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Susceptibility of Pacific white snook *Centropomus viridis* to *Vibrio* species

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ABSTRACT: To examine the pathogenicity of *Vibrio* strains, several doses of *Vibrio harveyi* (CAIM 1622 and CAIM 1508), *Vibrio ponticus* (CAIM 1751) and *Vibrio anguillarum* (CAIM 8) were used to challenge Pacific white snook *Centropomus viridis* Lockington, 1877 juveniles, and survival, gross signs and histological lesions were observed. Susceptibility of pathogenic vibrios CAIM 1508 and CAIM 1751 to antibiotics used in aquaculture was also evaluated. The growth ability of the tested strains was not related to their pathogenicity. One of the *V. harveyi* strains (CAIM 1508) was the most virulent, causing per-acute septicaemia in *C. viridis* even at a low dose (1.4×10^4 CFU g^{-1}). Although the *V. ponticus* strain (CAIM 1751) was less virulent, this is the first report of it as a pathogen of white snook. Fish challenged with *V. ponticus* displayed external, generalized haemorrhaging. Necrosis of the digestive tract and intravascular haemosiderosis were the most remarkable histological lesions in fish challenged with both strains. Multifocal necrosis of the internal organs and bacterial masses was also observed. The lowest minimum inhibitory concentration of the pathogenic strains (CAIM 1508 and CAIM 1751) was calculated for enrofloxacin (20 and 10 µg ml^{-1}, respectively), and both bacteria were resistant to amoxicillin, ampicillin and trimethoprim-sulfamethoxazole.

KEY WORDS: Vibriosis · White snook · Clinical signs · Histology

1. INTRODUCTION

Vibriosis, one of the most prevalent fish diseases, is widely responsible for mortality in aquaculture systems worldwide. Some *Vibrio* species, such as *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio campbellii* and *Vibrio parahaemolyticus*, are serious pathogens of aquatic organisms; a few *Vibrio* strains have been reported to act as primary pathogens, but most are opportunistic (Lavilla-Pitogo et al. 1990, Soto-Rodriguez et al. 2015). *Vibrio* species survive and multiply mainly in warm waters (>15°C) worldwide and disseminate rapidly among fish, rendering vibriosis a summer disease. As such, this disease is a particular threat to the aquaculture industry in tropical countries. *Vibrio harveyi* (*Vh*) is an important emerging pathogen of cultured fish and shellfish worldwide, and causes mass mortality in a wide variety of marine animal species (Ruwandeepika et al. 2010). As for most *Vibrio* species, the virulence of *Vh* depends on the strain, host susceptibility, dose and route of infection. For instance, a dose between 1.0×10^5 and 2.53×10^5 CFU $fish^{-1}$ of specific *Vh* strains caused 50% mortality in hybrid grouper (Shen et al. 2017), 80% mortality in *Lates calcarifer* (Dong et al. 2017) and 100% mortality in *Sparus aurata* (Haldar et al. 2010). Moreover, the clinical signs of infection with various *Vh* strains are diverse, causing gastroenteritis in cultured *Sciaenops ocellatus* with a lethal dose (LD_{50}) of 2.9×10^7 CFU $fish^{-1}$ (Liu et al. 2003), and 10^8 CFU g^{-1} was lethal in *Epinephelus coioides* (Lee et al. 2002). Moreover, 22 *Vh* strains

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were pathogenic in *Epinephelus fuscoguttatus*, and surviving individuals exhibited scale drop and deep dermal lesions (Zhu et al. 2018).

Vibrio ponticus (*Vp*) isolated in Spain was reported as a new *Vibrio* species in 2004 (Macián et al. 2004), and soon afterwards, *Vp* was also isolated from the kidney and liver of wild-caught, apparently healthy *Lutjanus guttatus* from northwestern Mexico (Gomez Gil et al. 2007). *Vp* isolates were later found to be pathogenic to *Lateolabrax japonicus* (Xie et al. 2007), and pathogenic *Vp* was recently isolated from diseased hybrid groupers in China, though pathogenicity was not confirmed (Liu et al. 2018).

