Isolation and experimental infection with *Vibrio alginolyticus* in the sea horse, *Hippocampus reidi* Ginsburg, 1933 (Osteichthyes: Syngnathidae) in Brazil

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(With 2 figures)

Abstract

The aim of this study was to evaluate the pathogenicity of *Vibrio alginolyticus* isolated from an outbreak of sea horse *Hippocampus reidi* reared in the State of Santa Catarina, Brazil, by experimental infection. Sea horses with necrosis on the mouth epithelium were collected from aquaria at the Aquaculture Department, UFSC and the bacterium isolated from the mouth, liver, heart and blood in tiosulphate citrate bilesalt sucrose agar broth. The strains were identified by API 20E kit with 99.1% probability as *Vibrio alginolyticus*. Twelve adult sea horses (9.63 ± 2.42 g and 15.12 ± 0.87 cm) were distributed in six aquaria of 10 L capacity with aerated sea water. Fish from three aquaria were submitted to an immersion bath in a solution containing 1.0×10^7 CFU of *V. alginolyticus*/mL for 15 minutes. Fish from the other three aquaria received the same procedure without bacteria. Twenty four hours after this challenge, 100% mortality was observed in the animals infected with *V. alginolyticus*. No mortality was observed in non-infected fish. Hyperplasia, displacement and fusion of secondary lamellae of the gills; leukocyte infiltration and necrotic foci in the kidney; hyperplasia, sinusoidal deformation and necrotic foci in the liver were observed in histopathological analysis. The *V. alginolyticus* isolated in this study was pathogenic to *H. reidi* and constitutes an important sanitary problem to its culture.

Keywords: experimental infection, vibriosis, histopathology.

Isolamento e infecção experimental de *Vibrio alginolyticus* em cavalo-marinho, *Hippocampus reidi* Ginsburg, 1933 (Osteichthyes: Syngnathidae) no Brasil

Resumo

Foi avaliado por meio de infecção experimental a patogenicidade de *Vibrio alginolyticus* isolado de um surto de enfermidade em cavalo-marinho *Hippocampus reidi* cultivado no Estado de Santa Catarina, Brasil. Os peixes com necroses no epitélio bucal foram coletados em aquários do Departamento de Aquicultura, UFSC e as bactérias isoladas da boca, fígado, coração e sangue em meio Agar tiossulfato citrato bile sacarose. Os isolados foram identificados pelo kit API 20E como *Vibrio alginolyticus* com 99,1% de probabilidade. Doze peixes adultos (9,63 ± 2,42 g e 15,12 ± 0,87 cm) foram distribuídos em seis aquários de 10 L com água marinha e aeração. Peixes de três aquários foram submetidos a um banho de imersão por 15 minutos em uma solução contendo $1,0 \times 10^7$ UFC de *V. alginolyticus*/mL. Nos outros três aquários realizou-se o mesmo procedimento sem a bactéria. Vinte e quatro horas após o desafio, 100% de mortalidade foi observada nos animais infectados com *V. alginolyticus*. Não houve mortalidade nos peixes não infectados. Nas análises histopatológicas, foi observado hiperplasia, deslocamento do epitélio e fusão das lamelas secundárias das brânquias; infiltração de leucócitos e necrose no rim; hiperplasia, deformação sinusoidal e necrose no fígado nos animais desafiados com *V. alginolyticus*. O *V. alginolyticus* isolado neste estudo foi patogênico para *H. reidi*, constituindo-se de um importante problema para seu cultivo.

Palavras-chave: infecção experimental, vibriose, histopatologia.

1. Introduction

The sea horse *Hippocampus reidi* is native to the Brazilian littoral (Dias and Rosa, 2003), and found fixed on mangrove vegetation, algae, Coelenterata and Bryozoa (Rosa et al., 2007). They can be found near the surface (Rosa et al., 2002) up to 55 m depth (Lourie et al., 1999), distributed along the Atlantic coast from Rio de Janeiro to the Gulf of Mexico (Rosa et al., 2002). The Brazilian Environmental Ministry (Normative instruction 05/2004) considers the species to be under threat. Due to lack of information on their biology in nature, they are classified as "Data Deficient" on the Red List of Threathened Species (IUNC, 2008).

