Vol. 115: 47–55, 2015
doi: 10.3354/dao02864

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published June 29

Induction of mild enterocolitis in zebrafish *Danio rerio* via ingestion of *Vibrio anguillarum* serovar O1

B. Randazzo[1], F. Abbate[1], F. Marino[1,*], M. Mancuso[2], M. C. Guerrera[2], U. Muglia[1], M. Navarra[3], A. Germanà[1]

[1]Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy
[2]Institute for Coastal Marine Environment, CNR, Spianata S. Raineri 86, 98122 Messina, Italy
[3]Department of Drug Sciences and Health Products, University of Messina, 98168 Messina, Italy

ABSTRACT: *Vibrio anguillarum* is the etiological agent of a fatal hemorrhagic disease known as vibriosis that affects a wide range of fish species, causing severe economic losses. Several investigations have been carried out to elucidate the virulence mechanisms of this pathogen and to develop rapid detection techniques and effective disease-prevention strategies. The aim of our study was to evaluate the most effective way to induce mild enteritis in a fish model, in order to allow further applications. The experiments were carried out using 2 methods of administration of *V. anguillarum* serotype O1 to adult zebrafish *Danio rerio*: via intraperitoneal injection and via ingestion of infected *Artemia* nauplii. The results showed that the intraperitoneal administration often caused massive fish death due to severe systemic involvement. In our experiments, the effect of intraperitoneal infection was evident 48 h post infection, with cumulative mortality within 7 d post infection with severe histopathological changes in kidney hematopoietic tissue and in the intestine. In contrast, oral infection via *Artemia* did not show systemic involvement and only a moderate degree of inflammatory influx of the mucosa, a partial recovery at 12 d post infection, and no mortality. For these reasons, oral infection with live food appears to be the most effective method to induce mild enteritis with a local inflammatory response.

KEY WORDS: Vibriosis · Enterocolitis · Zebrafish · Immunohistochemistry

INTRODUCTION

Vibrio anguillarum is an etiological agent of vibriosis causing typical hemorrhagic septicemia in several fish species of economic importance, including Pacific and Atlantic salmon (*Oncorhynchus* spp. and *Salmo salar*), rainbow trout *O. mykiss*, turbot *Scophthalmus maximus*, European seabass *Dicentrarchus labrax* L., gilthead sea bream *Sparus aurata*, striped bass *Morone saxatilis*, cod *Gadus morhua*, and Japanese and European eel *Anguilla japonica* and *A. anguilla* (Toranzo & Barja 1990, 1993, Actis et al. 1999, Toranzo et al. 2005).

V. anguillarum is divided in 23 O serotypes (O1–O23), with different pathogenicity and host specificity. Serotypes 01 and 02 of *V. anguillarum* are the main strains responsible for epizootic outbreaks (Tajima et al. 1985, Toranzo et al. 1987, Toranzo & Barja 1990, Pedersen et al. 1999).

Infection by *V. anguillarum* occurs through the skin or gills, or via oral intake of the pathogen in

*Corresponding author: marinof@unime.it

water or food (Chart & Munn 1980, Baudin-Laurencin & Germon 1987, Muroga et al. 1987, Kanno et al. 1989, Chair et al. 1994, Grisez et al. 1996, Olsson et al. 1996, Planas al. 2005, Weber et al. 2010), but to date, several different hypotheses about the infection route of *V. anguillarum* have been presented in the literature. In acute epizootics, the infection spreads so rapidly that most of the infected fish die without clinical signs (Actis et al. 1999, Toranzo et al. 2005, Austin & Austin 2007).

The pathogenesis of *V. anguillarum* is well known (Frans et al. 2011). At the beginning, the bacterium passes through the stomach and subsequently the gut becomes the site of adhesion, colonization, and proliferation, because the intestinal mucus provides an important nutrient source (Frans et al. 2011, Rekecki et al. 2012). *V. anguillarum* then moves through the intestinal epithelium by endocytosis, followed by release of the bacteria in the lamina propria. Afterwards, the pathogen enters the blood stream, resulting in septicemia or spreading to different organs, such as liver, spleen, and kidney (Grisez et al. 1996).

The zebrafish *Danio rerio* is an important model of vertebrate development and human disease because of its small size, rapid generation time, powerful genetic systems, and genomic resources. Zebrafish larvae have no adaptive immune system, thereby providing the opportunity to study the vertebrate innate immune system in isolation (Willett et al. 1999, Lam et al. 2004) and also providing an infection route for a number of virulence factors such as *V. anguillarum* (O'Toole et al. 2004, Zhang et al. 2012). In contrast, adults have a well-developed adaptive and innate cellular immune system, making this fish an ideal model for the study of infectious diseases and zoonotic aquatic pathogens (Phelps & Neely 2005, Rowe et al. 2014).

