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Mode of infection and spread of *Vibrio anguillarum* in turbot *Scophthalmus maximus* larvae after oral challenge through live feed

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ABSTRACT: The infection route of the marine fish pathogen *Vibrio anguillarum* was studied after oral challenge of juvenile turbot *Scophthalmus maximus* L. through a live feed. *Artemia* nauplii were incubated in a suspension of *V. anguillarum* cells, and subsequently fed twice to the fish. All challenged fish died within 4 d after the first challenge, while no mortality occurred in the non-challenged controls. The results of an immunohistochemical examination of the sectioned fish samples clearly demonstrated that *V. anguillarum* cells were ingested by the *Artemia* and that the latter were ingested by the fish. Bacteria were released from the *Artemia* mainly in the anterior part of the intestine. Most challenged fish started to show disease signs 24 h after the second challenge and died 2 d later. A histopathological analysis of moribund fish showed the development of septicaemia. Moreover, the sequential sampling, allowed the reconstruction of the infection route after oral challenge. Our results show that *V. anguillarum* was transported through the intestinal epithelium by endocytosis, after which the bacterium was released in the lamina propria. From there the bacterium was transported by the blood to the different organs, eventually leading to septicaemia and mortality.

KEY WORDS: *Vibrio anguillarum* · Infection route · Oral challenge · Live feed · Larviculture

INTRODUCTION

Vibriosis, caused by *Vibrio anguillarum*, is one of the most devastating infections of cultured marine fishes. In Southern Europe, sea bass *Dicentrarchus labrax* L. and turbot *Scophthalmus maximus* L. are affected by the disease (Vigneulle & Baudin Laurencin 1991). The disease is characterised as a typical haemorrhagic septicaemia, with large numbers of the pathogen present in the blood and the internal organs of the affected fish (Austin & Austin 1993). Although intensive research has been conducted on the virulence factors of *V. anguillarum*, the mode of entrance of the bacterium in fish remains unclear (Toranzo & Barja 1993).

In the literature, 2 different portals of entry have been documented. From their studies, Chart & Munn (1980), Baudin Laurencin & Germon (1987) and Kanno et al. (1989) concluded that *Vibrio anguillarum* invaded the fish through the skin, the gills or the anus. In contrast, Campbell & Bushwell (1983) and Muroga et al. (1987) suggested that *Vibrio* infections were predominantly established via the food chain.

Masumura et al. (1989) and Muroga et al. (1990) first demonstrated that intestinal necrosis of flounder larvae (INFL) could be effected in Japanese flounder through oral challenge with *Vibrio* sp. INFL incorporated into rotifers or brine shrimp. This experimental model was recently adapted for larval turbot and *V. anguillarum* by Chair et al. (1994). In their experiments Chair et al. obtained a cumulative mortality of 61% within 12 d after 1 administration of *V. anguillarum*-loaded *Artemia* to the fish.

This experimental model together with the immunohistochemical staining of sections of fish sampled at different times after challenge was used in the present study to investigate the infection route of *Vibrio anguillarum* after oral challenge, and to document the development of vibriosis in fish.

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MATERIAL AND METHODS

Fish. Juvenile turbot (36 d old) obtained from a commercial fish hatchery (France Turbot S.A., Noirmoutiers, France) were acclimated for 1 wk in a holding tank at 19°C. The water in the tank was continuously aerated and re-circulated via a biofilter. Daily, 10% of the total water volume was replaced by fresh aerated sea water. Twice daily, fish were fed with *Artemia* nauplii at a rate of 300 nauplii per fish. No mortality occurred during this acclimatisation period.

After acclimatisation, the larval turbot were distributed to four 5 l aquaria at a density of 20 individuals per aquarium. Each aquarium was continuously aerated and maintained at 19°C. Daily, faeces together with one-third of the water were siphoned off and replaced with fresh, aerated sea water.