Sea horses are appreciated in the ornamental industry (Giwojna, 2002), popular medicine and are souvenir objects (Foster and Vincent, 2004). Every year, thousands of sea horses are exported from Latin America to the United States and Europe (Baum and Vincent, 2005). According to Monteiro-Neto et al. (2003) and Rosa et al. (2005, 2006), Brazil is one of the most important exporters of *H. reidi*.

The majority of commercialised sea horses in Latin America are from capture (Baum and Vincent, 2005), which, allied to pollution and human interference in the ecosystem (Hodgson, 1999), cause reduction in the natural stocks of this fish (Baum and Vincent, 2005).

The reproduction of *H. reidi* in captivity is an alternative for international trade to avoid uncontrolled capture (Felício et al., 2006). On the other hand, their reproduction in captivity is difficult (Giwojna, 2002) and bacterial infections are the main obstacle to produce this fish species (Alcaide et al., 2001).

Among bacterial diseases in marine fishes, *Vibrio* ssp. is one of the most important causes of economic losses. This bacterium is normally found in the marine environment and the disease outbreaks occur when fish are exposed to infectious agents in the presence of stress factors (Austin and Austin, 2007). Septicemia induced by vibriosis is characterised by haemorrhages on the fin base, exoftalmia, loss of appetite and edematous lesions on the body surface (Toranzo et al., 2005). The studies on sea horse pathology are scarce and they have recently been affected by vibriosis as reported by Alcaide et al. (2001) with mortalities of more than 90%. These authors reported the main clinical signs as external haemorrhages, loss of skin colour, depressed abdomen, haemorrhagic liver, pale kidney and ascitic fluid in the intestines. In Brazil, nothing is known on sea horse pathology especially in experimental infection conditions.

This assay evaluated by experimental infection the pathogenicity of *Vibrio alginolyticus* to *H. reidi* isolated from an outbreak in sea horses reared in the State of Santa Catarina, south Brazil.

2. Material and Methods

Bacterial strains were isolated from an outbreak of sea horses reared at the Aquaculture Department, Federal University of Santa Catarina (UFSC) and the experimental infection occurred at the Research Laboratory of Aquatic Organisms Health, UFSC. Samples were collected from mouth lesions, blood, heart and liver for inoculation onto tiosulphate citrate bilesalt sucrose agar (TCBS, Difco™), selective to vibrionaceae. Plates were incubated for 24 hours at 30 °C and the colonies were isolated on tryptone soy agar (TSA) to obtain a pure culture for identification with the API 20E (Biomeriux®) kit.

Twelve adult sea horses (9.63 ± 2.42 g weight and 15.12 ± 0.87 cm length) were distributed in six aquaria with 10 L marine water in a completely randomised design. Lines of 2 cm diameter were fixed to the bottom of the aquaria to support the fish. Water temperature was maintained at 26 ± 1 °C with constant aeration. Fish from three aquaria were submitted to an immersion bath in a solution containing 1.0×10^7 colony forming units (CFU) of *V. alginolyticus*/mL for 15 minutes. Fish from the other three aquaria were submitted to the same procedure without bacteria.

Twenty four hours after challenged, the survival rate was noted and the surviving fishes (infected and non-

Table 1. Characteristics of Vibrio alginolyticus isolated from sea horse, *Hippocampus reidi* in the State of Santa Catarina, South Brazil. (+) positive reaction, (–) negative reaction.