The aim of our study was to use *V. anguillarum* as a colitogenic in adult zebrafish to produce non-lethal damage to the intestinal mucosa.

MATERIALS AND METHODS

Fish rearing

Adult zebrafish (40 males and 40 females), aged 12 mo with a mean weight of 0.4 g and mean length of 3.8 cm, were obtained from the Center for Experimental Fish Pathology of Sicily and reared at 26°C under natural photoperiod. Fish were housed in 1 l tanks (1 fish $tank^{-1}$) and fed twice a day ad libitum with *Artemia* nauplii.

The challenge period was 12 d long. Procedures were performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare and carried out in accordance with the EU directive 2010/63/EU.

Bacterial strain

A *Vibrio anguillarum* strain serotype O1 code 975/I, provided by the Experimental Zooprophylactic Institute (Istituto Zooprofilattico Sperimentale delle Venezie, IZS), Italy, isolated from bass specimens, was routinely cultured in tryptic soy broth (TSB-Oxoid) supplemented with 1.5% of NaCl (final concentration) and in thiosulfate citrate bile sucrose agar (TCBS-Oxoid) supplemented with 1.5% NaCl (final concentration), both incubated at 25°C for 24 h. Bacterial growth was monitored by a biophotometer (Eppendorf) at an optical density (OD) of 600 nm. At OD values of 0.1, 0.6, and 1, corresponding to lag, exponential, and early stationary phases, aliquots of the broth culture were collected for CFU count. The OD value corresponding to 10^4 CFU ml^{-1} was utilized for the intraperitoneal challenge (Rojo et al. 2007); a concentration of 10^9 CFU ml^{-1} was used for the infection via *Artemia* (Grisez et al. 1996). To assess the virulence of the bacterial strain, 4 fish, viz. 2 *Carassius auratus* (to assess virulence in a freshwater fish) and 2 *Dicentrarchus labrax*, were injected with aliquots of 100 µl of *V. anguillarum* suspension (10^6 cells ml^{-1}) using the protocol of Crisafi et al. (2011), and 4 other fish (2 for each species mentioned above) were inoculated with 100 µl of sterile saline solution (0.9% NaCl) and used as control group. After 48 h, the fish were sacrificed and samples of kidney were excised and spread onto marine agar and TCBS agar for the isolation of the *V. anguillarum* strain.

Intraperitoneal challenge

As a positive control, a group of 30 fish, previously anesthetized with MS-222 (0.1%) for at least 5 min, were intraperitoneally (i.p.) injected with 10 µl of 10^4 CFU ml^{-1} bacterial suspension using a 0.3 ml (30G) × 8 mm ultrafine sterile syringe. The needle was held parallel to the fish spine and

inserted cephalad into the midline of the abdomen just posterior to the pectoral fins up to 1.5 mm into the skin. A negative control group (10 specimens) was injected with an identical volume of sterile saline solution (0.9% NaCl).

Oral infection with live food

Fish (n = 30) received 3 doses of *V. anguillarum*-loaded *Artemia* nauplii: the first dose at the beginning of the experiment (T_0), the second dose 24 h later (T_{24}), and the third 48 h later (T_{48}). Dehydrated cysts of *Artemia* (SERA D 52518 Heisenberg), previously stored at 4°C, were placed in a 5 l cylindrical tank provided with artificial lighting and constant air input from below for 24 h. After hatching, nauplii were collected with a 145 µm sieve (Hobby Aquaristik no. 21620) and washed with sterile saline solution. About 6000 *Artemia* nauplii were incubated at 24°C for 1 h in 200 ml of 10^9 CFU ml^{-1} *V. anguillarum* suspension (following the protocol of Grisez et al. 1996), thoroughly rinsed with sterile saline solution, and resuspended in 30 ml of sterile saline solution (0.9% NaCl); a homogeneous concentration (1 ml aliquot) was dispensed into every tank (about 200 nauplii $tank^{-1}$) using a 3 ml sterile Pasteur pipette. In order to estimate the bacterial concentration, the same amount of nauplii was collected and rinsed following the procedure described above, transferred into a stomacher blender (SEAWARD®400 Circulator), and homogenized for 3 min. Serial dilutions were then prepared, and 0.1 ml of each dilution was spread-plated on TCBS agar (Oxoid) and incubated at 24°C for 24 h; the yellow colonies were counted, and the number of CFU was expressed as the approximate number of *V. anguillarum* bacteria per challenge.

To be sure that the whole amount of live feed was consumed by the fish, the number of *Artemia* was counted 2 h after the administration.