Bacterial challenge. The challenge involved 2 groups of fish, challenged and non-challenged controls, in 2 replicates each. Thus, 40 fish were challenged and 40 served as controls. Basically, the oral challenge of the juvenile turbot was conducted as outlined by Chair et al. (1994). For this, *Artemia* nauplii, at a density of 10^3 nauplii ml^{-1}, were incubated for 1 h in a bacterial suspension (approx. 10^9 cells ml^{-1}) of a serotype O1 *Vibrio anguillarum* strain (strain 43, B. Austin, Heriot-Watt University, Scotland) in 100 ml of sterile sea water.

After incubation in the bacterial suspension, the *Artemia* nauplii were collected on a sterile 145 µm filter, washed with 10 ml of sterile sea water and subsequently resuspended in 10 ml of sterile sea water. The density of *Artemia* nauplii was determined by counting, and approximately 6000 nauplii were added to each aquarium.

Challenged fish received 2 doses of *Vibrio anguillarum*-loaded *Artemia*: the first dose at the beginning of the experiment (T_0) and the second dose 24 h later (T_{24}). Following this, non-treated *Artemia* were fed at the same feeding rate. Control fishes received non-treated *Artemia* throughout the experiment at the same feeding rate.

To estimate the bacterial load of the *Artemia* used for challenge, the same amount of *Artemia* as used for challenge (6000 *Artemia* nauplii) was transferred into a Stomacher blender (Seward Medical) and homogenised for 3 min. Serial 10-fold dilutions were prepared from the homogenate in sterile sea water and 0.1 ml of each dilution was spread-plated onto TCBS agar (Oxoid). After 24 h incubation at 26°C, yellow colonies were counted and the number of yellow colony-forming-units was expressed as the approximate number of *Vibrio anguillarum* bacteria per challenge.

Production of antisera. Polyclonal antisera were raised in New Zealand White rabbits against the *Vibrio anguillarum* serotype O1 strain: 775 (Schiewe et al. 1977).

Cultures used for immunisation were grown on brain heart infusion agar (Difco) supplemented with 1% NaCl for 24 h at 26°C. Growth was harvested into a 50 mM phosphate buffer with 0.15 M NaCl (PBS) to which formalin was added to a final concentration of 0.3%. Somatic O-antigens were prepared by heating the bacterial suspension for 1 h at 100°C in a water bath, and washing the heated cells in 3 changes of PBS. Finally, the bacterial O-antigen preparations were resuspended in PBS at a MacFarland density standard No. 3. The somatic O-antigen preparations were aliquoted in 1 ml amounts and frozen (-20°C).

Rabbits were immunised by repeated injections with the O-antigen preparation: on Day 1, subcutaneous injection of 0.5 ml antigen suspension mixed with an equal volume of Freund's Incomplete Adjuvant (FIA) (Difco), and on Days 21, 22, 23, and 24 by intravenous injections of 0.25, 0.5, 1 and 1 ml of antigen suspension, without FIA, respectively.

One week after the last injection, the animals were bled by cardiac puncture. The blood was allowed to clot and the sera were separated and stored at -20°C until used.

Histological samples. Fish were sampled at different intervals after challenge, both from the challenged group and the control group. The first sample was collected 2 h after the first administration of *Vibrio anguillarum*-loaded *Artemia* (T_2). Subsequent samples were taken at 6, 20, 26 (2 h after the second challenge), 30, 48, 72 and 96 h. For each sample, 4 challenged and 4 non-challenged fish were taken, i.e. 2 from each aquarium. With the exception of samples T_{20} and T_{96}, only living fish were collected. Sample T_{20} contained 2 dead and 2 living fish; the final sample (T_{96}) contained only dead fish.

Entire fish were fixed in Bouin's fixative for 24 h; the fixative was then washed out under running tap water, and the fish were dehydrated through ethanol and dioxane and embedded in paraffin wax at 58°C. Cross-sections of 5 µm thickness were made through entire fish and the sections were attached to gelatin-coated slides.