Vibrio (*Listonella*) *anguillarum* (*Va*) causes haemorrhagic septicaemia in fish species, resulting in substantial economic losses in the aquaculture industry (Frans et al. 2011). The LD_{50} of *Va* 775 intraperitoneally injected into *Oncorhynchus mykiss* was 1×10^3 CFU fish^{-1} (Toranzo et al. 1987). For *Vh,* the pathogenic mechanisms involve the ability to attach and form biofilms, participate in quorum sensing, and secrete extracellular products that include haemolysins, although haemolysins are produced by most pathogenic and non-pathogenic *Vibrio* species. *Vh* has been reported as a pathogen of common snook *Centropomus undecimalis* in Florida, USA (Kraxberger-Beatty et al. 1990). In addition, because of antibiotic abuse in aquaculture, resistance to antibiotics occurs in *Vh*, complicating treatment. However, *Vp* has not yet been reported as a pathogen of the white snook *Centropomus viridis*, a euryhaline species with great potential for aquaculture. For example, these fish exhibit adaptability to confinement and can grow under high-density conditions using a commercial dry diet (Ibarra-Castro et al. 2017). *Centropomus viridis* has a tropical distribution from the eastern central Pacific region of Baja California, Mexico, and the Gulf of California to Peru, including the Galapagos Islands (Bussing 1995). Considering its value for commercial fisheries in Mexico, Pacific white snook was, for the first time in Latin American countries, successfully produced on a pilot scale for grow-out in ponds in 2017 and 2018. As *C. viridis* is important in the USA and Mexico, the present study was conducted to determine its susceptibility to different *Vibrio* species. We recorded the gross signs of infection and observed the histological damage in challenged fish.

2. MATERIALS AND METHODS

2.1. Bacterial inoculum

Bacterial strains were previously isolated from diverse marine species and tissues (Table 1), and *Escherichia coli* ATCC® 25922™ FDA CLSI (CAIM 21) was included as an innocuous bacterium. CAIM 1622 was identified as *Vibrio harveyi* by repetitive element palindromic PCR (rep-PCR; GTG_5) (Gomez Gil et al. 2007); CAIM 1508 and CAIM 1751 were identified as *V. harveyi* and *V. ponticus*, respectively, using whole genome sequences obtained in our laboratory (data not shown). CAIM 8 (*Vibrio anguillarum* 775 LMG 10939 NCIMB 2286) was kindly donated by Dr. Alicia Toranzo from Universidad de Santiago de Compostela. Bacterial inoculum was obtained as described previously (Soto-Rodriguez et al. 2018). Briefly, all strains were recovered from the cryovials; each was grown overnight at 30°C in 10 ml of trypticase soy broth (TSB; Bioxon) prepared with 2.0% NaCl (TSB+), except for CAIM 21, to which no NaCl was added. The bacterial suspension was adjusted to an optical density of 1.0 at 610 nm, and serially diluted to achieve densities ranging from 10^5 to 10^7 CFU ml^{-1}. To evaluate the growth of all tested strains, each bacterial suspension was incubated in 10 ml of TSB+ for 17 h at 100 rpm at 30°C. The bacterial suspension was adjusted to an optical density of 1.0 at 610 nm and serially diluted from 10^{-1} to 10^{-6} CFU ml^{-1} using 3 replicates. These suspensions were plated onto TSA+ (Bioxon) and thiosulfate-citrate-

Table 1. Strains used during challenges, cumulative mortality and time of death of juvenile *Centropomus viridis*. nd: no data; p.i.: post-infection; na: not applicable