Bacteriological assay

Samples of blood from head kidney were withdrawn both from *C. auratus* and *D. labrax* i.p. injected with *V. anguillarum* to re-isolate the pathogen. Blood samples were incubated for 24 h at 25°C in marine broth (Microbial Diagnostic) and incubated for the same time at the same temperature in marine agar (Microbial Diagnostics). The growth colonies were isolated in pure culture and then the bacteriological assays were performed to identify the pathogen using the protocol of Zaccone & Mancuso (2008).

Histological assay

From 2 to 6 fish were taken at 1, 3, 5, 7, and 12 d post infection (dpi), sacrificed by immersion in 1% MS-222 (Sigma), and fixed in Bouin's fixative. The number of sampled specimens varied according to the mortality in the group of i.p. challenged fish. Only surviving specimens were analyzed to avoid misinterpretations in histological outline due to post-mortem events such as autolytic changes, especially in the intestinal morphology. Four fish were sampled from the control group. The body cavities were carefully opened before immersion in Bouin's liquid to properly fix the intestine. After 24 h, the fixative was replaced with 70% ethanol and samples were processed through graded alcohol solutions and xylene and then embedded in paraffin. Sections of 5 µm were stained with hematoxylin–eosin for routine staining. Sections were examined using a Leica DMRB microscope, and photographs were taken using a Leica MPS 60 phototube connected to the microscope.

Immunohistochemistry

For the identification of *V. anguillarum* in tissues, sections of 5 µm were dewaxed in xylene, rehydrated in a series of ethanol baths, and washed in running tapwater. In order to prevent nonspecific antibody binding, sections were blocked using 5% BSA in Tris for 20 min. The absorbed polyclonal antisera anti *V. anguillarum* (provided by the Institute of Marine Research, IMR, of Bergen, Norway) were diluted in Tris buffer with 2.5% BSA 1:100 and sections were then incubated overnight at 4°C in a humid chamber with the primary antisera. Sections were washed in TBS for 10 min, and a goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) was used as a secondary antibody at a dilution of 1:100 in Tris buffer with 2.5% BSA. Sections were then incubated in secondary antibody at room temperature for 1 h and 30 min. The peroxidase reaction was developed in a solution of 3,3-diaminobenzidine tetrahydrochloride (Sigma; 0.04% w/v in Tris–HCl 0.05M, pH 7.4) and H_2O_2 (0.005%). Positive bacterial staining was indicated by brown coloration. Unchallenged larvae were used as negative controls.

RESULTS

Virulence in *Carassius auratus* and *Dicentrarchus labrax*

All specimens of *C. auratus* and *D. labrax* died 24 h after challenge, confirming the virulence of our *Vibrio* strain. Despite the rapidity of infection, typical symptoms of vibriosis were observed. In particular, fish appeared lethargic in the hours post infection, and erythema at the base of the pectoral fins appeared in both species. Moreover, after 24 to 48 h of incubation at 25°C, blood samples spread on marine agar showed colonies that were isolated in pure culture and subsequently identified by API 20E (Biomerieux), confirming that the bacteria isolates belonged to *V. anguillarum*.

Bacterial load for oral challenge and food consumption

The bacterial load of nauplii used for the oral challenge was 4×10^9 *V. anguillarum* cells per 6000 *Artemia*; assuming that each *Artemia* nauplius contained the same number of bacterial cells and that each fish ate the same number of nauplii, the infection dose was approximately 2×10^8 *V. anguillarum* cells per fish. Two hours after the administration of live feed, zebrafish ate almost 80% of the provided *Artemia* nauplii, with no differences between challenged and control groups.

Mortality and macroscopic observation

All specimens of the control groups showed no external signs of disease. Apart from a small hemorrhagic area, occasionally found around the site of injection, subjects injected i.p. with sterile saline solution appeared uninfected. In fish infected with i.p.-injected bacteria, occurrence of mortality was recorded beginning at 3 dpi. Clinical findings due to vibriosis and similar to those described in other species, such as hemorrhages spread on the ventral body surface and lethargy, were also observed.

In the group infected through *Artemia* nauplii, no mortality was recorded, and macroscopic symptoms of disease were not observed (results are summarized in Table 1).

