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Short Communication

Pathogenicity of *Aeromonas hydrophila* in High-value Native Pangasius Catfish, *Pangasius nasutus* (Bleeker)

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ABSTRACT

Pangasius catfish, *Pangasius nasutus*, is a promising candidate for aquaculture due to its high market value. However, the presence of pathogenic bacteria in *Aeromonas hydrophila* is a major concern in *P. nasutus* farming in this country. This study determines the pathogenicity of *A. hydrophila* in *P. nasutus*. A total of 80 *P. nasutus* juveniles were intraperitoneally injected with 0, 10^3, 10^5, and 10^7 CFU mL^{-1} of *A. hydrophila* and monitored until 240 hr. The infected moribund fish's kidneys, livers, and spleens were collected for histopathological analysis. The $LD_{50\text{-}240hr}$ value was found at 0.8×10^4 CFU/ml of *A. hydrophila*. The percentage of mortality in 0, 10^3, 10^5, and 10^7 CFU/ml infected groups were found to be at 0, 40, 60, and 90%, respectively. The infected fish showed congestion at the base of the fin, ascites, enlarged gall bladder, and swollen spleen. It is the earliest report on *A. hydrophila*'s pathogenicity in high-value native fish, *P. nasutus*.

Keywords: *Aeromonas hydrophila*, $LD_{50,}$ pangasius catfish, *Pangasius nasutus*, pathogenicity

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INTRODUCTION

Pangasius catfish, *Pangasius* sp., is one of the most significant fish species, particularly in Asian countries such as Vietnam, Bangladesh, India, Indonesia, Malaysia, Myanmar, Philippines, and Thailand (Sirimanapong et al., 2014). *Pangasius nasutus*, locally recognized as "patin buah" in Malaysia, was regarded as among the highest-valued fish, with an estimated price of RM 70-300/kg (Jamaludin & Ting, 2021; Yusof & Nakajima, 2019), threefold higher compared to the common local black Pangasius catfish, *Pangasius micronemus* (Hashim et al., 2015). There is a significant market demand for this species due to its sweet, savory, and more appealing than the other commercial species. This market preference is evident in Malaysia and regions that could serve as export markets, such as Asia, Europe, and North America (Rafi et al., 2022).

However, like other *Pangasius* sp. in Malaysia, *P. nasutus* is prone to *Aeromonas hydrophila* infections (Mansor et al., 2020). *Aeromonas hydrophila* is a rod-shaped, Gram-negative bacterium that lives in aquatic habitats, infects different fish and significantly harms the aquaculture industry (Jiang et al., 2016; Mazumder et al., 2021). The pathogen was first recognized as the causal agent of hemorrhagic septicemia and has been considered the dominant cause of motile aeromonad septicemia (MAS) (Tartor et al., 2021; Zhang et al., 2019). *Aeromonas hydrophila* causes various pathogenic severities in fish, including hemorrhagic septicemia, abdominal edema, exophthalmia, ulcers, and respiratory infections (Laith & Najiah, 2014; Zhang et al., 2016). In the USA alone, the outbreaks of MAS have cost 60–70 million dollars in loss a year (Bøgwald & Dalmo, 2019). At the same time, significant mortalities due to *A. hydrophila* infection were recorded in the South and South-East Asia farmed fish (Laith & Najiah, 2014). In Malaysia, the *A. hydrophila* outbreak was first recorded in diseased catfishes from a local farm, exhibiting MAS's common clinical and histological symptoms (Anjur et al., 2021).

Aeromonas hydrophila was reported to be commonly infecting *Pangasius* spp. at all life stages (Sarker & Faruk, 2016). Previous studies have been conducted on the infection of *A. hydrophila* in *Pangasianodon hypophthalmus*, *Pangasius bocourti*, and *Pangasius pangasius* (Doan et al., 2013; Hayati & Prihanto, 2020; Le et al., 2018). Their studies found that *A. hydrophila* could infect *Pangasius* sp. severely, with an average mortality of more than 80%. It is crucial to determine the pathogenicity of *A. hydrophila* in *P. nasutus*, as this fish species is currently being promoted in aquaculture. Understanding the disease development will help in future preventive measures. It is the earliest report on the infectivity of *A. hydrophila* in high-value native fish of Malaysia, *P. nasutus*.

