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Variations of immune parameters in the lined seahorse *Hippocampus erectus* after infection with enteritis pathogen of *Vibrio parahaemolyticus*

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ABSTRACT

Enteritis has been increasingly recognized as one of the major obstacles for the lined seahorse *Hippocampus erectus* mass culture success. In the present study, the intestinal bacteria strains of the lined seahorses *H. erectus* suffered from enteritis were isolated, then their pathogenicities were confirmed by artificial infection, and one pathogenic bacteria strain named DS3 was obtained. The median lethal dose (LD_{50}) of strain DS3 for 10 days was determined. The seahorses with different infection levels of uninfected (control), early stage of infection (ESI) and late stage of infection (LSI) were respectively sampled at 0, 3, 6 and 9 days post infection, and 12 immune parameters in the plasma were analyzed. The strain DS3 identified with a biochemical test combined with a molecular method was *Vibrio parahaemolyticus*, and its LD_{50} for 10 days was 1.3×10^3 cfu/fish. Six parameters including monocytes/leucocytes, leucocytes phagocytic rate, interleukin-2, interferon-α, lysozyme and immunoglobulin M exhibited a generally similar variation trend: highest in the control, second in the ESI and lowest in the LSI throughout the entire experiment. In view of the infection level of *V. parahaemolyticus* to *H. erectus* is largely decided by the seahorse's own immune capacity, therefore, these immune parameters were high in the non- or slightly infected seahorses, and low in the severely infected individuals may be an indicator for immune level. These immune parameters may be reliable indicators for the juvenile and broodstock quality assessment. Moreover, clarification of the enteritis pathogen also provides guidances for targeted medicine choice for the lined seahorse.

1. Introduction

Seahorses, *Hippocampus* spp., are highly specialized marine fishes. Their unique body morphology with horse shaped head and curvaceous trunk, unusual life history traits including male pregnancy and strict monogamy in the most species, and high traditional Chinese medicine value have made them charismatic icons to biologists, aquarium hobbyists and medicinal workers [1–3]. In the past ten years, wild seahorse population has been heavily reduced due to over-fishing and habitat destruction. From 2004, all seahorse species have been listed on the IUCN Red List and the Appendix II of CITES. Aquaculture of seahorses has been proposed as one solution to balance the wild population conservation and market demand. Seahorse aquaculture started in 1958 and has expanded greatly since 2000 due to the improved breeding and rearing protocols [3–6].

Although seahorse aquaculture has been greatly progressed, there are a few challenges affecting commercial seahorse culture, one of which is serious disease [7]. Common seahorse diseases include body surface ulcer, “hair” and “gas bubble”, swim bladder inflation, liver haemorrhage and gastrointestinal inflammation. These diseases may result from unqualified rearing environments [8], inferior diets [9] and pathogen infections including marine leeches, ciliates [10], microsporidians [11], fungi [12] and bacteria [13,14]. To date, except bacteriosis which can be treated to a great extent with antibiotics, the remaining diseases still have no effective measures to control. Therefore, provisions of an optimal rearing environment, a high-quality diet and a periodical quarantine are increasingly considered as fundamental and effective measures to prevent seahorse health problems [3].

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The lined seahorse *Hippocampus erectus*, an ideal species for commercial culture, has been reared in captivity successfully [8,15–17]. Unofficial source indicates that the annual cultured dried *H. erectus* has been more than 2.0 t in China since 2014, and mostly used for traditional Chinese medicine. However, captive-rearing *H. erectus* has suffered from diseases as well and the particular concern is enteritis. Like the enteritis in other seahorse species, such as *Hippocampus japonicus* [14], *H. erectus* enteritis also occurs primarily in the juveniles with the body height of 4–6 cm. In practice, seriously diseased seahorses with weak swimming capabilities, seldomly hold the holdfasts day and night, and their anal openings are obviously white. Anatomic symptoms of the diseased seahorse include liver haemorrhage, intestinal tract translucence, ascitic fluid hoarding and hindgut erosion. The mortality is quiet high (more than 80%), and the seahorses die in 3–5 days after the clinical symptoms appeared.

In the present study, the pathogenic bacteria causing enteritis in *H. erectus* was isolated and identified, thereby to provide guidances for targeted medicine choice. Thereafter, seahorses *H. erectus* were artificially infected with the pathogenic bacteria, and the variations of immune parameters post infection were analyzed, with the aim of screening out several immune indicators, thereby to provide reliable indicators for the juvenile and broodstock quality assessment.