Table 1. Experimental infection of zebrafish *Danio rerio* with *Vibrio anguillarum* serovar O1. Macroscopic results of intraperitoneal injection; p.i.: post infection

Day p.i.	Experimental group	Samples (n)	Haemorrhage		Lethargy	Found dead
			Injection site	Ventral body		
1	Control	2	1	0	0	0
	Treated	6	2	0	0	0
3	Control	2	2	0	0	0
	Treated	6	1	4	6	3
5	Control	2	0	0	0	0
	Treated	6	2	5	5	4
7	Control	2	0	0	0	0
	Treated	6	1	5	6	4
12	Control	2	0	0	0	0
	Treated	6	0	3	4	3

Histological observations

Tissue changes at different severity stages were found in all subjects analyzed in the 2 challenged groups. No histopathological alterations were observed in the control groups. Surviving fish were sampled at 1, 3, 5, 7, and 12 dpi. In i.p.-infected specimens, a degeneration of the parenchyma, a conspicuous infiltration of lymphocytes, and a general liquefactive necrosis was observed in the kidney after 3 dpi (Fig. 1e). General necrosis of the absorption epithelium was observed throughout the intestine. Lymphocytes were abundant in blood vessels adjacent to the intestinal wall and significantly present in the lamina propria of the villi (Fig. 1d). Fish treated with saline solution did not show any sign of disease in all examined organs.

In the oral challenge with *V. anguillarum*-loaded *Artemia*, histopathological analysis showed appreciable changes in the morphological characteristics of the intestine after 3 dpi and a variable degree of inflammatory infiltration after 24 h post infection. In particular at 3 dpi, typical changes in the intestinal epithelium, consisting of enterocyte degeneration and necrosis in association with a variable presence of lymphocytes infiltrating the lamina propria, were observed (Fig. 1f). At 8 dpi, damage similar to that observed at 5 dpi was present in the intestinal mucosa (data not shown), while a minor incidence of histological damage was found in the intestinal mucosa of fish sampled at 12 dpi. In the control group, no histological damage was demonstrated (results are summarized in Table 2).

