

First Report of *Aeromonas schubertii* Infection in Striped Snakehead *Channa striata* (Bloch, 1793) Fingerlings in Malaysia

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Abstract

Striped snakehead fingerlings *Channa striata* reared in a concrete pond with size 13.5 m³ (3 m × 3 m × 1.5 m) in a hatchery farm, in Labu, Negeri Sembilan, Malaysia had more than 70% mortality. The pond contained 1500 fingerlings of 5–7 cm in total length (TL). The purpose of this study was to investigate the high mortality event on the farm as part of a veterinary diagnostics service. Clinical signs observed were skin discoloration, lethargy, and anorexia. Necropsy was performed in 10 fish with gross lesions of 1–2 mm and multiple whitish nodules in the abdominal organs and most notably in the liver, and kidney. Following necropsy, bacteriology samples from the kidney and liver were inoculated onto Tryptic Soy Agar (TSA) and Blood Agar (BA). Pure colonies were observed and were Gram-negative with short rod morphology. The full-length 16S rRNA gene was amplified from the pure colonies followed by sequencing using the Nanopore MinION™ (Oxford Nanopore, UK). The isolated strain was identified as *Aeromonas schubertii* based on the assembled 16S rRNA sequence showing nucleotide identity of 99.73% (top hit) to *A. schubertii* (GenBank Accession: NR_037014.2) while only a nucleotide identity of 97.55% was observed against *Aeromonas hydrophila* (GenBank Accession: NR_119190.1-), respectively. Histopathological examination from the liver, kidney, spleen, and intestine revealed granulomatous nephritis, and degeneration of tubular epithelium, with granulomatous hepatitis, splenitis, and enteritis. The prognosis of this case was grave. No treatment was given as the owner had decided to dispose of all current stock. This is the first *A. schubertii* infection in *C. striata* described in Malaysia. The findings of the present study may serve as a reference for similar cases in *C. striata* in the future so rapid diagnosis and treatment can be made if required.

Keywords: *Aeromonas schubertii*, *Channa striata*, granuloma, nanopore, 16S rRNA

Received: 15 December 2023

Revised: 30 January 2024

Accepted: 2 March 2024

INTRODUCTION

Channa striata, commonly known as striped snakehead fish, belongs to the family Channidae and can be found in a wide range of freshwater habitats including rivers, swamps, ponds, canals, lakes, and rice fields (Song *et al.*, 2013). It is a popular protein food source in Malaysia due to its well-known nutraceutical and pharmaceutical properties and agreeable local flavor (Tan *et al.*, 2012). In Malaysia, natural healers and midwives have traditionally used preparations of *C. striata* to promote wound healing, especially during postpartum (bin Mohd Khatib and Jais, 2021). The culture of this fish has been intensified over the years to meet the growing demand and

consequently increases the susceptibility to diseases.

Aeromonas schubertii is a Gram-negative rod bacterium belonging to the *Aeromonas* complex group (Fernández-Bravo, 2020) and is present in freshwater, seawater, and soil. *A. schubertii* infection was first reported in human clinical cases from traumatic injuries in 1988 (Hickman-Brenner *et al.*, 1988). Other reports described *A. schubertii* infection in marine shrimps *Litopenaeus vannamei* and *Penaeus monodon* (Yano *et al.*, 2014), mussels (Latif-Eugenín *et al.*, 2016), and Nile tilapia *Oreochromis niloticus* (Ren *et al.*, 2020). In snakehead species, the infection has been reported in *C. argus*, *C. maculata*, and the hybrid snakeheads with gross lesions of multiple white

nodules in the spleen, liver, and kidney and known as the “white spots diseases” (Liu and Li, 2012). The mortality rate of *A. schubertii* infection can be high reaching 45% as reported by Liu *et al.* (2012) which can cause severe economic loss to the farmer.

The 16S rRNA sequencing has been routinely used in the identification of aquatic diseases (Dubey *et al.*, 2021). Advancements in sequencing technologies have now allowed for the rapid identification of pathogens in fish. The Nanopore MinION™ (Oxford Nanopore, UK) as utilized in the present study is capable of real-time analysis with less equipment, and possible applications in remote locations as a portable sequencing (Delamare-Deboutteville *et al.*, 2021) such as in aquaculture farms.