Bacterial species	Strain	Origin	Source	Dose (CFU g^{-1})	Fish mortality (%)	Hours p.i.	Hemolysis
Vibrio harveyi	CAIM 1622	*Spheroides annulatus*	External lesion	1.3×10^4	0	na	α
Vibrio harveyi	CAIM 1508	*Spheroides annulatus*	External lesion	1.4×10^4	100	4–10	β
Vibrio ponticus	CAIM 1751	*Hipocampus ingens*	Kidney	6.9×10^5	50	17–39	β
Vibrio anguillarum	CAIM 8	*Oncorhynchus kisutch*	nd	1.8×10^7	0	na	Negative
Esherichia coli ATCC® 25922™	CAIM 21	*Homo sapiens*	Clinical isolate	1.0×10^6	0	na	Negative

bile salts-sucrose agar (TCBS; Bioxon) and incubated overnight at 30°C, and then the bacterial density (in CFU ml^{-1}) was estimated.

2.2. Challenge

Juveniles of *Centropomus viris* were produced under standard protocols used at the Marine Finfish Hatchery, CIAD Mazatlan Unit. The fish were acclimated in 600 l round plastic tanks in an open-flow system that was aerated and filtered with 10 µm relative retention seawater. Juvenile fish were fed a commercial fish diet (Sketting 2 mm) daily *ad libitum* and observed for any abnormalities. Before challenge, some fish were randomly collected and analysed to ensure health. For experiments, 30 l aquaria were used, each containing 6 organisms weighing 7.32 ± 0.26 g, and 2 biological replicates were used for each experiment. The fish were intraperitoneally injected with 40 µl of bacterial inoculum or sterile PBS or CAIM 21 as negative controls. The doses used during the challenge were between 10^4 and 10^7 CFU g^{-1} (Table 1). The duration of the experiment was 246 h at 29°C.

2.3. Antibiotic susceptibility

Minimum inhibitory concentrations (MICs) against CAIM 1508 and 1751 were estimated following the method outlined by Hindler (1992). Seven antibiotics (oxytetracycline, enrofloxacin, florfenicol, norfloxacin, trimethoprim-sulfamethoxazole [TSX], amoxicillin and ampicillin, all from Sigma-Aldrich) were tested in triplicate at 7 concentrations ranging from 0 to 1000 µg ml^{-1}, along with a negative control (incubated for 24 h at 30°C). The quality control strain used as a reference strain was *E. coli* ATCC® 25922™ FDA CLSI (CAIM 21).

2.4. Histological analysis

To identify tissue damage, during the challenge trials, moribund fish or fish that had just died were collected, and their internal organs (spleen, liver, kidney and brain) were dissected and preserved in 10% buffered formalin. Fixed samples were processed following conventional histological methods and stained with haematoxylin and eosin (H&E) and Price's Giemsa stain, and examined under a light microscope.

3. RESULTS

All tested strain grew well on TSA+ and all strains grew as yellow colonies on TCBS. CAIM 1508 displayed sparse growth on TCBS, and CAIM 1751 showed the most abundant growth on both TSA+ and TCBS (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d134p189_supp.pdf). Regarding challenge, within the first few hours post-infection (hpi), all challenged organisms were anorexic, except those infected with CAIM 8, though almost every organism was feeding well by 24 hpi. At 3 hpi, organisms infected with CAIM 1508 exhibited lethargy, erratic swimming and rapid operculum movement, and mortality was observed at 4 hpi (Table 1). *Centropomus viridis* challenged with the CAIM 1508 strain displayed a short hyper- to peracute pathology, resulting in 100% mortality between 4 and 10 hpi. In contrast, fish challenged with the CAIM 1751 strain showed an acute course, with mortality starting at 17 hpi and reaching 50% at 39 hpi. Clinical signs of the fish included haemorrhaging around the operculum, pectoral fins and posterior ventral area (Fig. 1). For the duration of the experiment, no mortality was observed in fish infected with CAIM 1622 or CAIM 8 or in fish from the negative control group. For CAIM 1508, the lowest MIC found was 20 µg ml^{-1} for enrofloxacin or norfloxacin; for CAIM 1751, the lowest MIC was 10 µg ml^{-1} for enrofloxacin. The highest MICs for both strains were observed for TSX, amoxicillin and ampicillin (>1000 µg ml^{-1} each; see Table S2 in the Supplement).

