

CASE STUDY

***Vibrio ponticus* and *Vibrio harveyi* infection in snubnose pompano, *Trachinotus blochii*, from marine aquarium**

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Vibriosis in a mixed community with *Vibrio harveyi* and *Vibrio ponticus* in adult snubnose pompano, *Trachinotus blochii*, is documented from a marine aquarium. The disease was clinically manifested by reduced feeding activity, lethargy, abnormal swimming behaviour, extensive and focal superficial ulceration in dorsal region, and mild opaque eye with haemorrhage. Histopathological examinations revealed moderate to severe epidermal necrosis and ulceration with scale pocket and dermal oedema. Scales were often displaced, distorted or absent in skin sections. Cutaneous lesions were associated with a lymphocytic inflammatory infiltrate noted multifocally throughout the dermal layers. Grossly, the hepatopancreas had an area of pallor; on histological examination, the hepatopancreas displayed hepatocyte vacuolation and congestion.

Introduction

Identification of the causal agents of bacterial diseases in aquarium culture is an important part of the health care of ornamental fish, aimed at reducing infections. This applies to aquarium fish breeders as well as to public aquariums that enhance the attractiveness of zoos. The much-feared causative agents of acute bacterial diseases include the species of the genus *Vibrio*. According to Austin and Austin (2016), sixteen of 43 isolated species were classified as bacterial pathogens of freshwater and marine fish. Their pathogenicity was characterised as septicaemic lesions in acute cases. In sub-acute or chronic cases, ulcers may also be found.

Snubnose pompano is distributed throughout the Indian Ocean, and elsewhere in the Indo-West Pacific, from southern Japan to northern Australia and Lord Howe island, and eastwards to Samoa, Tonga, Marina and Marshall Islands (Smith-Vaniz, Collette, and Luckhurst 1999). According to Gopakumar (2011), this species has a high potential for aquaculture in India and is harvested from aquaculture in the Philippines and Malaysia (Ransangan, Lai, and Al-Harbi 2012; Amal et al. 2012).

An outbreak of vibriosis in a mixed community with *Vibrio harveyi* and *Vibrio ponticus* occurred in snubnose pompano in a marine aquarium of the zoo in the Czech Republic.

The present study is a first report describing co-infections by *Vibrio ponticus* and *Vibrio harveyi* in adult snubnose pompano, *Trachinotus blochii*. We recorded the gross signs of infection and observed the histological damage.

Material and methods

Fish collection

The fish came from a quarantine tank 2800 litres in size, where they were kept while the display tank was being refurbished. Water had the following physicochemical characteristics: temperature 25–26°C, pH 7.8–8.1, carbonate hardness 6.4 – 9.2 ° dKH, alkalinity 2.28 –3.3 mEq/L, orthophosphates (PO_4^{3-}) 1 – 2 mg/L, nitrates (NO_3^-) 50–100 mg/L, density of water 1.025 g/cm³.

Bacteriology

During the bacteriological examination, the methods Austin and Austin (2016) and Austin (2019) were respected. A total of six fish with clinical signs were sampled. Samples were taken from skin, eyes, kidney, liver and heart, and were streaked on blood agar (MKM 01011 Columbia agar; Trios) and tryptic soy agar (MKM 10061; Trios). Plates were then incubated at 24°C. Wet mounts of infected tissue from external lesions were checked under the microscope (×100 magnification).

CULTURE AND PHENOTYPIC IDENTIFICATION OF THE BACTERIAL STRAINS

Strains were inoculated on TCBS-selective medium for vibrios (thiosulphate-citrate-bile-sucrose agar) and Columbia blood agar (Oxoid). The commercial API 20 E kit and the apiweb software (BioMerieux, Marcy-l'Etoile, France), employed according to the manufacturer's instructions, were used for identification of the strains of the Gram-negative oxidase-positive rods. Complementary conventional tests described previously by Farmer, Janda, Birkhead, et al. (2003) were also used.