Immunohistochemistry. The immunohistochemical procedure applied was as described by Rimstad & Evensen (1993). Sections were deparaffinised by 2 xylene baths and rehydrated through an ethanol series (100, 96, 70 and 50%) and distilled water and subsequently washed in tris-buffered saline (TBS) (50 mM Tris-HCl, 0.8% NaCl, pH 7.6). Sections were blocked in a 5% bovine serum albumin (BSA) solution in TBS for 20 min. The primary antibody, raised against

the bacterial O-antigens, was incubated on the sections for 1 h at room temperature, diluted 1:5000 in TBS containing 2.5% BSA. After washing for 5 min in 2.5% BSA-TBS, the secondary antibody, biotinylated goat-anti-rabbit (IgG, Dakopatts, Denmark), was applied to the sections at a dilution of 1:300 in 2.5% BSA-TBS and incubated for 30 min. After washing for 5 min in 2.5% BSA-TBS, streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim, Germany) in 2.5% BSA-TBS was applied for 30 min at a dilution of 1:1000. The sections were washed in TBS for 5 min and incubated with Fast-Red (Sigma) (0.1 g l^{-1}) and naphthol-AS-MX phosphate (Sigma) (0.2 g l^{-1}) with 1 mM levamisole (Sigma) in 0.1 M Tris-HCl, pH 8.2, for 20 min.

After washing in tapwater, the sections were counterstained with Harris' haematoxylin for 2 min, washed again in tap water, and mounted with Aquamount Gurr (BDH Laboratory Supplies, UK). All steps were performed at room temperature.

As performance controls, the different steps in the staining procedure were omitted one by one, while all remaining steps were performed as usual (method specificity control). Antibody specificity controls were performed by the use of adsorbed primary antiserum prepared by the method of Mutharia et al. (1993).

RESULTS

Bacterial challenges and mortality

The bacterial loads of the *Artemia* nauplii used for the challenges were respectively: 2×10^6 *Vibrio anguillarum* cells per 6000 *Artemia* and 5×10^8 *V. anguillarum* cells per 6000 *Artemia*. Assuming that each *Artemia* nauplius contained the same number of *V. anguillarum* cells and that each turbot had eaten the same number of *Artemia* nauplii, the infection dose for each challenge would have been respectively 1×10^5 and 2.5×10^7 *V. anguillarum* cells per fish. However, it is unlikely that these assumptions are true, and variations in challenge dose between individual fish probably occurred.

Two dead fish were sampled 20 h after the first challenge (T_{20}), together with 2 apparently unaffected fish. All other fish in both challenged aquaria still appeared unaffected at that time. Clinical signs of disease were first observed at T_{48}, 24 h after the second challenge, and continued until T_{96}. From T_{48} onwards, all challenged fish appeared moribund but no additional mortalities occurred until T_{96}. At T_{96}, the remaining 12 challenged fish were dead. No mortality was observed in the non-challenged controls during the experimental period.

Immunohistochemical observations

The use of the Fast-Red TR salt as coupling salt and naphthol AS-MX phosphate as substrate for the alkaline phosphatase resulted in a bright red reaction when positive. Free bacteria thus appeared as bright red and sligthtly curved rods. The counter stain with haematoxylin allowed easy differentiation between positive (red) reactions and the blue background staining. Furthermore, the haematoxylin functioned as a normal histological stain, allowing identification of the various tissues and organs. Both the method specificity and antibody specificity controls performed were negative, demonstrating the reliability of the staining procedure and the specificity of the antiserum used.

Large numbers of *Artemia* nauplii were clearly present in the stomach of the fish sampled 2 h after the first challenge (T_2). In this sample a strong positive immunohistochemical reaction for *Vibrio anguillarum* was apparent in the gut of the *Artemia* (Fig. 1). Furthermore, positively stained bacteria were seen free in the intestinal lumen of the fish and attached to the brush border of the epithelium of the anterior part of the intestine (Fig. 2).

