

Challenge Experiment Endorses *Vibrio alginolyticus* as the Causative Agent in Three-week-old *Hippocampus kuda* Larvae

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Disease outbreaks caused by opportunistic marine bacterial pathogens represent a major constraint to the sustainability and productivity of seahorse aquaculture. Therefore, an immersion challenge was conducted to confirm the pathogenic role of *Vibrio alginolyticus* associated with a high juvenile mortality outbreak in *Hippocampus kuda* reported from a seahorse hatchery in Negombo, Sri Lanka. Healthy three-week-old larvae were obtained from the hatchery and adapted under controlled laboratory conditions that simulated hatchery parameters, including 28‰ salinity (280 PSU), 28 °C, and continuous gentle aeration. Larvae were maintained in tanks (n = 5 per tank, 2 L volume) and fed *Artemia* thrice daily for a duration of 7 days. Before the challenge experiment, the bacterium was confirmed as *V. alginolyticus* based on its characteristic biochemical profile, including Gram-negative, motile rods, positive oxidase and catalase activity, fermentative metabolism in the oxidative-fermentative test, growth in 6% sodium chloride, citrate utilization, nitrate reduction, and positive reactions for gelatin, casein, and starch hydrolysis, along with decarboxylase activities, which are consistent with standard identification keys for the species. An immersion challenge was performed by exposing larvae to a bacterial suspension of *V. alginolyticus* at a concentration of 1×10^6 CFU mL⁻¹, while a control group was maintained under identical conditions without bacterial exposure. Survival was monitored daily over 7 days. Mortality in the challenged group commenced on day 3 and increased progressively, reaching a significantly higher cumulative mortality compared to the control group, which exhibited only background mortality. Infected larvae displayed clinical signs including lethargy, reduced feeding, and abnormal swimming behaviour prior to death. The bacterium was successfully re-isolated from dead larvae using Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar, thereby fulfilling Koch's postulates and confirming its pathogenicity. The findings demonstrate that *V. alginolyticus* is capable of inducing disease and significant mortality in juvenile *H. kuda* under immersion challenge conditions, supporting its role as the causative agent of the observed outbreak and emphasizing its significance in seahorse aquaculture.

Keywords: *Hippocampus kuda*, Fish diseases, Larval mortality, *Vibrio alginolyticus*