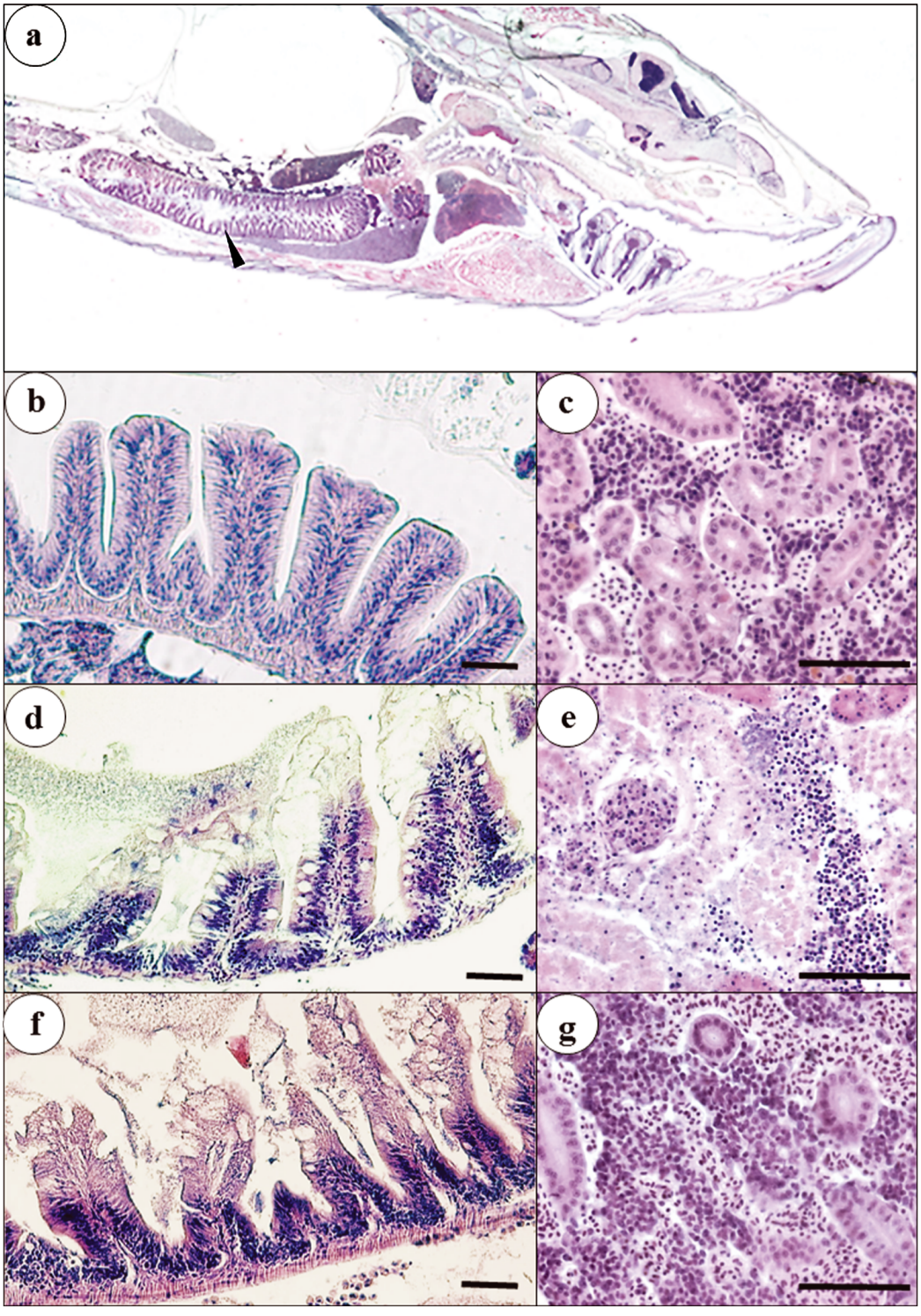

Fig. 1. (a) Sagittal section of a whole zebrafish *Danio rerio* (stained with H&E). The arrowhead indicates parts of the intestine. (b, c) Normal histological aspect of intestine (b) and kidney (c) in a zebrafish used as control. (d) Intestine of zebrafish sampled 5 d after intraperitoneal injection of *Vibrio anguillarum*, showing degeneration of the apical portion of intestinal folds and abundant amount of lymphocyte infiltration in the lamina propria. (e) Kidney of zebrafish sampled at the same time and showing general necrosis and lymphocyte infiltration. (f) Intestine of zebrafish 3 d after infection through *Artemia* nauplii, showing typical degeneration of the absorption epithelium with a small amount of lymphocytes. (g) No appreciable changes were observed in kidney of specimens infected with *V. anguillarum* through *Artemia* nauplii. Scale bars = 50 µm

Table 2. Experimental infection of zebrafish *Danio rerio* with *Vibrio anguillarum* serovar O1. Microscopy/immunohistochemistry results following an intraperitoneal injection of the serovar or feeding on infected *Artemia* nauplii. For each treatment at each timepoint, n = 6; all control fish (n = 2 per timepoint per treatment) had negative histological results; p.i.: post infection

Day p.i.	Treatment	Epithelial necrosis	Lymphocyte infiltration		*V. anguillarum* presence		
			Intestine	Kidney	Intestine	Kidney	Spleen
1	Injection	0	0	2	0	3	2
	Artemia	0	3	0	3	0	0
3	Injection	3	3	4	3	4	3
	Artemia	3	4	0	4	0	0
5	Injection	2	2	2	1	2	2
	Artemia	5	6	0	4	0	0
7	Injection	4	4	4	2	4	4
	Artemia	4	6	0	1	0	0
12	Injection	2	3	4	1	4	4
	Artemia	1	2	0	0	0	0

Immunohistochemistry

Immunohistological examinations were performed to verify the effectiveness of the infection and the real correlation between the damage observed and the adhesion of the bacteria to the epithelial mucosal layer of the intestinal folds, in particular in fish challenged through live feed. No samples belonging to the control group showed positive immunostaining (data not shown). In zebrafish challenged through i.p. injection, the occurrence of the bacteria was observed in different sites of the peritoneal cavity. The reaction was especially notable in the intertubular spaces of the cranial kidney (Fig. 2b). Moreover, a positive reaction for *V. anguillarum* was also found scattered in all intestinal segments with no specific localization (Fig. 2a). In fish challenged through live *Artemia* nauplii, no infiltration with bacteria was observed in the kidney (Fig. 2f) or other organs. In contrast, a clear presence of bacteria was observed strictly associated with the absorption epithelium (Fig. 2d,e), and this finding was still evident after 5 dpi (results are summarized in Table 2).

DISCUSSION

The fish pathogen *Vibrio anguillarum* is an excellent candidate for developing a natural enteric infection model in zebrafish. Several studies have been carried out to detect the route of entry of *V. anguillarum* into fish, and have shown that vibriosis is inducible by a water-borne route (O'Toole et al. 2004, Oehlers et al. 2010).

Rojo et al. (2007) developed an effective way of infection using i.p. injection of a strain of *V. anguillarum* to study the gene expression of innate immunity by choosing the highest bacterial concentration corresponding to 10^6 CFU ml^{-1} to induce overt enteritis. In contrast, we used a lower concentration (10^4 CFU ml^{-1}) to induce minor effects both in terms of mortality and damage to the mucosa. Nevertheless, the high mortality, probably due to a systemic involvement, does not allow applicability to testing the real effectiveness of drugs, natural extracts, or probiotics. Moreover, the results demonstrated that the virulence of our strain, when i.p. injected, is also maintained in a freshwater fish such as *Carassius auratus*.