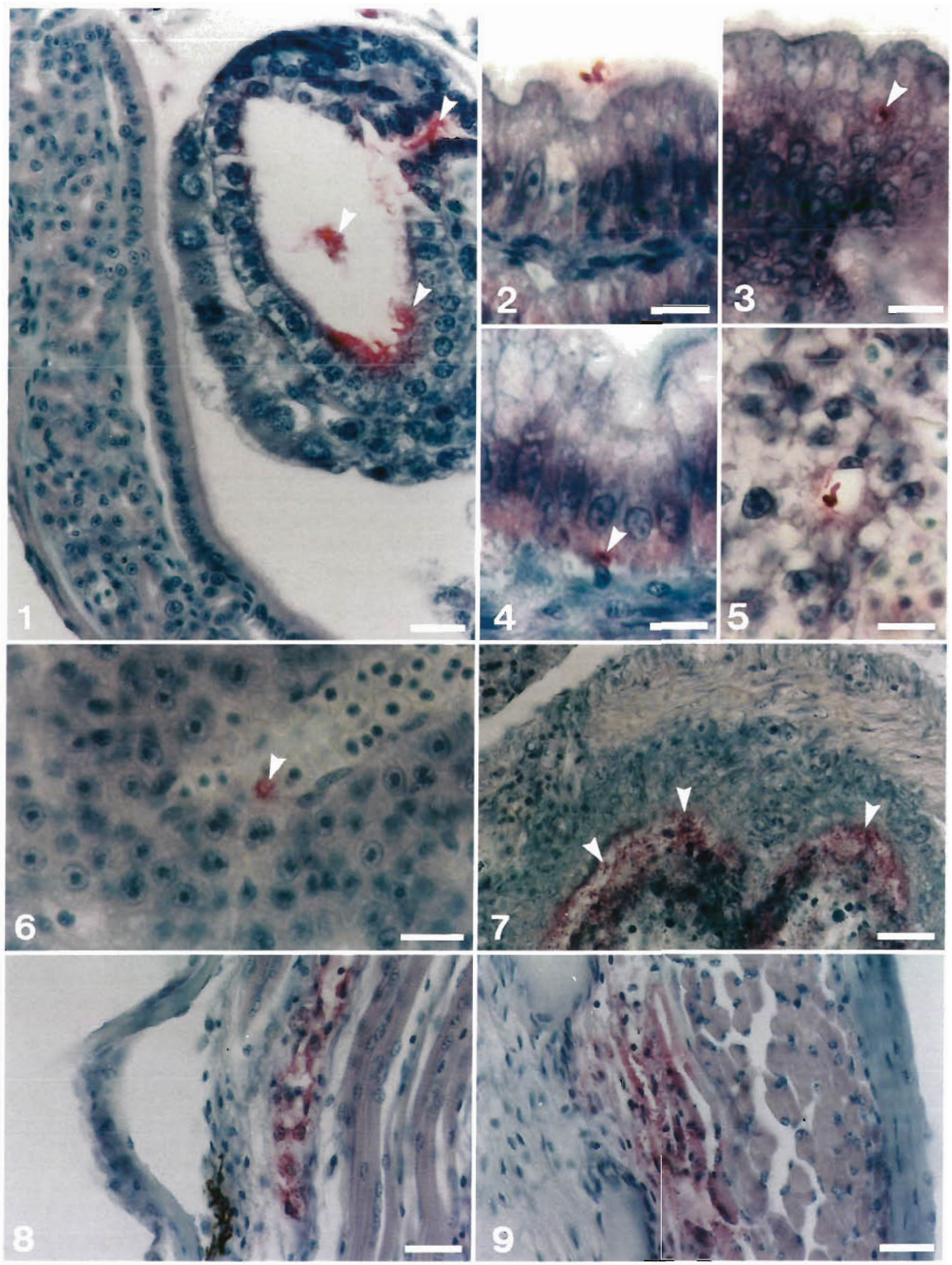
In the mucosa, bacteria were seen occasionally in epithelial cells (Fig. 3) and free in the lamina propria (Fig. 4). Intracellular bacteria, surrounded by a clear halo, probably corresponding to endosomes, could be observed in apical, more central and basal locations. Overall, the intestinal wall showed no signs of damage.

In the samples taken 6, 20 and 26 h after the first challenge (T_6, T_{20}, T_{26}), the same picture as described above was observed together with the appearance of free circulating bacteria in the capillaries of the liver (Fig. 5). The first free circulating bacteria were noticed in the liver sinusoids 6 h after challenge. In these samples a gradual shift of the *Artemia* from the stomach to the posterior intestine could be observed.