MALDI-TOF MASS SPECTROMETRY IDENTIFICATION

For the analysis, cultures were grown on Columbia blood agar and transferred with a bamboo toothpick onto an MSP 96 polished steel target (Bruker Daltonics). The samples were covered with 1.0 µl matrix solution: a saturated solution of α -cyano-4-hydroxy-cinnamic acid (Sigma) in 50% acetonitrile and 2.5% trifluoroacetic acid. The samples were analysed using a MALDI-TOF mass spectrometer (Microflex LT, Bruker Daltonics, Germany). The recorded spectra were processed using the Flex Analysis (version 3.3) and BioTyper (version 3.0) software with the version 3.2.1.0 database and Security Relevant Library.

The *Vibrio* strains were deposited in the collection of cultures of the NRL for *E. coli* and *Shigella* (NRL/ECS), NIPH, Prague. *Vibrio harveyi* was deposited under number NRL/ECS 16/156, and *Vibrio ponticus* under number NRL/ECS 16/157. MALDI-TOF identification results of strains No. NRL/ECS 16/156 (E16-156) and NRL/ECS 16/157 (E16-157) (Flex Analysis Output Form and BioTyper software) are shown in [Table 1](#).

MOLECULAR IDENTIFICATION

The results of phenotypic identification and MALDI-TOF mass spectrometry identification were verified by Sanger sequencing. Total DNA was isolated using QIAamp DNA Mini Kit (Qiagen, DE) according to the instructions of the manufacturer. 16S rDNA fragment was PCR-amplified using primers PSL for: AGGATTAGATACCCTGGTAGTCCA and P13P rev: AGGCCCGGGAACGTATTTCAC. The obtained PCR products were directly sequenced on the ABI3500 genetic analyser (ThermoFisher Scientific, USA), using the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, USA), according to the recommendations of the manufacturer. Sequences of both *Vibrio ponticus* and *Vibrio harveyi* 16S rDNA diagnostic fragments have been deposited in NCBI gene bank under the accession numbers OQ451892 and OQ451893. The hybridization positions of both PSL and P13 primers as per their respective reference sequences were 826 bp - 1470 bp (producing a 643 bp fragment) for *Vibrio ponticus* (RefSeq NR_029032.1), and 785 bp – 1436 bp (producing a 652 bp fragment) for *Vibrio harveyi* (RefSeq NR_043165.1).

Parasitology

Parasitological examination was carried out according to Ergens and Lom (1970), Lom and Dyková (1992) and Moravec (2023). In the taxonomic placement of the isolated ciliate protozoan, we took into consideration that all *Cryptocaryon* isolates currently are considered members of one species (Colorni and Burgess 1997; Yambot, Song, and Sung 2003). The specimen was carefully examined for morphological characters following the previous descriptions of Wright and Colorni (2002) and Dyková and Lom (2007).

Sampling procedure for histopathology

Necropsy procedures were carried out according to Roberts (2012) and Noga (2010). After anaesthesia with Menocain (3-aminobenzoic acid ethyl ester sodium hydrogen sulphate at a concentration of 0.06g/L (Král 1988) and subsequent killing of the moribund fish using the cranial concussion, tissue samples of skin, gill, hepatopancreas, kidney and spleen were fixed in modified Davidson's fixative, embedded in paraffin and sectioned at 5–7 µm. Histological sections were stained with haematoxylin-eosin, Sudan black B, PAS method (periodic acid-Shiff's reagent), Gram method, Van Gieson stains,

Masson's trichrome staining, melan-A (MART-1) and Ki-67 (MKI67). The BX53 microscope (Olympus) with the digital camera DP73 (Olympus) was used for microscopic examination and photo documentation.

Results

Clinical and histopathological findings

Six of the fifteen affected fish with body weights ranging from 1.4 to 2.1 kg were examined and a complete necropsy was performed. Disease signs included reduced feeding activity, lethargy, abnormal swimming behaviour. Clinical signs included extensive and focal superficial skin lesions on dorsal region ([Figure 1a](#)) and unilateral or bilateral mild opaque eye coverings/eyeballs with haemorrhage into the anterior chamber ([Figure 1b](#)). External haemorrhages and petechia on the skin were not evident. Necropsy in moribund fish revealed a poorly defined area of pallor on the caudal aspect of the hepatopancreas ([Figure 2a](#)).