Histological analysis of *C. viridis* challenged with CAIM 1508 and CAIM 1751 indicated peritonitis with damage to organs associated with proliferation of the bacteria. All analysed fish challenged with

Fig. 1. Juvenile *Centropomus viridis* infected with *Vibrio ponticus* strain CAIM 1751 at 17 h post-infection, showing severe haemorrhage around the operculum, pectoral fins and posterior ventral area, including the anus

CAIM 1508 exhibited severe vascular congestion and intravascular haemosiderosis (Fig. 2a), and macrophages were found surrounding the organs of the abdominal cavity. The connective tissue of the peritoneum and intestinal mesentery was necrotic, and bacterial clusters were observed (Fig. 2b). Capillary congestion and multifocal necrosis was observed for the epithelium of the digestive tract, liver (Fig. 2c), pancreas, spleen and kidney, with bacteria adjacent to necrotic foci (Fig. 2d). *Centropomus viridis* challenged with CAIM 1751 also developed widespread septicaemia in all internal organs and tissues. Fish that died between 17 and 39 hpi exhibited reduced congestion in organs and tissues than fish challenged with CAIM 1508; however, intravascular haemosiderosis with a greater number of macrophages was also observed. In the peritoneum and mesentery, greater tissue necrosis was observed, along with dispersed bacteria in the connective lax tissue. Severe necrosis of the intestinal epithelium (Fig. 3a), exocrine pancreas (Fig. 3b), spleen and heart was also observed. Bacterial clusters were also observed in the circulatory system of the abdominal cavity, pancreas, kidney and brain (Fig. 3c), along with surrounding erythrocytes. An increase in melano-macrophage centres (Fig. 3b) and bacterial clusters was also recorded in the intervisceral connective tissue (Fig. 3d).

To observe the course of the disease, infected organisms surviving to the end of the challenge (240 hpi) were examined, revealing less damage in the abdominal organs; nonetheless, granulomas (with abundant bacteria inside) were observed in the mesentery and in the lax connective tissue adjacent