Occasionally, a positive immunohistochemical staining reaction was obtained in circulating phagocytes (Fig. 6). Examination of these positively stained phagocytes at high magnification did not reveal distinct bacterial forms.

Two dead fish, collected 20 h after the first challenge showed complete desquamation of the intestinal epithelium with numerous *Vibrio anguillarum* cells covering the luminal side of the lamina propria (Fig. 7). Furthermore, large numbers of positively stained bacteria were found in the pancreatic duct although the pancreatic parenchyma appeared normal. In these fish, no bacteria were found in other tissues or in the blood.

The second challenge manifested itself as a second massive wave of invasion, and the same infection steps as described above were observed again. Due to the higher challenge dose, many more bacteria were apparent compared to the first challenge.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Twenty-four hours after the second challenge (T_{48}), the first moribund fish were noted. From then onwards, the immunohistochemical staining of the sections showed typical signs of a bacterial septicaemia with large numbers of *Vibrio anguillarum* cells present in the blood (Fig. 8) and in various organs, including the brain (not shown).

Histopathological alterations included: the presence of fluid with numerous bacteria in the body cavities, focal muscle necrosis (Fig. 9), desquamation of the intestinal epithelium, congestion of blood vessels, and extensive liver necrosis typically characterized by karyorhexis.

Phagocytes were seen engorged with bacteria or bacterial products in virtually all internal organs. In the last sample (T_{96}), consisting only of dead fish, all tissues were heavily infected and extensive cell necrosis was noted in the tissues.

DISCUSSION

As already mentioned, the mode of infection with *Vibrio anguillarum* in fish is unclear from the literature, and several theories for the route of entry of the pathogen have been put forward based on different experimental challenge designs. Chair et al. (1994) demonstrated that the infection of larval turbot by the food chain could indeed lead to high mortality and our present results substantiate these findings. In our study, fish died within 4 d after 2 oral challenges with *V. anguillarum* delivered via *Artemia* nauplii. The immunohistochemical results clearly showed that *V. anguillarum* cells were ingested by *Artemia* and that these infected *Artemia* were in turn ingested by the turbot juveniles.

A possible explanation for the different outcomes of oral challenge by the food (as reported here and in the literature) and oral challenge by intubation could be that the *Vibrio anguillarum* cells were protected against the acidity of the stomach by the surrounding *Artemia*. As shown in our results, *V. anguillarum* cells are mainly released from the decaying *Artemia* in the anterior intestine, and not in the stomach.

An overview of our results allowed us to reconstruct the infection route followed by *Vibrio anguillarum*. The infection itself, i.e. the invasion of the internal organs, started with the attachment of *V. anguillarum* cells to the brush border of the epithelium of the anterior intestine. Subsequently these membrane-bound *V. anguillarum* cells were taken up by endocytosis and the endosomes were transported from the apical site of the epithelial cells to the basal site. At the basal site, the bacteria were released from the endosomes into the lamina propria. The passage of the intestinal epithelium by *V. anguillarum* cells proceeded very quickly. The entire sequence, from the uptake of infected *Artemia* to the release of *V. anguillarum* cells into the lamina propria occurred within 2 h, since these different steps were all observed in the first sample, taken 2 h after the administration of *V. anguillarum*-loaded *Artemia* to the fish.

The trans-epithelial transport of 'dead' bacterial cells by endocytosis has previously been described in studies on the uptake of bacterial antigens by the gut epithelium (Vigneulle & Baudin Laurencin 1991, Olafson & Hansen 1992). Because the process observed in our samples with 'live' *Vibrio anguillarum* cells was not different from that of 'dead' bacterial cells described in previously mentioned reports, it seems likely that the bacteria took no active part in this transport. The binding of *V. anguillarum* to the brush border itself, however, might be a feature which determines the virulence of *V. anguillarum*, as demonstrated by Horne & Baxendale (1983).