Histopathological examinations revealed moderate to severe epidermal necrosis; enlarged scale pockets were mild, and there were marked scale pocked oedema, disarrangement and irregularity of the stratum compactum and lifting of its superficial layers into the oedematous stratum spongiosum. Scales were often displaced, distorted or absent in skin sections. Lesions were associated with inflammatory responses and included mixed lymphocytic cellular aggregates distributed, for the most part, focally throughout the dermal layers ([Figure 1c-1e](#)). Hypodermis and subcuticular muscle were not affected. The hepatopancreas displayed vacuolation of hepatocytes and hepatic vein and artery congestion ([Figures 2a, 2b](#)). In some sections of the primary lamellae, the loss of the secondary lamellae and the presence of early of trophonts of *Cryptocoryon irritans* in the epithelium were seen ([Figure 1f](#)).

Bacteriology

On the primary plates, both *Vibrio* species isolated in mixed culture from all the fish were recovered from the edges of skin lesions as sparse to rich cultures. *V. harveyi* recovered from the ocular lesions was present as sparse culture in two fish and as rich culture in four fish. *V. ponticus* obtained from liver was isolated as sparse culture in four fish and as rich culture in two fish. In a microscopic wet mount examination the detected gliding movement of these bacteria, which occasionally forms filaments, was not observed. The bacteria were also not found in imprint preparations and in histological sections as gram – rods..