MATERIALS AND METHODS

Experimental Fish

One hundred (100) Pangasius catfish, *Pangasius nasutus* (8.95 ± 2.50 g), were purchased and transferred to the Aquatic

Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia from a commercial fish farm in Rawang, Selangor. Fish were maintained in an aerated recirculating water system at a temperature of 26.23 ± 0.59°C, pH at 6.48 ± 0.24, dissolved oxygen at 6.38 ± 0.48 mg/L, and ammonia at 0.01 ± 0.00 mg/L. The fish were acclimatized under laboratory conditions for ten days before the experimental challenges. Twenty (20) fish were randomly selected to check for bacteria and parasitic infection. The fish was found healthy, and no clinical signs were ever observed.

Bacterial Culture and Confirmation

Aeromonas hydrophila strain Ah1sa5 was isolated and obtained from diseased tilapia (*Oreochromis* sp.) on a local farm in Tasik Kenyir Terengganu, Malaysia (Matusin, 2015). The isolate was sub-cultured on tryptic soy agar (Oxoid, United Kingdom) and incubated at 30°C for 16 hr. Pure colonies were inoculated in tryptone soy broth (TSB; Oxoid, United Kingdom) and incubated overnight at 30°C and 150 rpm.

Aeromonas hydrophila used in this study were subjected to polymerase chain reaction (PCR) identification following Azzam-Sayuti, Ina-Salwany, Zamri-saad, Yusof, et al. (2021). The isolate was cultured on tryptone soy agar (TSA, Oxoid, United Kingdom) on plates for 24 hr at 30°C. Afterward, the *A. hydrophila* isolates were grown into 200 ml of TSB broth and cultured for 24 hr with gentle shaking at 30°C.

Genetic DNA from each sample was extracted from a pure bacterial colony using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. The extracted DNA was subjected to PCR amplification using 16S rRNA (F: GGTTACCTTGTTACGACTT and R: AGAGTTTGATCCTGGCTCAG) and DNA gyrase B subunit (*gyr*B) (F: TCCGGCGGTCTGCACGCGGT and R: TTGTCCGGGTTGTACTCGTC) primers to detect the target region of the bacterial strain with a PCR product size of approximately 1,541 and 1,100 bp, respectively. The PCR reactions were made using REDiant 2× PCR Master Mix (FirstBase, Malaysia) in a final volume of 25 µl containing 2× PCR master mix, 1 µM of each primer, and 100 ng of template DNA. Each master mix of 50 µl contains 1× PCR buffer, 200 mmol/L of each dNTP, 2.0 mmol/L magnesium chloride ($MgCl_2$), 1 U *Taq* DNA polymerase, and 50 pmol of every primer. The thermal cycling was carried out on a Thermal cycler (Bio-Rad Laboratories, USA) using the amplification conditions as follows: 1 cycle of 95°C for 5 s (initial denaturation), 33 cycles of 95°C for 1 min, 59°C for 2 min 15 s and 72°C for 1 min 15 s, and then a final extension at 72°C for 10 s. The PCR products were sequenced at Firstbase (Malaysia), and the phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) (version 10.1.8) bioinformatics software with 100 bootstrap trials and the neighbor-joining method.

Lethal Dose of *A. hydrophila* Against *P. nasutus*

Preparation of *A. hydrophila* Cultures. Briefly, 10 colonies of *A. hydrophila* were randomly selected and inoculated into 500 ml of tryptic soy broth (TSB, Oxoid, United Kingdom) and incubated at 30°C and 150 rpm for 16 hr. Ten-fold serial dilutions of the bacterial culture were conducted to determine the concentration of the bacterial stocks, and 100 µl of TSB from every dilution were plated on tryptic soy agar (TSA; Oxoid, United Kingdom) in duplicate. The culture was then incubated at 30°C overnight. Standard plate count was used to calculate the colony forming unit per milliliter (CFU/ml), according to Wohlsen et al. (2006). The bacteria were then harvested through centrifugation and washed thrice with phosphate buffer saline (PBS, pH 7.4). Finally, the bacterial pellets were suspended in PBS (pH 7.4) before being diluted to the desired concentrations.