A. schubertii infection in cultured snakeheads can be an important pathogen with the intensification of the culture of this species contributing losses to farmers. Furthermore, no previous cases of *A. schubertii* were reported in *C. striata* in Malaysia, to the best of the authors' knowledge. Therefore, the clinical description of the gross lesion, histopathological diagnosis, bacteria isolation, and identification with the 16S rRNA sequencing discussed in the present study can aid future diagnoses should similar cases be observed and prompt further epidemiological investigation for prevention and mitigation of this disease.

MATERIALS AND METHODS

Ethical Approval

This study did not require ethical approval.

Study Period and Location

The study was conducted at one single sampling in August 2022 at a hatchery farm, in Labu, Negeri Sembilan, Malaysia as part of veterinary diagnostic services.

Clinical Description

Striped snakehead fingerlings *C. striata* reared in a concrete pond with size 13.5 m³ (3 m × 3 m × 1.5 m) in a hatchery farm, in Labu, Negeri Sembilan, Malaysia had more than 70% mortality

in May 2022. The pond contained 1500 fingerlings of 5–7 cm in total length (TL). The farmer reported the fish being lethargic and inappetence.

Twelve moribund fish from the pond were selected for further examination. The fish were collected by fish nets and placed on an examination table. Collection of fish samples was also on physical examination, two fish observed showed discoloration with a darker color of the skin. A wet mount of the skin and gills for ectoparasites was performed with no significant findings.

Upon necropsy, multiple whitish nodules of 1–2 mm were observed in the liver, gastrointestinal organs, and kidney (Figure 1). Swab samples from the liver and kidney were taken and inoculated onto Tryptic Soy Agar (TSA) and blood agar (BA) and incubated for 24 hours. The kidney, liver, and intestine were taken for histopathological examination with the samples preserved in 10% buffered formalin and processed by standard methods for histology. Histological slides were stained using hematoxylin and eosin (H&E) stain.

After 24 hours of incubation of the samples on the TSA media, pure 1–2 mm white colonies can be observed. Gram staining was performed which revealed gram-negative short rods. Two colonies were taken for 16S rRNA Nanopore (Oxford Nanopore, UK) sequencing for identification. Microbial full-length 16S rRNA sequence was amplified using the 27F (TTCTGTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG) and 1492R (ACTTGCTGTCGCTCTATCTTCTACGGYTACCTTGTTACGACTT) primers with Nanopore partial adapter on the primer 5' end (Matsuo *et al.*, 2021). Sequencing was performed on a Nanopore Flongle Flowcell for 24 hours and basecalled using Guppy v5.0.7 (super accuracy mode). The demultiplexed sequences were clustered and polished using NanoCLUST (Rodríguez-Perez *et al.*, 2020). Taxonomic assignment of the consensus sequence used blastN against the latest GTDB release r202 16S rRNA database. For phylogenetic analysis, the cluster sequences and their top blast hits were combined and aligned

with MAFFT v7 (Kato *et al.*, 2002). A maximum likelihood tree was constructed from the aligned nucleotide sequences using FastTree2 (-nt -gtr setting) and visualized using Figtree.

RESULTS AND DISCUSSION

Microscopic findings in this case were consistent with previous reports of *A. schubertii* infections in snakeheads. The first reported infection of *A. schubertii* in China (Liu and Li, 2012), in *Ophiocephalus argus* showed similar findings of multiple, ivory-white, firm nodules of 0.5–1 mm in diameter in the kidney. Additionally in the present case, similar lesions were observed in other internal organs such as the liver, intestine, and spleen. Similar clinical signs were also observed in this case with Liu and Li, (2012) including lethargy and reduced feeding activity.