Once the *Vibrio anguillarum* cells were released in the lamina propria, they were probably transported by the vena porta to the liver, and thence to the other tissues via the blood. When present in the blood stream, the bacterium faces the immune system of the fish, including circulating phagocytes which scavenge the circulating bacteria. Several virulence factors have been described for *V. anguillarum* that enable them to survive and multiply in the blood of non-immune fish. Trust et al. (1981) demonstrated that virulent *V. anguillarum* strains are resistant to the bactericidal effect of fish serum, and a highly efficient iron sequestering

Fig. 1 to 9. *Vibrio anguillarum* infection in *Scophthalmus maximus* after oral challenge. LM. Immunohistochemical staining. Infection route of *V. anguillarum* in turbot after oral challenge through the live feed. Fig. 1. *Artemia* nauplii in the stomach of turbot, 2 h after infection. A strong positive immunohistochemical reaction in the gut of these *Artemia* (arrows) illustrates the presence of *V. anguillarum*. Fig. 2. *V. anguillarum* attached to the microvilli (brush border) of the intestinal epithelium. Fig. 3. Intracellular *V. anguillarum* cell surrounded by a clear halo (arrow), representing an endosome in the intestinal epithelium. Fig. 4. Release of *V. anguillarum* from an endosome into the lamina propria (arrow). Fig. 5. Free circulating *V. anguillarum* cells in a liver sinusoid (arrow). Fig. 6. Positive reaction obtained in a circulating phagocyte in the liver. Fig. 7. Dead fish sampled 20 h after infection showing severe desquamation of the intestinal epithelium and large numbers of *V. anguillarum* cells at the luminal side of the lamina propria (arrows). Fig. 8. Congestion of a blood vessel, with *V. anguillarum* cells associated with the blood vessel wall. Fig. 9. Focal muscle necrosis, showing numerous *V. anguillarum* cells. Figs. 1, 7, 8 & 9: scale bar = 20 µm. Figs. 2 to 6: scale bar = 10 µm

system has been described for this pathogen (Crosa 1980). Both attributes probably enable the pathogen to survive and to proliferate in the blood, thus eventually leading to septicaemia and death of the host.

The positive staining obtained in circulating phagocytes indicates that *Vibrio anguillarum* was phagocytosed. Because no bacterial forms could be distinguished in these cells, the results suggest that *V. anguillarum* cells were broken down and that the remaining cell wall fragments reacted with the antiserum.

In the 2 fish that died 20 h after the first challenge the histopathological changes were characterised by a severe desquamation of the intestinal epithelium together with the presence of bacteria at the luminal side of the lamina propria and in the ductus pancreaticus. No systemic spread of *Vibrio anguillarum* was noted in these fish. It is unclear why only these 2 fish showed this extensive epithelial necrosis, while all other fish sampled at that time did not show any cell damage. Two possible explanations must be considered. The epithelial necrosis might be a post mortem phenomenon or might indeed be related to massive infection. As it is unlikely that each fish would have eaten the same amount of *Artemia*, some fish would have been exposed to a larger challenge dose than others. Based on the massive epithelial desquamation and the presence of large numbers of *V. anguillarum* cells at the luminal side of the lamina propria, we assume that these 2 particular fish had eaten a large number of *Artemia* nauplii, and as a result were suddenly exposed to large numbers of *V. anguillarum* cells attacking the intestinal epithelium. The epithelial desquamation and the subsequent loss of body fluids possibly caused the sudden mortality.

All other fish started to show visible disease signs only after the second challenge. The immunohistology of these fishes clearly demonstrated all typical signs of a bacterial septicaemia, i.e. the proliferation of *Vibrio anguillarum* in the blood and the internal organs. The secretion of bacterial extracellular enzymes (reviewed by Toranzo & Barja 1993) in the congested capillaries probably accounted for the observed tissue necrosis and eventual death of the fish. The histological results obtained during the septicaemic phase are in agreement with the study of Chart & Munn (1980).

In this study, the infection route of *Vibrio anguillarum* in larval turbot after oral challenge by the food chain has been elucidated. Our results clearly show that *V. anguillarum* is taken up by the intestinal epithelium by endocytosis and that it is subsequently released into the lamina propria. From the lamina propria the bacterium is transported via the blood to the liver and other internal organs. The presence of *V. anguillarum* in the blood can lead to septicaemia if the infective dose is sufficiently high.

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