Challenge Test. The median lethal dose (LD_{50}) of *A. hydrophila* was determined with 80 juvenile *P. nasutus*. The fish were equally disseminated in four tanks in duplicate (10 fish per tank) and not given any feed a day before the challenge trial. Fish were intraperitoneally injected (i.p.) with 0.1 ml of *A. hydrophila* suspension with the respective concentrations: 0 CFUml (control, PBS only), 10^3, 10^5, and 10^7 CFU/ml. The mortality pattern and gross lesion were monitored until 240 h. The $LD_{50–240hr}$ values were calculated as described by Reed and Munch (1938) and were performed using Statistical Product and Service Solutions (SPSS) Statistics software (ver. 26) (SPSS Inc., USA). Bacterial isolation from the challenged moribund fish and 16S rRNA PCR, and nucleotide Basic Local Alignment Search Tool (BLAST) analysis was done afterward to confirm the cause of their death from *A. hydrophila* infection and phylogenetic analyses using the neighbor-Joining method were conducted afterward in MEGA X (Kumar et al., 2018).

Histopathological Analysis

The infected moribund fish underwent histological examination of the kidneys, livers, and spleens to check for any gross lesions in the internal organs. The collected samples were fixed in 10% neutral-buffered formalin for at least 24 hr, dehydrated using a series of increasing concentrations of ethanol (50–100%), cleared in xylene, and embedded in paraffin. The paraffin sections (4 µm thick) were prepared using a microtome (Leica RM 2155, Germany) and stained with hematoxylin and eosin (H & E) dyes before being inspected microscopically (Rahman et al., 2022).

RESULTS

Bacterial Culture and Confirmation

As shown in Figure 1, the phylogenetic tree does not delineate the reference strains into separate groups. The bacteria isolated from the infected fish were distantly related to the *A. hydrophila* reference strain, MG984625.1 ATCC (Figure 1A) and AY987520.1 (Figure 1B). Amplification products for 16S rRNA