A number of studies have been carried out on the effectiveness of oral challenge in inducing vibriosis in fish. Planas et al. (2005) proposed a useful model for experimental infections with *V. anguillarum* via rotifers in turbot *Scophthalmus maximus* larvae; moreover, intestinal necrosis, obtained by oral challenge with *Vibrio* sp., was demonstrated in flounder larvae (Masumura et al. 1989, Muroga et al. 1990). The effectiveness of *Artemia* as a vehicle of infection was already demonstrated in saltwater fish, resulting in a cumulative mortality of 61% within 120 d after the first administration of brine shrimp in turbot larvae (Chair et al. 1994). Previous studies have shown the efficiency of *Artemia* nauplii in bioencapsulating bacteria (Gomez-Gil et al. 1998). The use of *Artemia* probably could have avoided the effect of a salinity close to 0‰, which is lethal to *V. anguillarum* harvested in the late-exponential growth phase (Hoff 1989). We modified the administration doses used by Grisez et al. (1996), because previous experiments performed in our laboratory showed that this protocol did not often cause damage to the intestinal mucosa in zebrafish with our bacterial strain (data

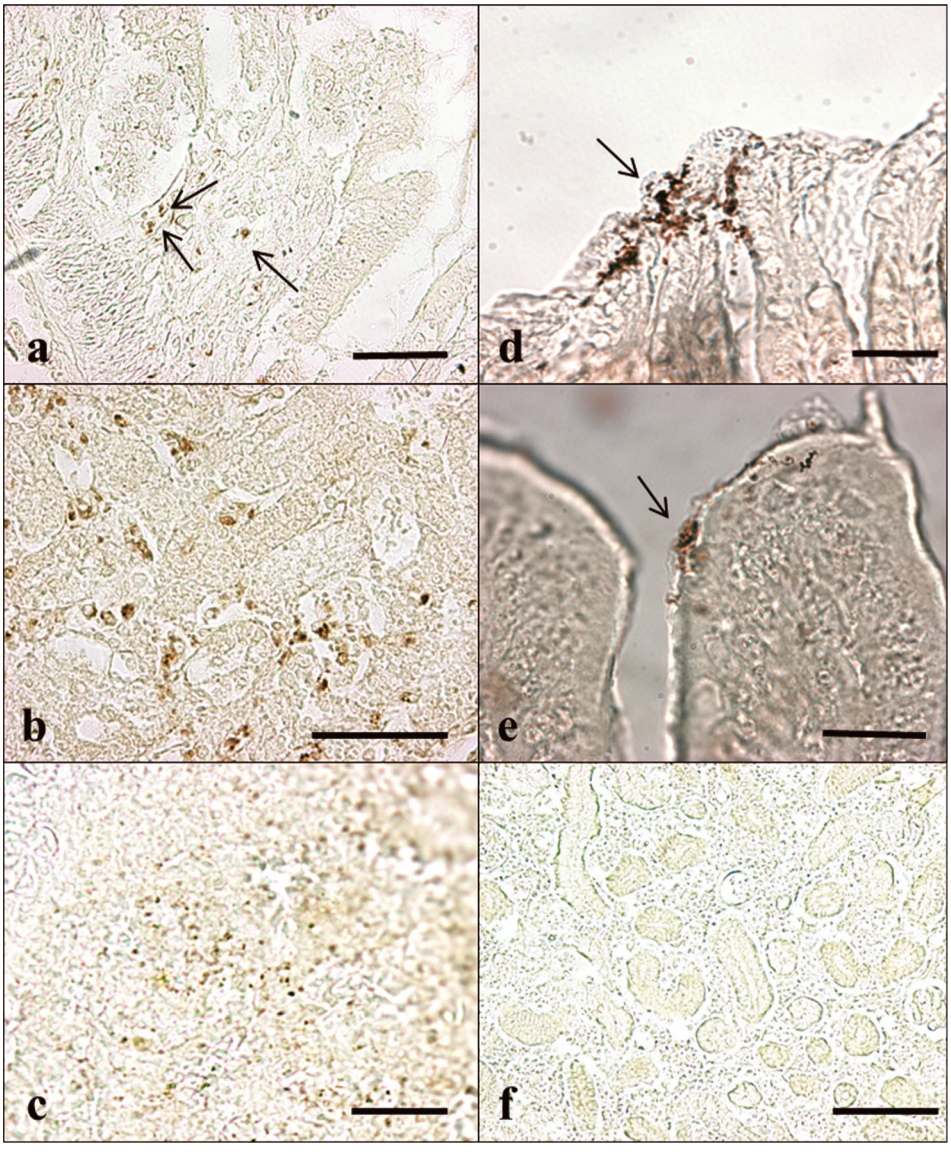

Fig. 2. Immunohistochemical staining of zebrafish *Danio rerio* intestine using rabbit anti-*Vibrio anguillarum*. Intestine of zebrafish sampled 5 d after intraperitoneal infection with *V. anguillarum* showing positive immunoreactivity of the bacteria (arrows) scattered in (a) the intestinal folds, (b) kidney, and (c) spleen. (d) Intestine of zebrafish 1 d after administration of *V. anguillarum*-loaded *Artemia* showing the presence of bacteria close to fold tips. (e) Immunohistochemistry reveals the adhesion of bacteria to the intestinal brush border in a zebrafish sampled 3 d post infection. (f) Kidney of a zebrafish after administration of infected *Artemia* with no bacteria. Scale bars = (a,b,c,d,f) 50 µm, (e) 25 µm

not shown), and for this reason we considered it more useful to reiterate the infection with *V. anguillarum*-loaded *Artemia* for 3 consecutive days. In the present study, this challenge was effective in inducing damage to the mucosa correlated with the activity of *V. anguillarum* and compatible with the life of the host, in contrast to what happens in saltwater fish such as sea bass, in particular at the larval stages where mortality was registered at 2 dpi (Rekecki et al. 2012). This research highlights that in zebrafish, the local inflammatory response due to this mode of infection is not supported by a systemic involvement, suggesting that the infection has not progressed and, therefore, does not affect other organs that are usually the targets of this pathogen. Intestinal epithelial damage is thought to be important for the development and maintenance of colitis in animal models (Sansonetti 2004). The intestinal epithelium plays an important role in balancing the need for nutrient and waste exchange with maintaining immunological integrity;