Test	Reaction	Test	Reaction
Fermentation/oxidation		Lysine decarboxylase	+
Glucose	+	Ornithine decarboxylase	+
Mannitol	+	Citrate utilization	+
Sucrose	+	Tryptophane deaminase	+
Amygdalin	+	Indole production	+
Inositol	–	Gelatinase	+
Sorbitol	–	Oxidase	+
Rhammose	–	β-galactosidase	–
Melibiose	–	H_2S production	–
Arabinose	–	Urease	–
Acetoin production (Voges Proskauer)	–	Nitrate/Nitrite reduction	–

infected) were anesthetised in a benzocaine solution (50 mg.L^{-1}), sacrificed and immediately fixed for histology by placing the organs in 10% buffered formalin. All samples were embedded in paraffin wax and processed using standard histological procedures and the 5 µm sections (microtome Olympus CUT 4055) stained with haematoxylin and eosin (H and E).

3. Results and Discussion

Four bacterial strains were isolated from the outbreak of sea horses (lesions on the mouth, liver, heart and blood). All those isolated were identified as *Vibrio alginolyticus* with 99.1% of probability by the API test (Table 1).

Bacteria from the genus *Vibrio* are frequently isolated from outbreaks in marine fish such as *V. alginolyticus* in the gilthead seabream *Sparus aurata* (Akaayli et al., 2008), *V. harveyi* in seabass *Lates calcarifer* (Tendencia, 2002) and in the summer flounder *Paralichthys dentatus* (Gauger et al., 2006), *V. pelagius* in turbot *Scophthalmus maximus* (Villamil et al., 2003), *V. splendidus* and *V. scophthalmi* in common dentex *Dentex dentex* (Sitjà-Bobadilla et al., 2007). In sea horses, Alcaide et al. (2001) reported *V. harveyi* from an outbreak in *Hippocampus kuda*.

Twenty four hours after the challenge, 100% mortality was found in seahorses that proved the pathogenicity of the isolated strains. In this assay, fish presented necrosis on the mouth epithelium similar to the animals in which the strains were originally isolated. On the other hand, in uninfected fish, neither mortality nor bacterial lesions were found corroborating the results of Alcaide et al. (2001) after infection with *V. harveyi*.

Histopathological analyses of uninfected fish showed normal tissues (Figures 1c,d). After infection, the gills were characterised by loss of cellular integrity (Figure 1e) such as hyperplastic epithelial cells (Figure 1f), displacement (Figure 1g) and fusion predominantly affected the secondary lamellae (Figure 1h). Similar gill changes were found in Atlantic salmon *Salmo salar* experimentally infected with *V. anguillarum* (Morrison et al., 2001) and turbot (Villamil et al., 2003). Mucous cell proliferation is a host response to altered environmental conditions, exposure to toxicants (Mallat, 1985), parasites (Azevedo et al., 2006) and bacterial infections (Villamil et al., 2003). These gill alterations might harm the ionic changes in fish (Morrison et al., 2001) and constitute a portal of entry to other diseases (Moraes and Martins, 2004).

Normal histological structure in uninfected sea horses was observed (Figure 2a,b). On the contrary, in infected fish, the kidney was characterised by necrotic