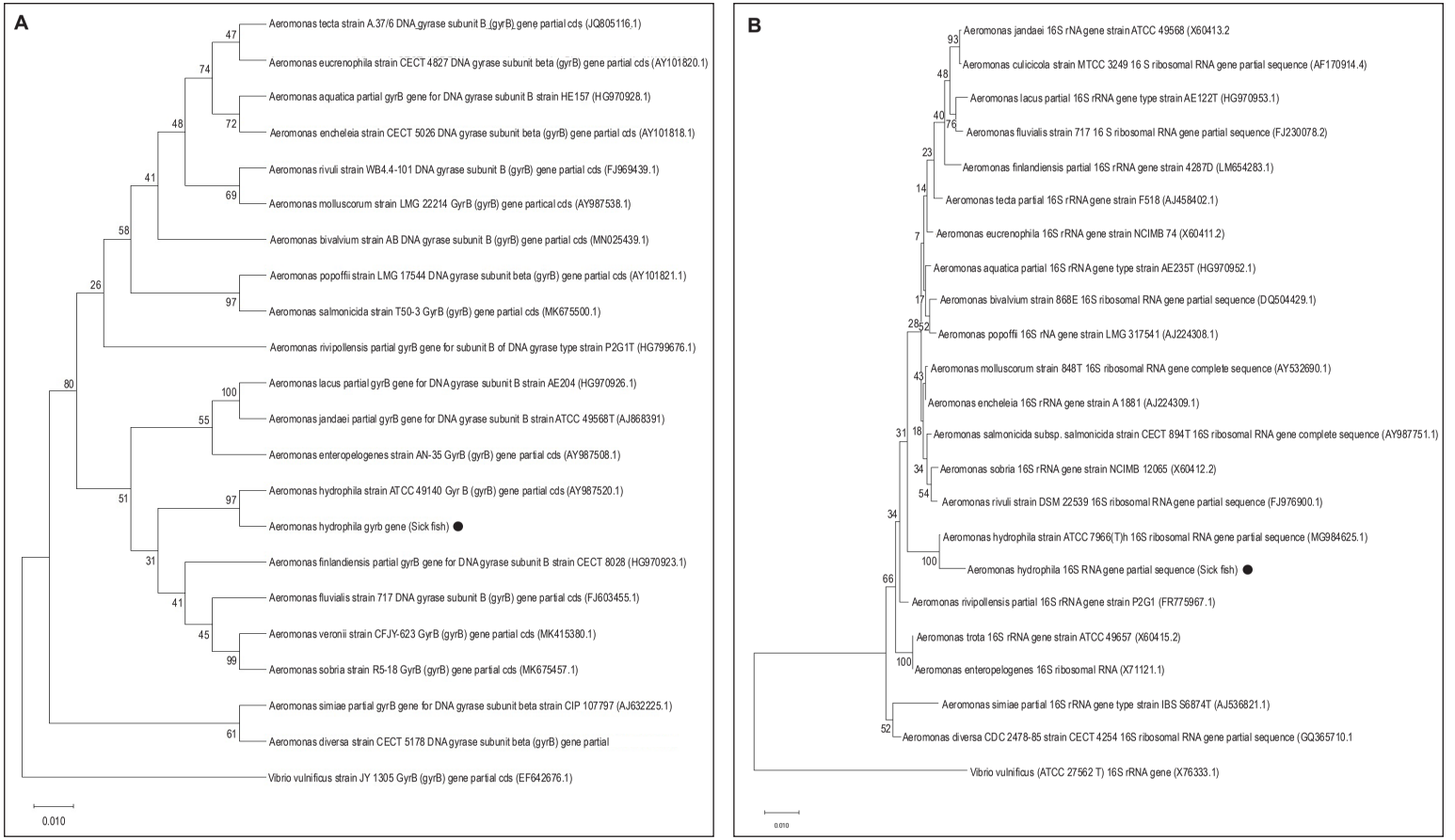

Figure 1. Phylogenetic analysis of *Aeromonas hydrophila* based on 16S rRNA (A) and *gyr*B (B) genes inferred using the neighbor-joining method with other *Aeromonas* sp., as a reference and one non-*Aeromonas* sp., *Vibrio vulnificus*, as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The bacterial strain used in this study was marked with "●"

and *gyr*B genes were sequenced using the BLAST Nucleotide algorithm, and the similar sequences from the GenBank non-redundant (NR) data source were determined as *A. hydrophila* with 98% (Accession no.: JN711800.1) and 99% (Accession no.: MK937644.1) similarities, respectively.

Mortality Pattern and $LD_{50\text{-}240h}$ of *A. hydrophila* Against *P. nasutus*

After 24 hr post-challenge (hpc), mortality of *P. nasutus* was seen in concentration 10^7 CFU/ml and at 48 hpc for 10^3, 10^5, and 10^7 CFU/ml. After 24 hpc, 40% mortality was recorded in the 10^5 CFU/ml group, while at 72 hpc, the mortalities were the highest for concentrations of 10^3 and 10^5 CFU/ml at 20 and 30%, respectively. The mortalities lasted for 96 hpc for concentrations of 10^3, 10^5, and 10^7 CFU/ml, and 120 hpc for group 10^7 CFU/ml. By 240 hpc, the mortality rate was observed as 40, 60, and 90% for 10^3, 10^5, and 10^7 CFU/ml, respectively (Figure 2). No mortality was observed in the control samples throughout the experiment. Probit analysis revealed that the lethal dose ($LD_{50\text{-}240hr}$) of *A. hydrophila* for *P. nasutus* was observed at 0.8×10^4 CFU/ml. Nevertheless, the re-isolated bacteria from the experimentally infected *P. nasutus* was confirmed phenotypically and molecularly as *A. hydrophila*.