furthermore, the hallmark of intestinal inflammation is the infiltration of the intestine by leukocytes (Abraham & Cho 2009). In our study, the damage to the mucosa was considered one of the more important characteristics of enteritis and the amount of inflammatory cell infiltration was regarded as a measure of the degree of inflammation.

Our results suggest that mild enteritis in adult zebrafish can be induced using *Artemia* as the vehicle for the pathogen, providing the possibility to test the potential efficacy of drugs or natural extracts both in aquaculture and in human clinical fields.

Acknowledgements. We thank Dr. Amedeo Manfrin (IZS) for providing the bacterial strain and Dr. Nina Sandlund (IMR) for providing the antisera.

LITERATURE CITED

Abraham C, Cho JH (2009) Inflammatory bowel disease. N Engl J Med 361:2066–2078

Actis LA, Tolmasky ME, Crosa JH (1999) Vibriosis. In: Woo P, Bruno D (eds) Fish diseases and disorders: viral, bacterial and fungal infections. CAB International Publishing, Wallingford, p 523–558

Austin B, Austin DA (2007) Bacterial fish pathogens. Diseases in farmed and wild fish. Ellis Horwood, Chichester, p 265–307

Baudin Laurencin F, Germon E (1987) Experimental infection of rainbow trout, *Salmo gairdneri* R., by dipping in suspension of *Vibrio anguillarum*: ways of bacterial penetration; influence of temperature and salinity. Aquaculture 67:203–205

Chair M, Dehasque M, Van Poucke S, Nelis H, Sorgeloos P, De Leenheer AP (1994) An oral challenge for turbot with *Vibrio anguillarum.* Aquacult Int 2:270–272

Chart H, Munn CB (1980) Experimental vibriosis in the eel (*Anguilla anguilla*). In: Ahne W (ed) Fish diseases. 3rd COPRAQ Session. Springer, Heidelberg, p 39–44

Crisafi F, Denaro R, Genovese M, Cappello S, Mancuso M, Genovese L (2011) Comparison of 16Sr DNA and toxR genes as targets for detection of *Vibrio anguillarum* in *Dicentrarchus labrax* kidney and liver. Res Microbiol 162:223–230

Frans I, Michiels CW, Bossier P, Willems KA, Lievens B, Rediers H (2011) *Vibrio anguillarum* as a fish pathogen: virulence factor, diagnosis and prevention. J Fish Dis 34: 643–661

Gomez-Gil B, Herrera-Vega MA, Abreu-Grobois FA, Roque A (1998) Bioencapsulation of two different *Vibrio* species in nauplii of the brine shrimp (*Artemia franciscana*). Appl Environ Microbiol 64:2318–2322

Grisez L, Chair M, Sorgeloos P, Ollevier F (1996) Mode of infection and spread of *Vibrio anguillarum* in turbot *Scophthalmus maximus* larvae after oral challenge through live feed. Dis Aquat Org 26:181–187

Hoff KA (1989) Survival of *Vibrio anguillarum* and *Vibrio salmonicida* at different salinities. Appl Environ Microbiol 55:1775–1786