2. Materials and methods

2.1. Experimental seahorses

Different batches of the healthy cultured juvenile *H. erectus* (body height: 5.63 ± 0.34 cm, wet body weigh: 0.74 ± 0.12 g) were collected from Qionghai Research Center of East China Sea Fisheries Research Institute, Hainan, China. The seahorses were acclimatized in the large tanks (120 × 60 × 30 cm), each with 150 individuals. All tanks plumbed to a central filtration system featuring mechanical, biological filtration, and ultraviolet sterilizer. The cultured conditions were salinity of 32.0 ± 1.0‰, temperature of 27 ± 0.5 °C, light intensity of 2000 ± 300 lx, and a photoperiod of 14 h L:10 h D, respectively. Plastic plants were provided for holdfasts. The seahorses were fed twice a day (08:00 and 15:00) with the sterile copepods, and the feces in the tanks were siphoned out 3 h after each feeding.

2.2. Bacterial isolation

The moribund seahorses *H. erectus* with typical clinical symptoms were sampled from an enteritis epidemic area in Dongshan, Fujian, China. After shipping to Qionghai Research Center, the diseased seahorses were rinsed with sterile 0.01 M phosphate buffered saline (PBS) at pH 7.2, then were dissected in a clean bench. The intestinal tracts were collected and cut open, and the inclusions were inoculated in a TCBS agar medium. Single bacterial colonies were isolated from visible colonies after 24 h of incubation at 28 °C, and were transferred to fresh medium. Clear colonies were picked up and transferred again, until pure colonies were finally obtained. In this way, 3 strains named DS1, DS2 and DS3 were isolated.

2.3. Pathogenicity confirmation

Three isolated strains were diluted into three concentration gradients of 10^7, 10^5 and 10^3 cfu/mL with sterile PBS, respectively. Each strain and each gradient was intraperitoneally injected into 20 healthy seahorses, and each seahorse with 20 μl of bacterial dilution. Injected seahorses were cultured in the small tanks (50 × 30 × 30 cm) with the same cultured conditions as for the acclimatized seahorses, and their incidence and mortality were monitored and recorded daily for 10 days. As a result, strain DS3 with a high mortality in gradients of 10^7 and 10^5 cfu/mL were observed, besides, the moribund and dead individuals in DS3 treatments also presented highly similar symptoms of the enteritis (Fig. 1). As for the remaining two strains, no clinical signs of enteritis and no death occurred. The bacterial strain in the moribund individuals in DS3 treatment was also isolated.

2.4. Pathogenic bacteria identification

Strain DS3 isolated from the epidemic area of Dongshan together with the bacterial strain isolated from artificially infected seahorses in pathogenicity confirmation experiment was identified by gram staining, NaCl tolerance test, API 20E strip (BioMeÂrieux, S.A. France), and 16S rRNA gene sequence analysis, respectively. The detailed 16S rRNA analysis procedure was referenced the previous report in *H. japonicus* [14].

2.5. Median lethal dose determination

Strain DS3 was diluted into five concentrations (5×10^6, 5×10^5, 5×10^4, 5×10^3 and 5×10^2 cfu/mL) with sterile PBS. The healthy seahorses were divided into six groups, one group injected with sterile PBS as the control and the other five groups were injected with different bacterial dilutions, respectively. Each group had 20 seahorses and each seahorse injected with 20 μl of bacterial dilution or PBS. Injected seahorses were cultured in the small tanks (50 × 30 × 30 cm) with the same cultured conditions as for the acclimatized seahorses, and their incidence and mortality were monitored and recorded daily for 10 days. The median lethal dose (LD_{50}) was calculated by the modified Karber's method [18].

2.6. Variation analysis of immune parameters after bacterial infection

2.6.1. Bacterial infection

The healthy seahorses were divided into two groups: the control group injected with sterile PBS and the bacterial group injected with LD_{50} of strain DS3. Each group had 5 replicates and each replicate with 40 seahorses. Injected seahorses were cultured in the medium tanks (60 × 60 × 30 cm) with the same cultured conditions as for the acclimatized seahorses for 10 days. Dead individuals were taken out immediately as soon as possible. The injected seahorses were sampled at 0, 3, 6 and 9 days post injection at night when the seahorses usually stopped swimming and held the holdfasts. For the control group, 8 seahorses were sampled at each sampling time. While for the bacterial group, 4 seahorses held the holdfasts (indicating they were slightly infected and classified as early stage of infection (ESI)) and 4 seahorses swam weakly and unable to hold the holdfasts (indicating they were severely infected and classified as late stage of infection (LSI)) were collected at each sampling time.

2.6.2. Blood sampling and processing

The sampled seahorse was placed into a bucket containing a solution of 0.035% MS-222 (Sigma-Aldrich, Castle Hill, NSW, Australia) in seawater and anaesthetized for 2 min, then 1/3 of the tail was cut. The remaining tail of amputated seahorse was immediately inserted into a 1.5-mL sterile centrifuge tube containing 0.4 mL of anticoagulant (citric acid 0.48 g, sodium citrate 1.32 g, glucose 1.47 g, and distilled water 100 mL) and dipped into the anticoagulant. Blood was spontaneously collected from the caudal artery and mixed with anticoagulant, after about 2 min, the