Pathological Symptoms of *P. nasutus* Post-infection with *A. hydrophila*

After intraperitoneal injection of *A. hydrophila*, all infected fish showed varying clinical signs and gross lesions typical of *Aeromonas* infection. They include reduced feed intake, isolation, irregular breathing, and swimming near the surface, which was not observed in the control group. Gross lesions include hemorrhagic foci around

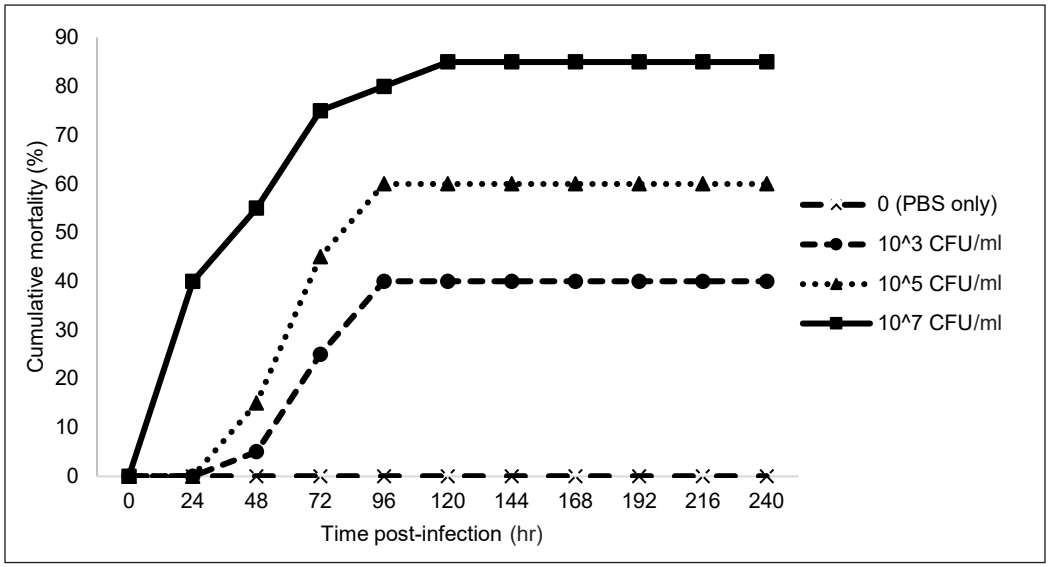

Figure 2. Cumulative mortality of *Pangasius nasutus* infected with different concentrations of *Aeromonas hydrophila*

the anal region and under the pectoral fin (Figure 3). The post-mortem examination showed swollen gall bladder, bloody-colored fluid in the abdominal cavity, and an enlarged spleen. These were not observed in control fish.

Histopathological Analysis

Histopathological lesions following *A. hydrophila* infection in the kidneys of *P. nasutus* showed varying degrees of necrosis and degeneration in the tubular epithelium and interstitial nephritis with inflammatory cell infiltration (Figure 4). Desquamated tubular epithelial cells with cell lysis and expansion in the glomerular cavity were also observed in infected kidneys. The infected livers showed multifocal congestion, dilatation of the sinusoids with vacuolar degeneration, and hepatocellular necrosis. The infected spleen showed subcapsular necrosis, multifocal necrosis, splenic hemorrhage with numerous red blood cells, and aggregation of marked melano-macrophage centers (MMC) with hemosiderin deposition. The spleens of the control fish showed normal architecture with the absence of MMCs. Indistinct pathological changes were observed after injection with PBS in the control group's kidney, liver, and spleen.

DISCUSSION

Pangasius nasutus was recently introduced as a new candidate for aquaculture (Tahapari et al., 2020). The fish is highly consumed in Malaysia, particularly in Pahang, where it is popularly cooked in a special local delicacy. The supply of *P. nasutus* primarily comes from the wild stock (Jaapar et al., 2021). The challenge experiment revealed typical *A. hydrophila* infection signs and gross lesions