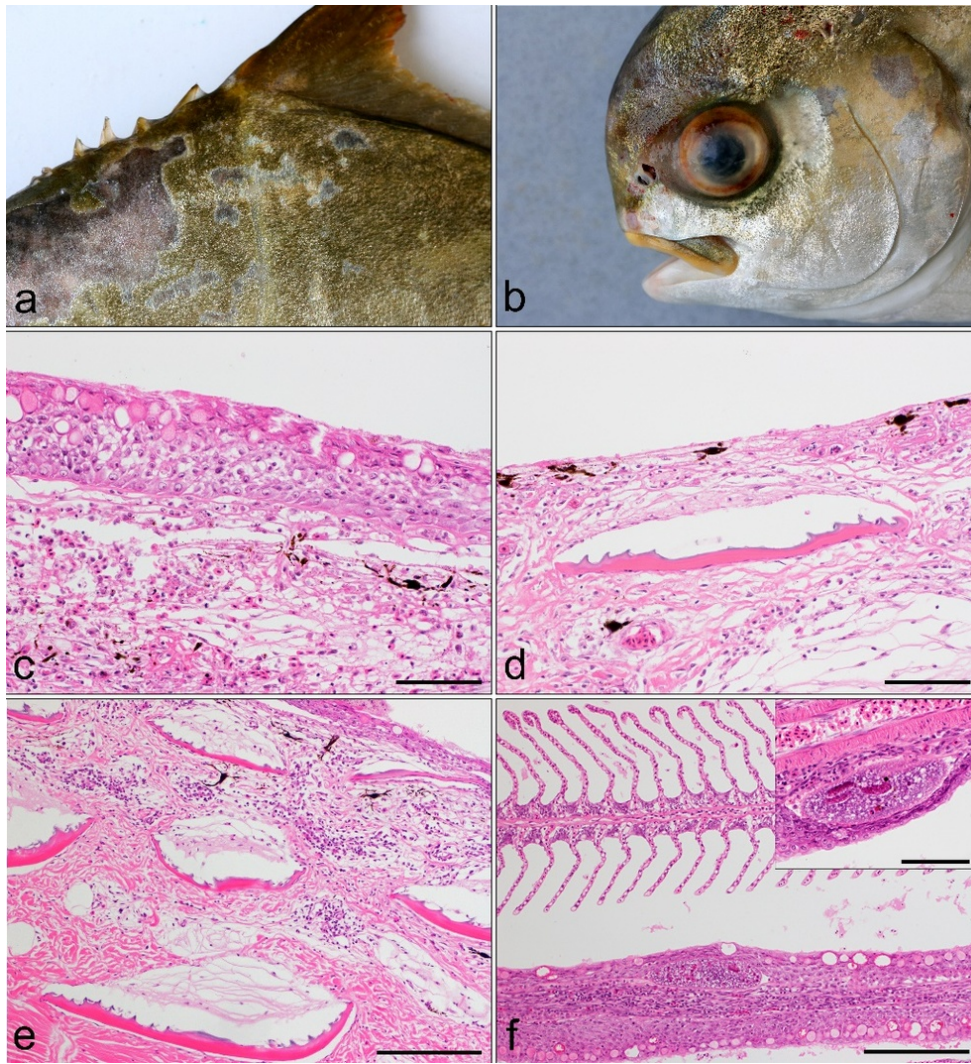


Figure 1. Clinical and histopathological changes in snubnose pompano with a mixed infection of *Vibrio harveyi* and *Vibrio ponticus* and a *Cryptocaryon irritans* ciliate infection.

(a) Extensive and focal superficial ulceration on dorsal region. (b) Opaque eye covering/eyeballs with periocular haemorrhaging. (c) Mild spongiosis in epidermis, accumulation of erythrocytes (haemorrhage) and mild mixed inflammatory infiltration of lymphocytes and neutrophils within the oedematous *stratum spongiosum*. H-E. Scale bar = 60 µm. (d) Loss of epidermis was distributed over the affected area. H-E. Scale bar = 60 µm. (e) Scale pockets are enlarged, and there is multifocal inflammatory infiltration with oedema of all dermal layers. H-E. Scale bar = 150 µm. (f) Normal gill and early trophont of *Cryptocaryon irritans* growing in epithelium of gill filament. H-E. Scale bar = 150 µm, scale bar of insert = 60 µm.

Characteristics of the bacterial strains

PHENOTYPIC BIOCHEMICAL CHARACTERISTICS

Both the strains, no. NRL/ECS 16/156 and no. NRL/ECS 16/157, were oxidase-positive, well grown on TCBS agar (and Columbia blood agar) after incubation at 25°C and with no growth observed in nutrient broth of 8% NaCl.

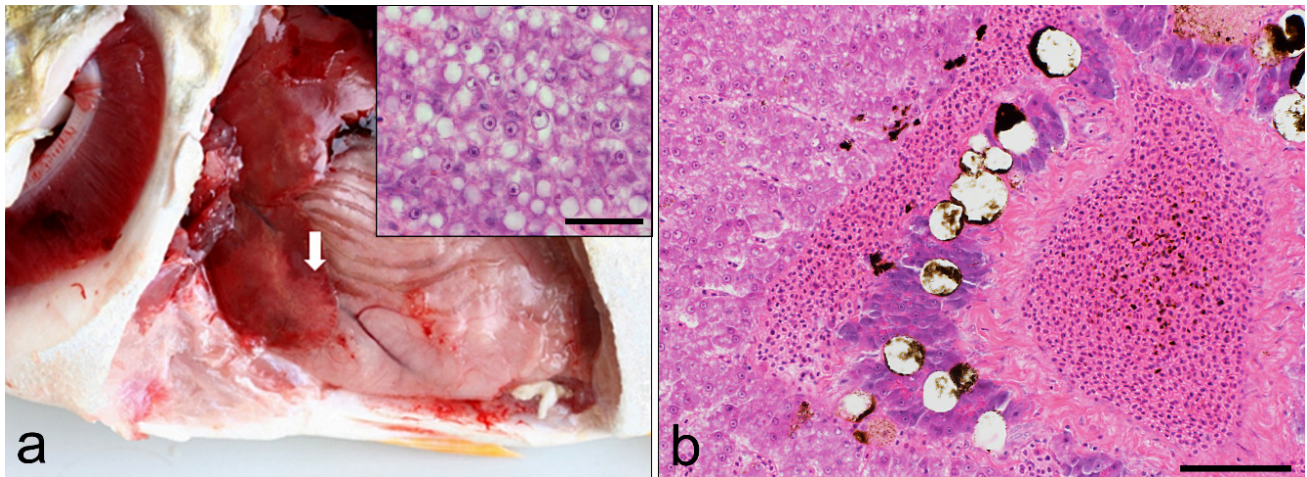


Figure 2. Clinical and histopathological changes in internal organs in snubnose pompano with a mixed infection of *Vibrio harveyi* and *Vibrio ponticus*.