Histopathological examination from the liver, kidney, spleen, and intestine revealed granulomatous nephritis and degeneration of tubular epithelium (Figure 2), with granulomatous hepatitis (Figure 3), splenitis (Figure 4), and enteritis (Figure 5). Additionally, bacteria colonies were also observed encapsulated in the granuloma in the present study (Figure 3). Granulomas enclosing acid-fast rods (AFR) after Ziehl–Neelsen (ZN) staining within various host tissues are a typical characteristic of mycobacterium infection (Novotny *et al.*, 2010). Thus, during necropsy, kidney, and liver were sampled from two fish for acid-fast staining to remove the possibility of *Nocardia* sp. or *Mycobacterium* sp. infection. All samples stained with ZN did not indicate acid-fast organisms thus no further molecular assays were done. *A. schubertii* pathogenic mechanisms in aquatic organisms remain unclear (Cao *et al.*, 2021). However, the granulomatous lesion which is due to a chronic response of the immune system is an attempt to prevent the proliferation of the pathogen, therefore encapsulating them in the granulomas (Rajme-Manzur *et al.*, 2021). The lesion begins with the accumulation of inflammatory cells mainly mononuclear cells which differentiate into macrophages and then

into multinucleated giant cells (Manrique *et al.*, 2017).

Bacteria isolated from the diseased snakeheads were identical in morphology, and histology to *A. schubertii*. The 16S rRNA sequencing result and phylogenetic analysis (Figure 6) further support the diagnosis of *A. schubertii* as the aetiological agent in this case. In the analysis, 10 top BLAST hits were compared to the current sequences with 99.73% nucleotide identity to *A. schubertii* while only 97.55% nucleotide identity was observed for *A. hydrophila*. The full-length 16S rRNA sequence of the bacterial strain has been submitted to NCBI GenBank under the accession number OQ154977. The rapid turnaround time (< 24 hours) from pure culture to DNA sequencing result suggests that MinION/Flongle sequencing platform (Oxford Nanopore, UK) can yield quick, accurate bacterial identification results with a simple low-cost portable device for generating real-time sequence data and has the potential application in remote farm areas (Jerome-Deboutville *et al.*, 2021).

The farm epizootic had lasted approximately three weeks with a severe loss of 70% of fish. No further treatment was done as the owner had decided to cull all fish and dry the tank for several weeks prior to restocking. This is the first clinical case of *A. schubertii* described in *C. striata* in this region. The findings of the present study may serve as a basis for future diagnosis of the disease and present a potential study for One Health epidemiological investigation not only for prevention and mitigation of this disease in fish but also in humans. Farmers are advised to seek veterinary assistance as treatments for *A. schubertii* infection should be done following an antibiotic susceptibility test on the isolates. Commonly used antibiotics in freshwater culture in Malaysia such as amikacin, ampicillin, cefotaxime, amoxicillin, gentamicin, chloramphenicol, tetracycline, and levofloxacin should be tested (Azzam-Sayuti *et al.*, 2021) before administration of treatment. The mentioned study investigated the antibiotic resistance profiles of *Aeromonas* spp. isolated from cultures of freshwater fishes such as striped

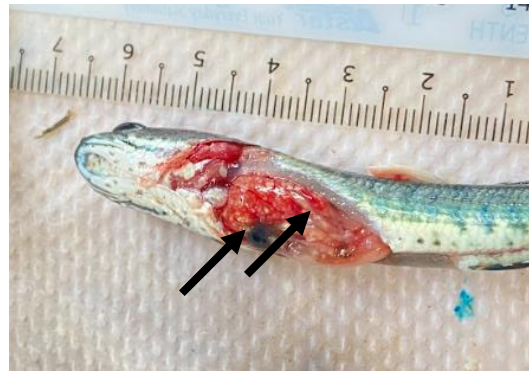


Figure 1. Multiple whitish nodules with size 1–2 mm can be observed in the liver and gastrointestinal organs (→). This lesion was observed in all twelve striped snakehead fingerlings *C. striata* fish sampled.