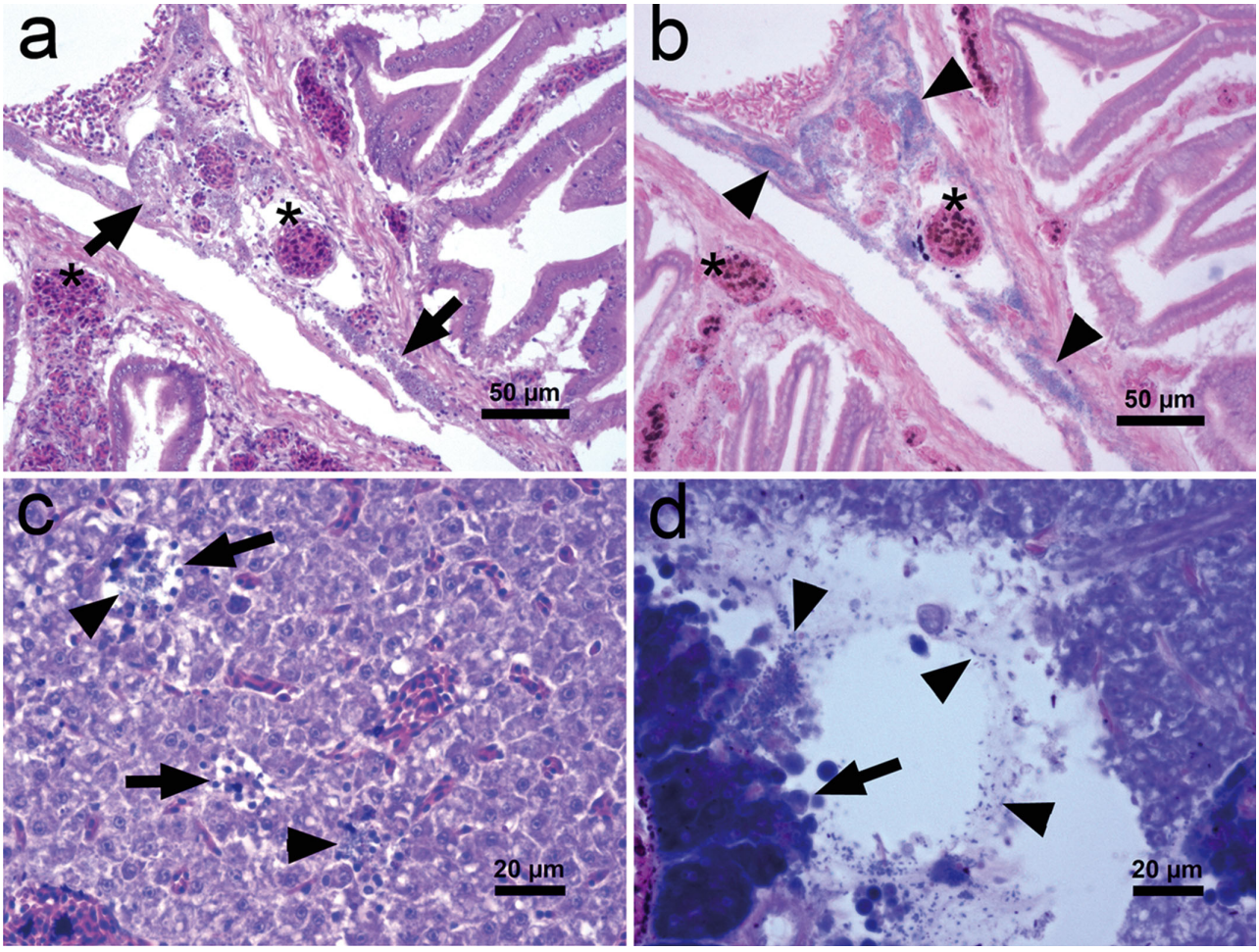

Fig. 2. Microphotographs of *Centropomus viridis* challenged with *Vibrio harveyi* strain CAIM 1508. (a,b) Necrotic tissue of the visceral mesentery (arrows) and intestine with severe hemosiderosis (*), capillary congestion and bacterial proliferation (arrowheads) in the inter-visceral tissue. (c) Liver with necrotic foci (arrows) and bacterial clusters (arrowheads). (d) Necrotic pancreas (arrow) and hepatocyte cells with bacterial proliferation (arrowheads). (a,c) H&E stain, (b,d) Giemsa stain