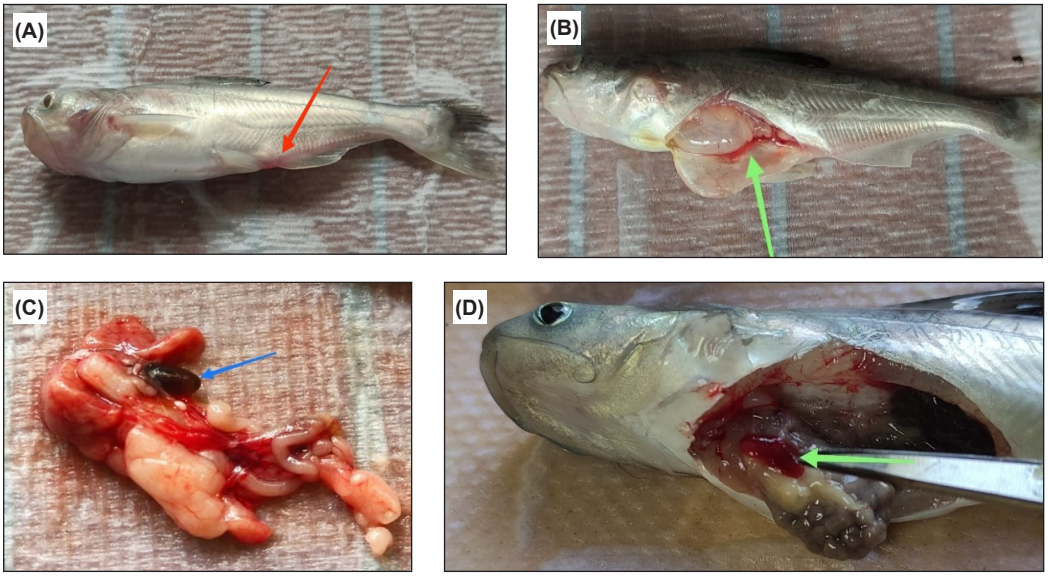

Figure 3. Gross lesions were found on the *Pangasius nasutus* infected with *Aeromonas hydrophila*. (A) Congestion at the base of the fin (orange arrow); (B) Fluid accumulation in the abdominal cavity (green arrow); (C) Enlarged gall bladder (blue arrow); (D) Swollen spleen (green arrow) in challenged *P. nasutus*