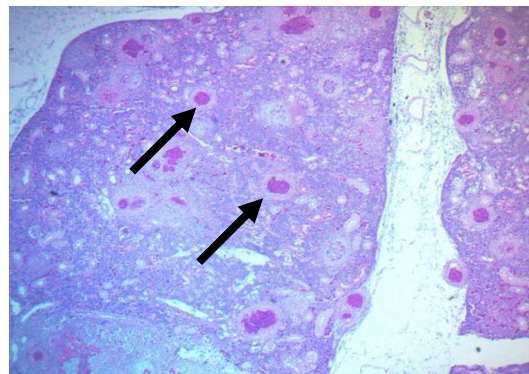


Figure 2. Kidney of *C. striata* showing granulomatous nephritis with diffuse granulation formation (→).

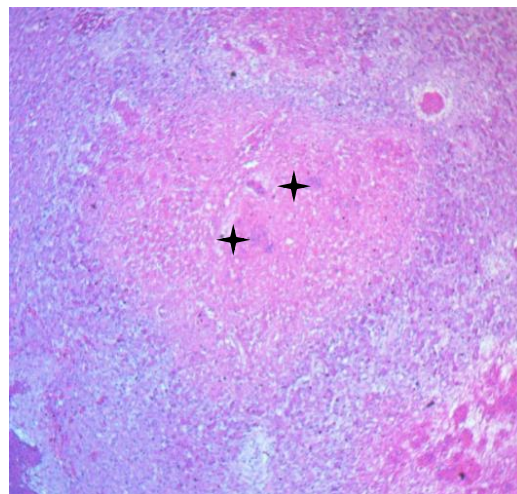


Figure 3. Liver of *C. striata* showing diffuse granuloma formation with basophilic bacterial colony in the granuloma (*).

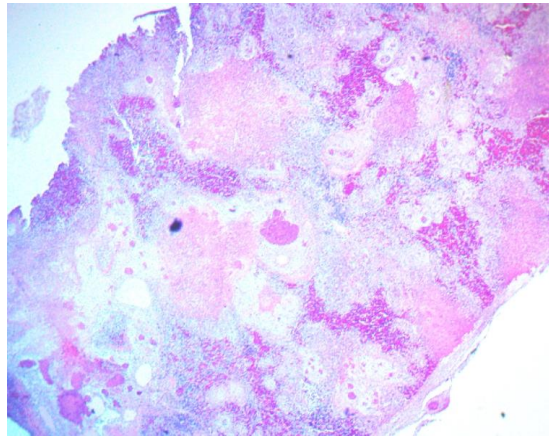


Figure 4. Spleen of *C. striata* showing diffuse granuloma formation.

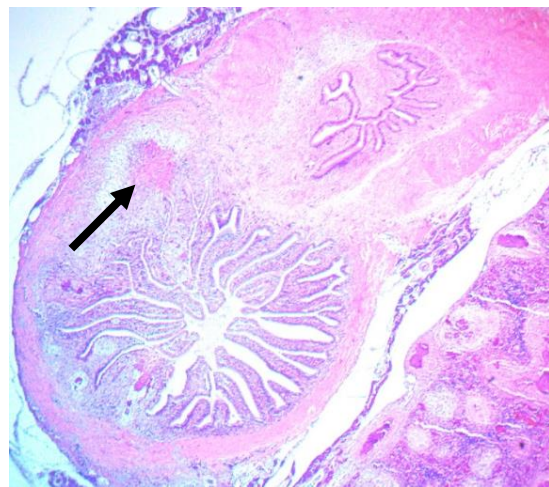


Figure 5. Intestine of *C. striata* showing diffuse granuloma formation (→).