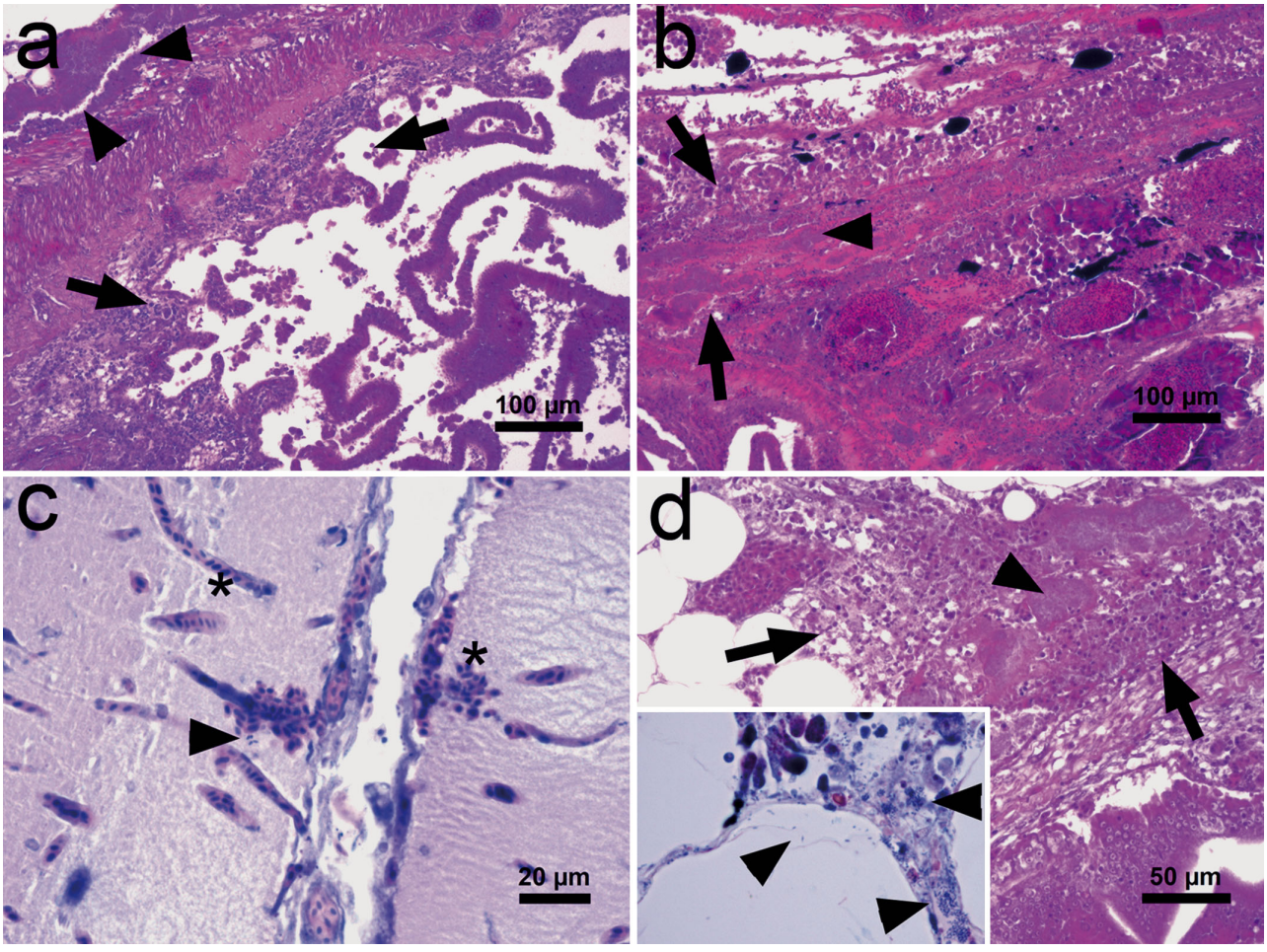

Fig. 3. Microphotographs of *Centropomus viridis* challenged with *Vibrio ponticus* strain CAIM 1751. (a) Necrotic intestine (arrows) with inflammatory cells and bacterial proliferation (arrowheads). (b) Intervisceral tissue with severe necrosis (arrows) of the exocrine pancreas, melanomacrophage centers and bacteria (arrowhead). (c) Bacteria (arrowhead) and capillary congestion (*) in the brain. (d) Severe necrosis of the mesentery (arrow) with bacterial proliferation and development of septic nodules (arrowhead). Inset: bacterial rods in the mesentery (Giemsa stain). (a,b,d) H&E stain, (c) Giemsa stain

to the intestine. Overall, surviving organisms in the negative control did not show any pathological changes in abdominal tissues.

4. DISCUSSION

In this study, the growth ability of the *Vibrio* species was not related to their pathogenicity; CAIM 1751 showed high growth on both media but was not highly pathogenic to *Centropomus viridis* juveniles. *Vh* virulence was found to be dependent on the strain: only CAIM 1508 caused 100% mortality in white snook during the first hour post-infection. Additionally, the virulence of *Vibrio* species may also dependent on the species and host. *Vibrio anguillarum* (CAIM 8) did not cause mortality in snook or in juveniles of the snapper *Lutjanus guttatus* (data not shown), but this bacterial species is reported to be a pathogen of warm- and cold-water fish (Toranzo et al. 2005). Pujalte et al. (2003) reported that all *Vh* strains tested were pathogenic to *Dicentrarchus labrax*, with 9 causing mortality at treatment levels of approximately 10^4–10^5 CFU g^{-1} and with exposed fish mostly dying within the first 24 hpi. In contrast, no adverse effects on *Sparus aurata* were observed, even at high doses.

