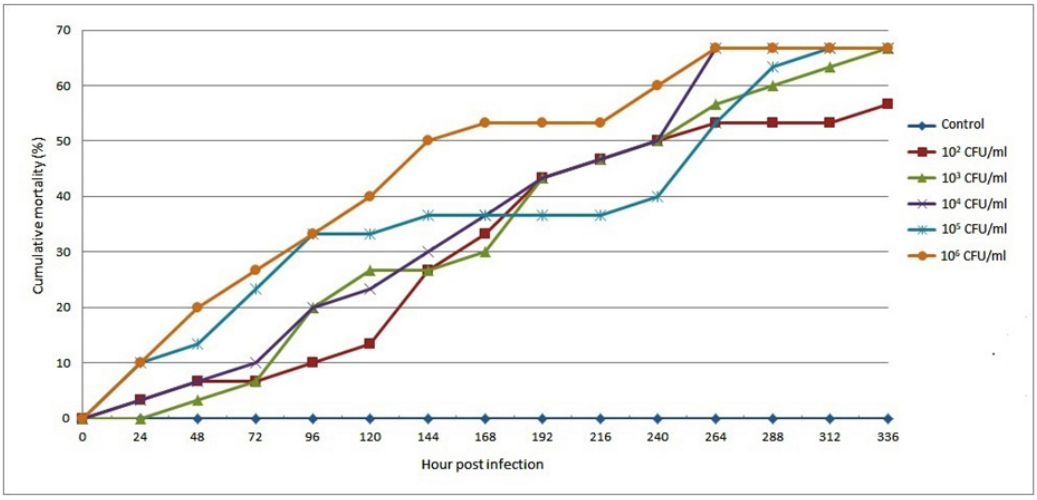

Figure 1. Cumulative mortality patterns of red hybrid tilapia juveniles following intraperitoneal challenge with various concentration of *Aeromonas veronii* 5L up to 336 hours

showed distended abdomen, occasional detachment of scales and ulceration on the body surface, pale, inflammation around the operculum and based on fins, skin hemorrhages and ulceration of fins (Figure 2). Post-mortem examination revealed hemorrhagic liver, enlarged gall bladder, accumulation of fluid in abdominal cavity, inflammation and hemorrhage of the brain.

The histopathological assessment showed generalized congestion of the brain (Figure 3). Kidneys showed generalized tubular necrosis with mild haemorrhage. The histopathological lesions occasionally observed in the liver include necrosis of hepatopancreatic cells and generalized necrosis of the hepatocytes. Generalized multifocal necrosis and multifocal melano macrophage centre were observed in the spleen. Besides, hyperplasia of red pulp and splenic infarctions were also observed.