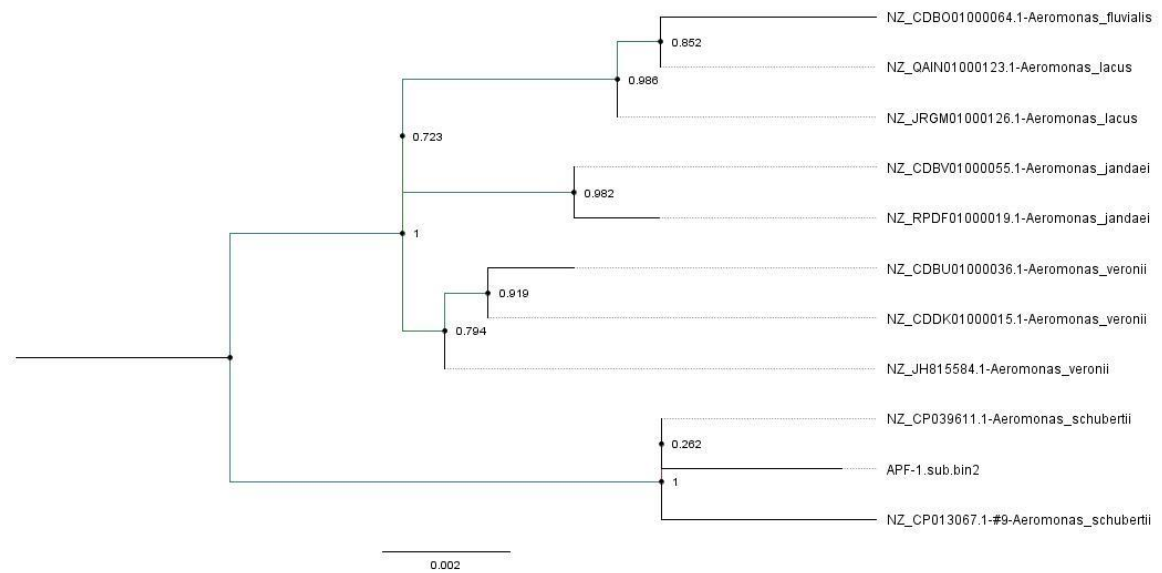


Figure 6. Maximum likelihood trees constructed in figtree based on the nucleotide consensus sequences alignment of *A. schubertii* consensus with 10 reference top blast hit sequences. The branch lengths indicate the number of substitutions per site, and node labels indicate bootstrap support values in percentage. Tree rooted using the mid-point rooting method.

catfish (*Pangasianodon hypophthalmus*) and walking catfish (*Clarias batrachus*) in peninsular Malaysia. Additionally, the study revealed among the *Aeromonas* spp. isolated included *A. dhakensis* (2%) and *A. hydrophila* (20%) with a high percentage of isolates (69%) with multiple antibiotic resistance (MAR). Another recent study on *A. schubertii* on the same species in the Mekong Delta, Vietnam revealed all isolates (n=25) were multidrug-resistant, with resistance detected towards amoxicillin (96%), florfenicol (92%), and ciprofloxacin (92%) (Pham Thi *et al.*, 2023). Similar antimicrobial susceptibility testing studies should also be investigated in the region to see trends of resistance and the best treatment options if possible. The effective treatment can reduce losses and avoid the emergence of potential *Aeromonas*-resistant strains (Anjur *et al.*, 2021). Proper disinfection of tanks and reducing the stress levels in fish by providing adequate space, feed, and maintaining good water quality are vital in reducing risks of diseases in aquaculture settings (Hine *et al.*, 2010; Fikri *et al.*, 2022). Competent authorities (CAs) can play a role by educating farmers on good aquatic animal health practices and judicious use of antibiotics with systematic response and management of suspected outbreaks to minimize the impacts of the disease (Baliao *et al.*, 2020). Additional studies of *A. schubertii* in snakeheads would include comparing the different isolates obtained from different regions in Malaysia for phylogenetic analysis, antimicrobial susceptibility tests, and a pathogenicity challenge experiment from the isolates obtained. Whole genome sequencing should also be explored for key genetic information such as multi-locus sequence type, species identity, virulence factor, and antimicrobial resistance genes.

CONCLUSION

The study reports *A. schubertii* infection in snakeheads through consistent histopathological patterns and molecular analysis, particularly utilizing the MinION/Flongle sequencing platform for rapid identification. Emphasizing responsible antibiotic use and One Health

approaches, the findings underscore the need for proper diagnosis to mitigate *A. schubertii* infections in both fish and humans.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Faculty of Veterinary Medicine, Universiti Putra Malaysia, for supporting this study.

AUTHORS' CONTRIBUTIONS

NDMT: Conceptualization and drafted the manuscript. NDMT, MFM, and HMG: Performed sample evaluation. NDMT, MFM, and HMG: Prepared figures. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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