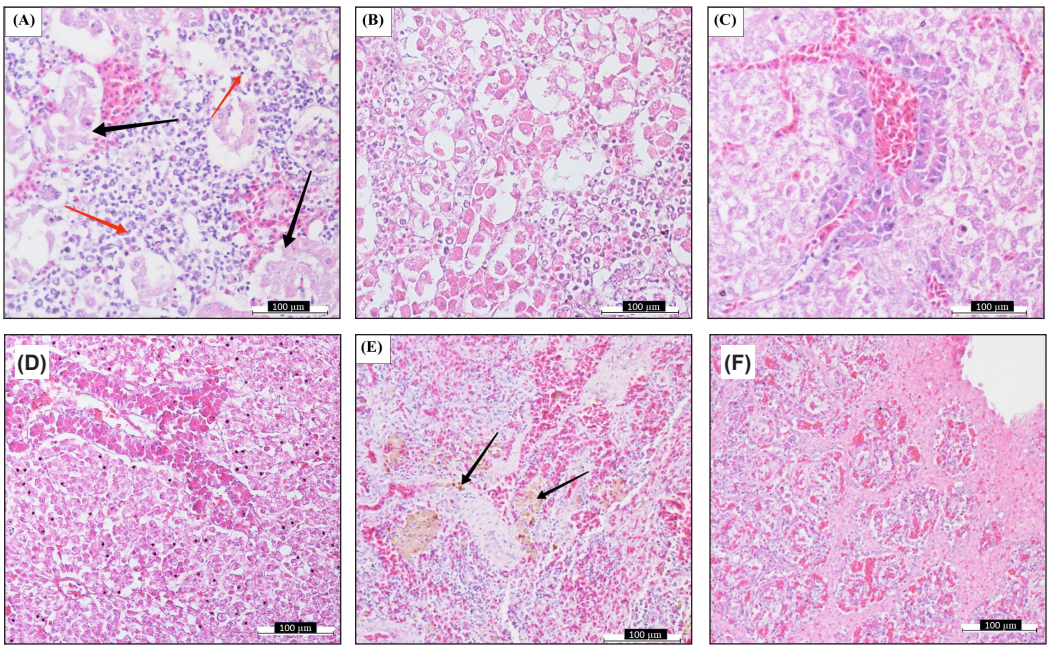

Figure 4. Histological detections of infected kidney, liver, and spleen and control *Pangasius nasutus* following *Aeromonas hydrophila* challenge. (A) Kidneys of infected fish showed necrosis with interstitial nephritis, especially with the tubular epithelial cells (black arrow) with inflammatory cell infiltration (red arrow) (400×); (B) Normal architecture with intact, healthy tubular epithelia in the kidneys of control fish (400×); (C) Infected liver showing dilatation of the sinusoids with vacuolar degeneration and necrosis (200×); (D) Liver of the control fish showed normal liver architecture and hepatocytes (200×); (E) Infected spleen showed subcapsular necrosis, splenic hemorrhage, and aggregation of hemosiderin (black arrows) (100×); (F) The spleen of the control fish showed normal splenic architecture (100×)

as reported in Asian catfish (*P. bocourti*) and snakehead (*Channa striata*) (Doan et al., 2013; Samayanpaulraj et al., 2019). With increasing bacterial concentration, the mortality rate increased due to more toxins being released, potentially damaging the fish's internal organs (Dong et al., 2017). Early mortality in the infected fish was observed at 24 hpc in 10^7 CFU/ml treatments (40%), while in 10^3 (5%) and 10^5 CFU/ml (15%) treatments, mortality started after 48 hpc, demonstrating the pathogenicity of the bacteria to cause acute-to-chronic illness in *P. nasutus*. While after 72 hpc, 25, 45, and 75% cumulative mortalities were recorded in 10^3, 10^5, and 10^7 CFU/ml treatments, respectively. The $LD_{50\text{-}240hr}$ of *A. hydrophila* strain Ah1sa5 for *P. nasutus* was found at 0.8×10^4 CFU/ml. In this study, the LD_{50} of *A. hydrophila* was lower than in Basa, *P. bocourti* (Doan et al., 2013), and in striped catfish, *P. hypophthalmus* (Sirimanapong et al., 2014), which both reported 10^8 and 10^5 CFU/ml, respectively. The differences in the LD_{50} value may cause by the *A. hydrophila* strains used in each study and the challenged host. *P. nasutus* was reported to have a higher sensitivity to environmental stressors and was difficult to be farmed (Zulkiflee et al., 2020).

The clinical manifestations seen in this study after the *A. hydrophila* infection in *P. nasutus* were mainly behavioral pattern changes and respiratory difficulties. The same clinical signs have been described in the intradermal or intraperitoneal infection of *A. hydrophila* and other fish species (Azzam-Sayuti, Ina-Salwany, Zamri-Saad, Annas et al., 2021; de Oliveira et al., 2011; Dias et al., 2016). It is believed that the effect of acetylcholinesterase secreted by *A. hydrophila* may lead to these signs due to its narcotic effects on the central nervous system (Dias et al., 2016). However, other reported macroscopic lesions, such as hemorrhages at the site of infection, scale loss, and dermal necrosis (Azzam-Sayuti, Ina-Salwany, Zamri-Saad, Annas et al., 2021; Dias et al., 2016), were not observed in *P. nasutus*. After *A. hydrophila* infection, the histopathological variations observed in the internal organs of *P. nasutus* were similar and common to the lesions found in other infected fish species (Abdelhamed et al., 2017; Chen et al., 2018; Saharia et al., 2018). However, polymorphonucleated cell infiltration and glomerular cell proliferation have also been reported in *A. hydrophila* infections in fish (Chen et al., 2018; Saharia et al., 2018).

CONCLUSION

It is the first report on the pathogenicity of *A. hydrophila* in high-value native fish of Malaysia, *P. nasutus*. This study indicates that *P. nasutus* is susceptible to *A. hydrophila* infection and could be a potential disease threat to cultured and wild *P. nasutus*. Further studies need to be conducted on the severity of *A. hydrophila* infection in *P. nasutus* vital organs either via histopathological changes scoring or at the molecular level. Besides, the possible transmission of *A. hydrophila* infection to the fish should be considered. This valuable information could result in significant disease preventive measures such as biosecurity and vaccine development programs.

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