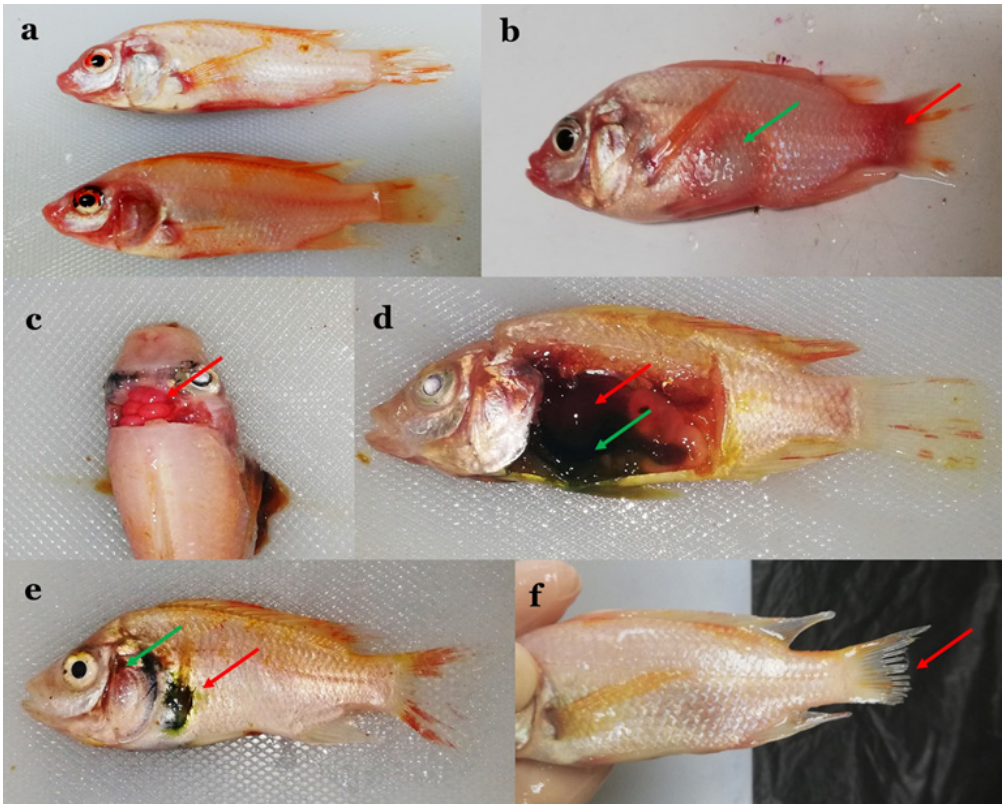

Figure 2. Gross lesions of juvenile red hybrid tilapia following intraperitoneal infection by *Aeromonas veronii* 5L. a) Inflammation around the operculum area and fins base; b) Ulceration around the abdomen (green arrow) and inflammation at the base of caudal fin (red arrow); c) Inflammation and haemorrhage of the brain (red arrow); d) Haemorrhage liver (red arrow) and enlarged gall bladder (green arrow); e) Inflammation around operculum area (green arrow) and distension of abdominal area (red arrow) due to fluid accumulation; f) Severe ulceration of caudal fin (red arrow)