The clinical signs of fish naturally and experimentally infected with various *Vibrio* strains are not specific, but rather depend on the fish species. The common signs of vibriosis in fish are also the classical signs of any disease in fish and include anorexia,

lethargy, erratic swimming, skin discoloration and exophthalmia; *Centropomus undecimalis* naturally infected with *Vh* also developed corneal opacity (Kraxberger-Beatty et al. 1990). Regardless, reports about the pathogenicity of *Vp* to marine fish are scarce. Moribund cage-cultured *Lateolabrax japonicus* infected with *Vp* showed various skin lesions, ranging from red blots to visible ulcerations on their bodies (Xie et al. 2007). Haemorrhagic lesions on fins and body surfaces and exophthalmia were observed in cultured cobia infected with *Vp* (Sharma & Dube 2017). Moreover, scale drop and muscle necrosis syndrome was observed in hybrid groupers (Zhu et al. 2018) and barramundi naturally diseased or intramuscularly injected with pathogenic *Vh* (Dong et al. 2017).

Fish challenged with CAIM 1751 displayed external, generalized haemorrhaging as the main gross sign. Curiously, external haemorrhaging in snook infected with *Vibrio* was also observed in tilapia experimentally infected with *Aeromonas* species (Dong et al. 2017, Soto-Rodriguez et al. 2018). Histological analysis revealed that infection caused by CAIM 1508 and CAIM 1751 resulted in peritonitis at different stages of the disease. In both cases, the presence of surrounding macrophages indicated an immunological response to the pathogens. CAIM 1508 caused per-acute vibriosis in *C. viridis*, whereas an acute stage (moderate septicaemia) occurred in fish challenged with CAIM 1751. Similar damage has also been reported in *Paralichthys dentatus* naturally infected with *Vh*, with the fish exhibiting acute peritonitis and necrosis of the posterior intestine and inflammation of the lymphoid tissue in the submucosa of the intestine (Gauger et al. 2006). Furthermore, *Takifugu rubripes* intramuscularly injected with *Vh* showed production of granulation tissue containing many suppurated foci, which replaced the necrotic dermis and lateral musculature (Mohi et al. 2010).

As there are a variety of gross signs and histological lesions reported for marine fish infected with *Vh*, bacterial virulence is dependent on the strain, fish species, stage of the disease and the type of infection (natural or experimental). Because *C. viridis* is a catadromous organism, research is required to evaluate the effect of temperature and salinity on susceptibility. Considering its virulence in cultured white snook, the isolation and molecular identification of *Vp* and its role in causing mortality in marine fishes requires further investigation, as does the LD_{50}. More importantly, the discovery of virulence factors is crucial to understanding the pathologies of the bacteria and their interactions with hosts. In the present study, the highest MICs for both pathogenic strains (CAIM 1508 and 1751) were found for the beta-lactam antibiotics amoxicillin and ampicillin, as well as TSX. Furthermore, genes expressing 8 different haemolysins that might be responsible for the generalized haemorrhaging observed in challenged snook were detected in the genome of CAIM 1508 (data not shown). As the pathogenicity of *Vh* is heavily dependent on the strain, the specific virulence factors harboured by CAIM 1508, possibly including some type of haemolysin and siderophore, might be responsible for the internal lesions observed in the affected *C. viridis* juveniles. Vibriosis is a difficult disease to control in fish culture, although there are prevention measures against vibriosis that should be considered. For instance, disinfection of the fish eggs (which may help prevent infection) and the appropriate use of antibiotics. Due to their effectiveness and safety to humans and the environment, farmers may be able to utilize a proper vaccine and bacteriophages.

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