(a) Pale area on hepatopancreas (arrows). Inset: Detail of vacuolation of hepatocytes. H-E. Scale bar = 30 μ m. (b) Hepatic vein congestion. H-E. Scale bar = 60 μ m.

Strain no. NRL/ECS 16/156: acid was produced from sucrose, trehalose, salicin and glucose (without gas), but not from lactose, sorbitol, xylose and mucate. Lysine decarboxylase, ornithine decarboxylase and indol were produced, but not H₂S, beta-galactosidase and arginine dihydrolase. Nitrates were reduced, Voges-Proskauer reaction and methyl red test were negative.

Strain no. NRL/ECS 16/157: Acid was produced from mannitol, sucrose, trehalose, xylose and glucose (without gas), but not from adonitol, arabinose, inositol, melibiose, rhamnose, lactose, sorbitol, salicin and mucate. Indol was produced, but not lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, H₂S and beta-galactosidase. Nitrates were also reduced, Voges-Proskauer reaction and methyl red test were negative, and strain could not utilize citrate, gelatine and urea. *V. ponticus* was suspected as probable species.

MALDI-TOF MASS SPECTROMETRY RESULTS

Strain no. NRL/ECS 16/156 was measured twice and identified as *Vibrio harveyi* with a best log score of 2.232. It was therefore identified at the genus level and probable species level ([Table 1](#)).

Strain no. NRL/ECS 16/157 was measured twice and identified as *Vibrio ponticus* with a best log score of 1.926. It was therefore identified at probable genus level ([Table 1](#)).

MOLECULAR CHARACTERIZATION OF THE STRAINS

Using the 16S rDNA sequencing it is only possible to precisely identify the type of bacteria (100% homology with the respective reference sequences). The exact identity of the bacterial strain / isolate involved should be identified using more detailed genetic mapping, not performed in this study.

Table 1. Results of MALDI-TOF identification of strains no. NRL/ECS 16/156 (E16-156) and NRL/ECS 16/157 (E16-157) (Output form Flex Analysis and BioTyper software)

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
H8 (++) (A)	E16-156	<i>Vibrio harveyi</i>	2.206	<i>Vibrio mytili</i>	1.877
H9 (++) (A)	E16-156	<i>Vibrio harveyi</i>	2.232	<i>Vibrio parahaemolyticus</i>	1.855
H10 (+) (B)	E16-157	<i>Vibrio ponticus</i>	1.926	identification not reliable	1.536
H11 (-) (C)	E16-157	identification not reliable	1.645	identification not reliable	1.453

Discussion

The first serious health problems observed in snubnose pompano were associated with the occurrence of nocardiosis, caused by *Nocardia seriolae*, in Malaysia and China (Labrie et al. 2008) and in Vietnam (Vu-Khac et al. 2016). Mass mortality of 58-day-old snubnose pompano fry exhibiting clinical signs typical of VNN (viral nervous necrosis) in a hatchery in the Philippines is described by Pakingking et al. (2011). An outbreak of *Streptococcus agalactiae* infection in cage-cultured golden pompano in Malaysia was reported by Amal et al. (2012), and outbreaks in skin flukes *Neobenedenia girellae* infection in snubnose pompano from a public aquarium in Korea are described by Nam et al. (2020).