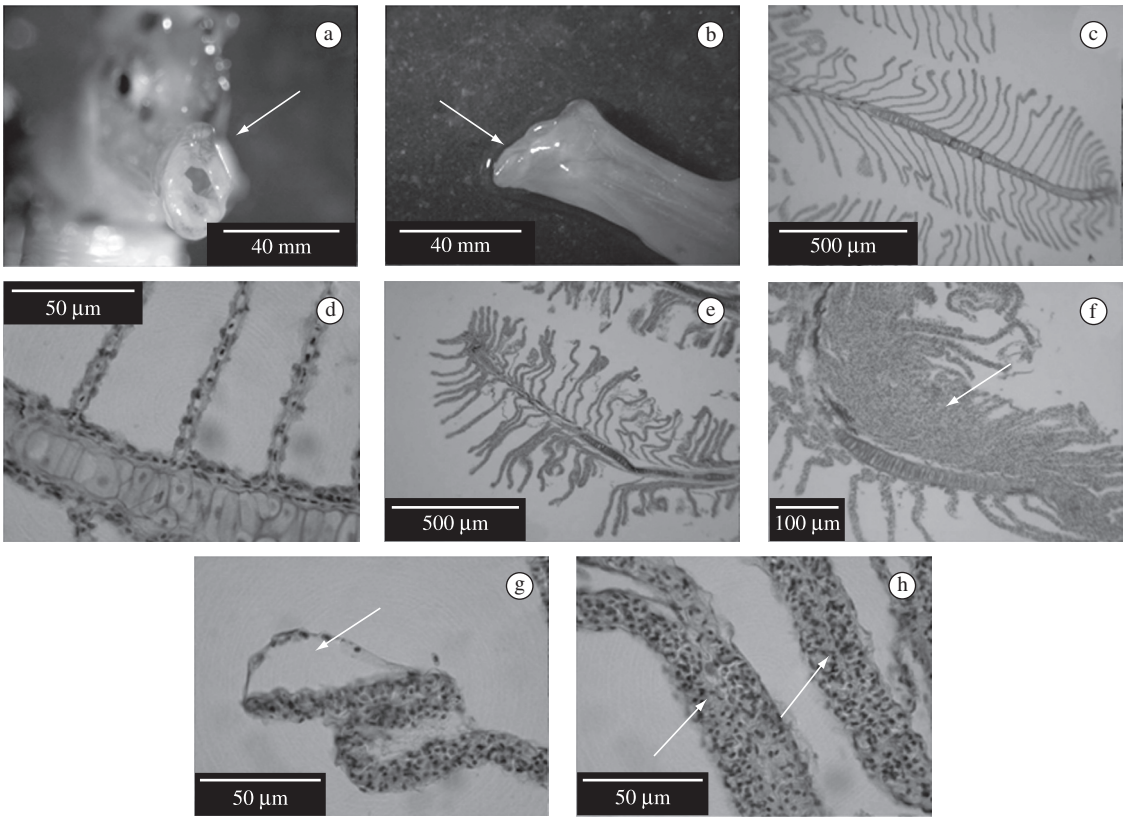

Figure 1. a-b) Mouth lesion (arrows) in *Hippocampus reidi* infected by *Vibrio alginolyticus*; c-d) gill section of uninfected fish; e-h) gill filaments of infected fish showing alterations in the primary and secondary lamellae e), hyperplasia (arrows) f), displacement (arrows) g) and fusion of secondary lamellae (arrows) (h). H and E.