Kanno T, Nakai T, Muroga K (1989) Mode of transmission of vibriosis among ayu, *Plecoglossus altivelis.* J Aquat Anim Health 1:2–6

Lam SH, Chua HL, Gong Z, Lam TJ, Sin YM (2004) Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28:9–28

Masumura K, Yasunobu H, Okada N, Muroga K (1989) Isolation of *Vibrio* sp., the causative bacterium of intestinal necrosis of Japanese flounder larvae. Fish Pathol 24: 135–141

Muroga K, Higashi M, Keetoku H (1987) The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. Aquaculture 65:79–88

Muroga K, Yasunobu H, Okada N, Masumara K (1990) Bacterial enteritis of cultured flounder *Paralichthys olivaceus* larvae. Dis Aquat Org 9:121–125

O'Toole R, von Hofsten J, Rosqvist R, Olsson PE, Wolf-Watz H (2004) Visualization of zebrafish infection by GFP-labelled *Vibrio anguillarum.* Microb Pathog 37:41–46

Oehlers SH, Flores MV, Hall CJ, O'Toole R, Swift S, Crosier KE, Crosier PS (2010) Expression of zebrafish cxcl8 (interleukin 8) and its receptors during development and in response to immune stimulation. Dev Comp Immunol 34:352–359

Olsson JC, Jöborn A, Westerdahl A, Blomberg L, Kjelleberg S, Conway PL (1996) Is the turbot *Scophthalmus maximus* (L) intestine a portal of entry for the fish pathogen *Vibrio anguillarum?* J Fish Dis 19:225–234

Pedersen K, Grisez L, Van Houdt R, Tiainen T, Ollivier F, Larsen JL (1999) Extended serotyping scheme for *Vibrio anguillarum* with the definition and characterization of seven provisional O-serogroups. Curr Microbiol 38: 183–189

Phelps HA, Neely MN (2005) Evolution of the zebrafish model: from development to immunity and infectious disease. Zebrafish 2:87–103

Planas M, Pérez-Lorenzo M, Vázquez JA, Pintado J (2005) A model for the experimental infections with *Vibrio* (*Listonella*) *anguillarum* in first feeding turbot (*Scophthalmus maximus L.*) larvae under hatchery conditions. Aquaculture 250:232–243

Rekecki A, Gunasekara RA, Dierckens K, Laureau S and others (2012) Bacterial host interaction of GFP-labelled *Vibrio anguillarum* HI-610 with gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae. J Fish Dis 35: 265–273

Rojo I, de Ilárduya OM, Estonba A, Pardo MA (2007) Innate immune gene expression in individual zebrafish after *Listonella anguillarum* inoculation. Fish Shellfish Immunol 23:1285–1293

Rowe HM, Withey JH, Neely MN (2014) Zebrafish as a model for zoonotic aquatic pathogens. Dev Comp Immunol 46:96–107

Sansonetti PJ (2004) War and peace at mucosal surfaces. Nat Rev Immunol 4:953–964

Sieburth JM (1975) Microbial seascapes. University Park Press, Baltimore, MD

Tajima K, Ezura Y, Kirnura T (1985) Studies on the taxonomy and serology of causative organisms of fish vibriosis. Fish Pathol 20:131–142

Toranzo AE, Barja JL (1990) A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special

reference to aquaculture in the northwest of Spain. Dis Aquat Org 9:73–82

Toranzo AE, Barja JL (1993) Virulence factors of bacteria pathogenic for coldwater fish. Annu Rev Fish Dis 3:5–36

Toranzo AE, Santos Y, Lemos ML, Ledo A, Bolinches J (1987) Homology of *Vibrio anguillarum* strains causing epizootics in turbot, salmon and trout reared on the Atlantic coast of Spain. Aquaculture 67:41–52

Toranzo AE, Magarinos B, Romalde JL (2005) A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246:37–61

Weber B, Chen C, Milton DL (2010) Colonization of fish skin is vital for *Vibrio anguillarum* to cause disease. Environ Microbiol Rep 2:133–139

Willett CE, Cortes A, Zuasti A, Zapata AG (1999) Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn 214:323–336

Zaccone R, Mancuso M (2008) First report on antibody response of *Seriola dumerilii* (Risso 1810) challenged with *Listonella anguillarum*. Fish Shellfish Immunol 25: 689–692

Zhang Z, Wu H, Xiao J , Wang Q , Liu Q, Zhang Y (2012) Immune responses of zebrafish (*Danio rerio*) induced by bath-vaccination with a live attenuated *Vibrio anguillarum* vaccine candidate. Fish Shellfish Immunol 33: 36–41

Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: July 30, 2014; Accepted: March 10, 2015
Proofs received from author(s): June 12, 2015