Ocular lesions caused by *V. harveyi* in cage-cultured pompano broodstock are described by Pakingking et al. (2018). They noted that gross examinations of diseased fish samples were typified by obvious haemorrhagic lesions and mild to severe exophthalmia. Hispano, Nebra, and Blanch (1997) describe the isolation of *V. harveyi* associated with an ocular lesion in short sunfish (*Mola mola*) held in captivity in display tanks in Barcelona. Muroga et al. (1984) isolated *Vibrio* sp. from milkfish (*Chanos chanos*) juveniles with unilateral and bilateral opaque eye covering/eyeballs, exophthalmia and haemorrhagic eyes. Pakingking and Bautista (2018) attempted to elucidate the putative aetiology of the exophthalmia among net-cage-cultured pompano broodfish by examining the identity and pathogenicity of the bacterial strains isolated in pure culture from the exophthalmic eyes and brains of pompano broodfish infected with *Lepeophtherius spinifer* through biochemical characterisation and genetic analysis of the 16SrRNA sequence and infection challenges, respectively. Yu et al. (2018) describe skin and muscle ulceration, internal organs swelling and congestion in naturally diseased cultured golden pompano (*Trachinotus ovatus*).

Except of negative lysine decarboxylase and beta-galactosidase biochemical characteristics of the strain no. NRL/ECS 16/157 lead us to identification *V. ponticus*, as described in Gai et al. (2022). However further approaches as MALDI TOF and 16S rRNA were necessary to support and confirm species level identification.

As a new pathogenic agent, *V. ponticus*, which caused vibriosis in cultured Japanese sea bass (*Lateolabrax japonicus*) in China, is described by Xie et al. (2007). Moribund fish swam slowly on the surface of the water and had different degrees of ulcerations, ranging from a red blot to visible holes in some places on the fishes' bodies (e.g., the cauda). Pathological signs in diseased golden pompano due to *Vibrio ponticus* are described by Liu et al. (2018). They state that the diseased fish had swollen and hyperaemic liver and spleen, yellowish effusion in the gut, and different degrees of ulcerations on the bases of the dorsal and pelvic fins. Some of the fish had hyperaemia of the eyes.

The condition described above is a case of co-infection with two dominant bacterial pathogens of marine fish (Austin and Austin 2016), which, combined with a parasitic infection, contributed to the severe course of the disease. Occurrence of mixed bacterial infections is not uncommon even in freshwater fish: for example, farmed rainbow trout were infected with *Aeromonas hydrophila* and *Flavobacterium columnare* (Řehulka and Mráz 1982) or with coagulase-negative staphylococci (CoNS), of which seven (*S. capitis*, *S. cohnii* subsp. *urealyticus*, *S. haemolyticus*, *S. petrasii* subsp. *pragensis*, *S. saprophyticus*, *S. warneri*, *S. xylosus*) were isolated from co-infections in fish with bacterial, viral or parasitic infections (Řehulka, Marešková, and Petráš 2020). A mixed infection with *A. hydrophila* isolated from fish with columnaris is also reported by Scott and Bollinger (2014). In addition, *Pseudomonas anguilliseptica* was found with *Delftia acidovorans* in European eel (Andree et al. 2013), and *Moritella viscosa* and *Aliivibrio wodanis* occurred in salmon with winter ulcer disease (Hjerde et al. 2015).

Our results provide an opportunity to assess coinfections with *Cryptocaryon irritans*. *V. harveyi* co-infection with *C. irritans* in orange-spotted grouper *Epinephelus coioides* is described by Lai et al. (2023). These authors established a *C. irritans* high-dose local-infected model, causing mortality of groupers, which had a low vitality; histopathological analysis demonstrated an inflammatory response and degeneration in infected skin, gill and liver. Based on the morbidity symptom occurring in non-infected organs, the authors hypothesized that the results of morbidity and mortality were due to secondary bacterial infection after parasitism by *C. irritans*. We found *C. irritans* trophonts only in the gills growing mainly in the epithelium at the top of the gill filaments.

In our study, we observed many of the signs described by the authors mentioned above, and our results confirm that the two vibrios are among the feared pathogens that cause the gross signs whose primary manifestations include ocular lesions or dermal ulceration. Our results also enhance the knowledge concerning mixed vibrio infections in snubnose pompano under stress conditions associated with mortalities. The involvement of *V. ponticus* in the development of pathological changes in these organs will have to be verified experimentally.

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