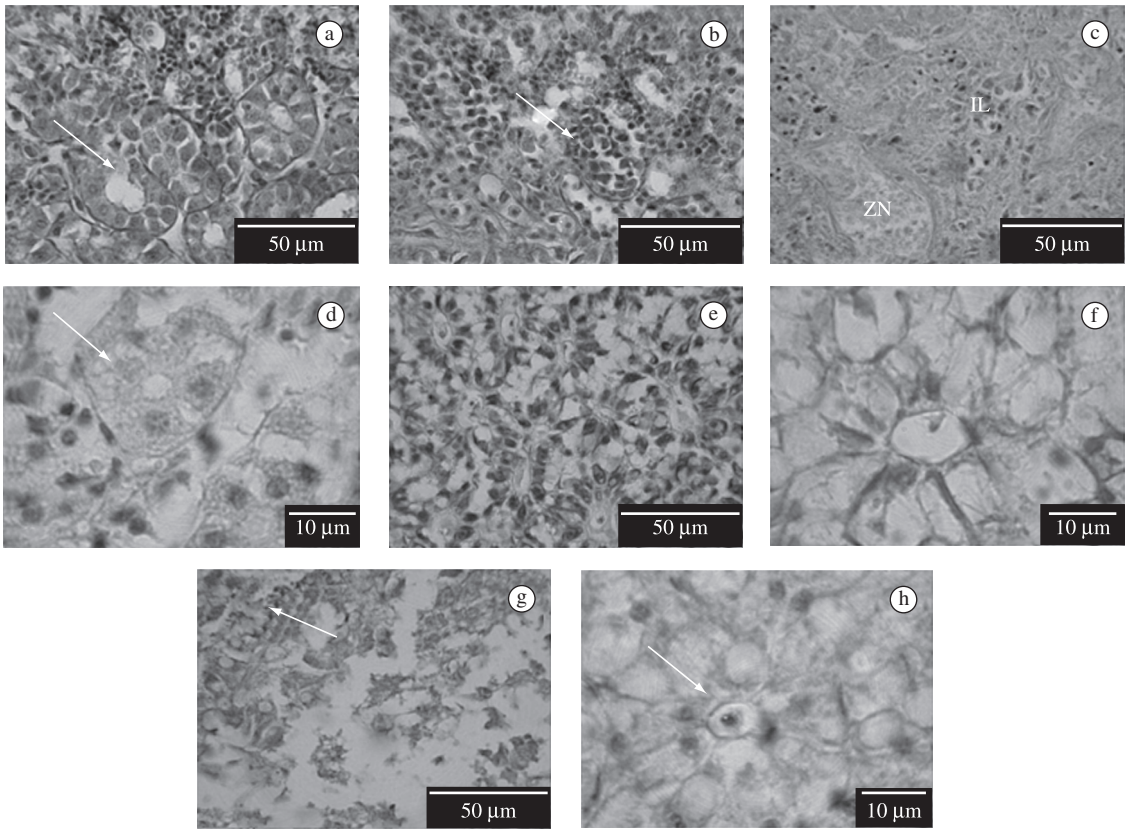

Figure 2. a-b) Kidney section of uninfected *Hippocampus reidi,* transversal section of tubule (arrow), tubule lumen (a) and glomerule (arrow) (b); c-d) kidney of infected fish. Necrosis (ZN), leukocytic infiltration (IL) (c) and altered tubule (arrow) (d); e-f) liver of uninfected fish (f); g-h) liver of infected fish showing loss of cellular integrity, necrosis (arrow) (g) and injured hepatocyte (arrow) (h). H and E.

foci and leukocytic infiltration (Figure 2c,d). In these fish, deformation and cellular injury of glomerulus, contorted tubules and collector tubules were also observed (Figure 2c,d). Leukocytic infiltration indicates an inflammatory reaction according to the observations of Benjamin et al. (2000). The kidney's alterations here observed were similar to the ones in turbot infected with *V. pelagius* (Villamil et al., 2003) and in turbot *Colistium nudipinnis* with septicemia caused by *V. splendidus* (Diggles et al., 2000). Diggles et al. (2000) related extensive haemorrhage and necrosis in the kidney and assumed the evidence of pathogenic vibriosis in turbot.

The liver of uninfected sea horses showed normal histology of hepatocytes and bile ducts (Figure 2e,f). In infected fish hyperplasia, necrotic foci, sinusoidal and bile duct deformation (Figure 2g,h) were found. Our results corroborated the observations in brill *Colistium guntheri* suffering from septicemic bacterial by *V. campbellii* (Diggles et al., 2000) and in turbot infected by *Moritella viscosa* (Björnsdóttir et al., 2004). The liver is an organ that may be used as an indicator of alterations in nutritional or physiological status as commented by Segner and Juario (1986). However, general metabolism of fish is compromised in cases of infectious diseases.

Vibrio alginolyticus is capable of producing toxins as serum proteases (Lee et al., 1997; Chen et al., 1999), which may be responsible for alterations in the gills, kidney and liver in infected fishes. These events might cause organ dysfunction by committing metabolic activities, culminating in fish death.

In conclusion, the strain of *V. alginolyticus* isolated was, in fact, pathogenic to *H. reidi*, as there was a high mortality in experimental infection and the observation of lesions on the mouth, gills, liver and kidney. It is important to emphasise that vibriosis can affect the development of new technologies of sea horse culture, appreciated fish in the aquarium trade. To minimise the effects of vibriosis in sea horse, an adequate prophylaxis combined with the use of immunostimulants or probiotic bacteria must be considered.

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