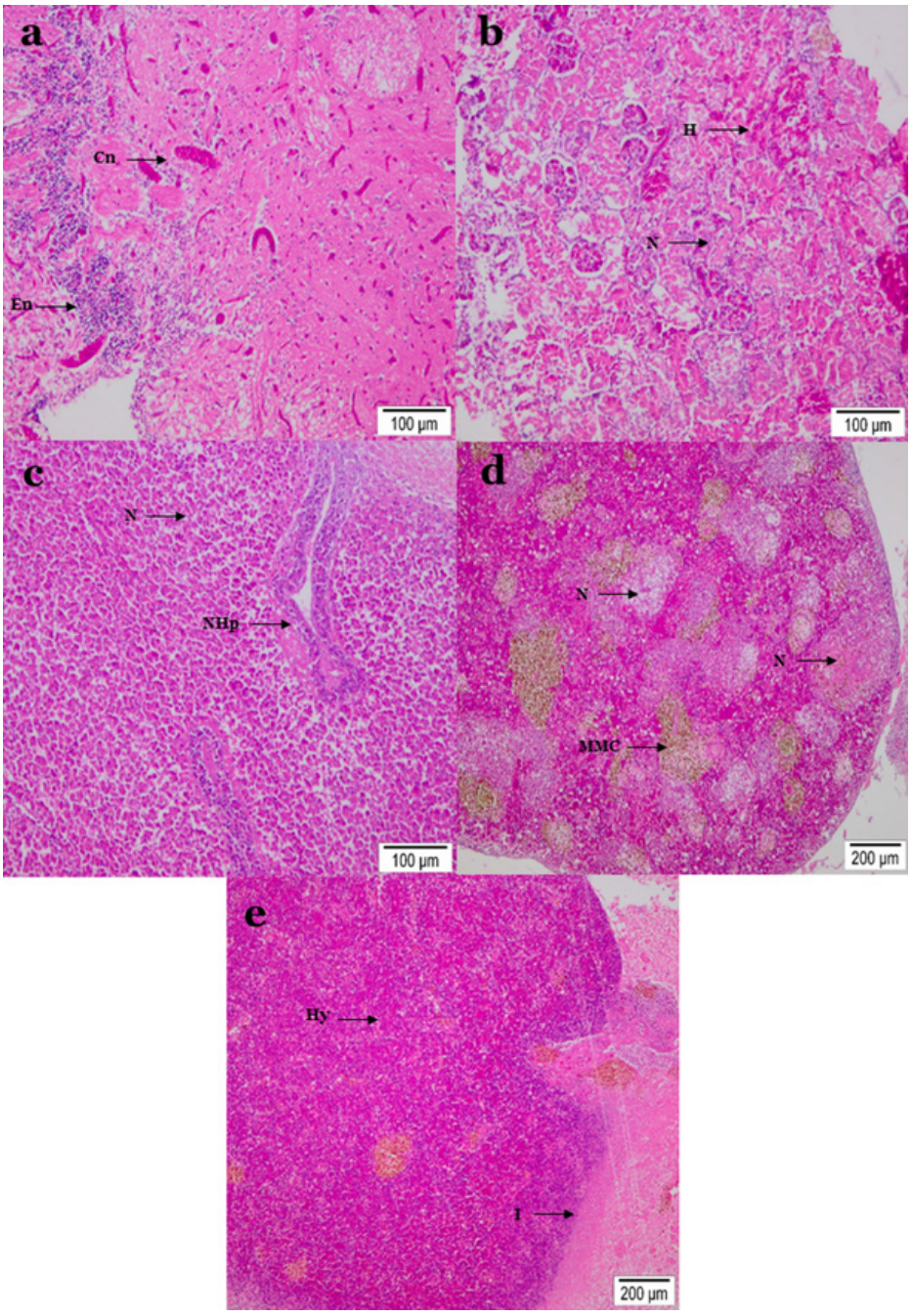

Figure 3. Histopathological lesions of juvenile red hybrid tilapia following intraperitoneal injection by *Aeromonas veronii* 5L. a) Encephilitis (En) with presence of generalized congestion (Cn), brain, HE, ×100. b) Generalized tubular necrosis (N) with mild haemorrhage (H), kidney, HE, ×100. c) Necrosis of hepatopancreatic cells (NHp) and generalized necrosis (N), liver, HE, ×100. d) Generalized multifocal necrosis (N) and increased in sizes and number of multifocal melano macrophage centre (MMC), spleen, HE, ×100. e) Hyperplasia of red pulp (Hy) and splenic infarction (I), spleen, HE, ×100

DISCUSSION

Clinical infection by *A. veronii* in fish has not been reported in Malaysia. However, disease outbreak causing daily mortality between 300 and 1800 cultured red hybrid tilapia juveniles was recorded in 2018, resulting in approximately 25% mortality (Amal et al., 2018). The outbreak was due to concurrent infection involving TiLV infection and *A. veronii*. In this study, experimental infection resulted in mortality as early as 24 hpi, with peak cumulative mortality rate of 66.7%. With low $LD_{50\text{-}336h}$ at $1.9 \times 10^{3.73}$ CFU/mL, this *A. veronii* 5L isolate was considered virulent. The virulence of *A. veronii* was also observed in Nile tilapia juvenile in Thailand, where *A. veronii* at 8.9×10^6 CFU/fish killed 100% of fish within 24 hpi (Dong et al., 2017).

In this study, infected fish displayed typical aeromonad infection (Eissa et al., 2015; Sreedharan et al., 2011; Yu et al., 2010; Zhu et al., 2016), suggesting common signs of haemorrhagic septicemic as in the virulent motile aeromonad septicaemic strain (Dias et al., 2016). In Saudi Arabia, experimentally infected Nile tilapia by *A. veronii* showed hemorrhage at the base of all fins, generalized external hemorrhage on the body skin, congestion and enlargement of internal organs, enteritis, and intestine filled with transparent fluids (Hassan et al., 2017). The results of histopathological changes in this study also indicated septicaemia, which are in agreement with previous study in Thailand (Dong et al., 2017).

Antibiotic sensitivity study indicated that *A. veronii* 5L was isolated from the fish that previously had high-risk exposure to the tested antibiotics. Nevertheless, in this study, cefotaxime, cefepime, cephalothin, gentamycin, tetracycline, ciprofloxacin, nalidixic acid, nitrofurantoin and chloramphenicol could still be used to treat infections by *A. veronii*. Similarly, Hassan et al. (2017) also indicated that the capability of chloramphenicol, nitrofurantoin and nalidixic acid to control *A. veronii* infection in tilapia.

CONCLUSION

This study revealed the virulent and capability of *A. veronii* as another important pathogen in tilapia culture in Malaysia in the future, besides *S. agalactiae*, *S. iniae* and *A. hydrophila*. Moreover, while the production of tilapia keeps increasing in this country, the results should also alarm the tilapia industry, especially to the farm operators and authorities.

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