First report of *Vibrio anguillarum* isolation from diseased big scale sand smelt *Atherina boyeri*

M. Yiagnisis [1]*, I.N. Vatsos [2], C. Kyriakou [3] and M. Alexis [1]

[1] Fish Pathology and Nutrition Laboratory, Aquaculture Institute, Hellenic Center for Marine Research, Agios Kosmas, 16604 Hellinikon, Athens, Greece; [2] Department of Animal Production, Ichthyology Ecology and Protection of Environment, Faculty of Veterinary Medicine Aristotle University of Thessaloniki, Thessaloniki, Greece; [3] Eparcheio Limnou, Department of Agricultural Development, Limnos, Greece.

Abstract

Vibriosis is an important disease of farmed and wild fish, caused by species of the genus *Vibrio*. During December 2000, high mortalities were observed in a wild population of big scale sand smelt *Atherina boyeri* in Limnos, Greece. The microbiological analysis of the moribund fish resulted in the isolation of a pure culture of *Vibrio anguillarum*. The bacterium was identified by bacteriological procedures and slide agglutination reaction. Focal or more extensive necrosis was found in almost all organs, in all fish examined and the retina of the eyes appeared corrugated. The pathogenicity of the strain for sea bass was confirmed by bath challenge causing mortalities up to 97%. Factors contributing to the outbreak of the disease were considered to be the presence of parasites (Platyhelminthes) in the intestinal tract. No simultaneous infection at a fish farm in the vicinity had been reported. However, since there are disease interactions between wild and cultured fish, infected wild fish, can act as a reservoir of the pathogen.

Introduction

Several v*ibrios* cause diseases, referred to in the literature as "vibriosis", in marine fish, both wild and cultured (Colwell & Grimes, 1984). *Vibrio anguillarum,* a gram-negative, facultative anaerobic, non-spore forming bacterium, oxidase positive, is considered to be the main causative agent of vibriosis. (Sørensen & Larsen, 1986; Egidius, 1987; Austin & Austin, 1999). Canestrini (1893) was the first to isolate this bacterium in eel (*Anguilla anguilla*), and subsequently this pathogen has been identified in approximately 50 species of fresh- and salt-water fish in many geographical areas (Actis et al., 1999). In wild fish, *V. anguillarum* has been associated with mortalities in sea mullet, (*Mugil cephalus*) (Burke, 1981), saithe (*Pollachius virens*) (Håstein & Smith, 1977; Myhr et al., 1991), wild turbot (*Scophthalmus maximus*) (Toranzo et al., 1985), golden grey mullet (*Mugil auratus*) (Blanch & Jofre, 1992) and ayu (*Plecoglossus altivelis*) (Muroga et al., 1984). In the Mediterranean area *V. anguillarum* causes vibriosis mainly in cultured sea bass (*Dicentrarchus labrax*), sea bream *(Sparus aurata*) and snarpsnout sea bream (*Puntazzo puntazzo)* (Yiagnisis et al., 1999). Susceptibility to *Vibrio* infections is related to several environmental and host factors, which can

* Corresponding author's E-mail: mayia@ath.hcmr.gr

cause stress to fish (Anderson, 1990). This paper reports on the first isolation of *V. anguillarum* from a wild population of diseased big scale sand smelt (*Atherina boyeri*) in Greece.

Materials and methods

History

In December 2000, 10 days after intensive raining, an outbreak of disease occurred in wild stocks of sand smelt around the island of Limnos, Greece. The incident took place in the area around the city of Mirina (Figure 1). The seawater temperature was between 11^{0}C and 12^{0}C. Sand smelts were reported to be lethargic with dark coloration and white spots at the base of the dorsal fin and/or the tail. In some cases, these spots became red (bleeding) and there were also petechiae at the base of the fins. Concerning the number of dead fish found in the different sites, more dead fish were found at sites B and D, while a few dead fish were reported at sites A, C, E and F. Figure 1 shows the sites of the appearance of the incidence with the diseased sand smelts and the sites of the samplings. Moribund fish were sampled twice from B and D sites with an interval of one week and transported on ice to the laboratory. On arrival, they underwent a full postmortem examination, which involved parasitological, histological and microbiological examinations. The fish weighed between 5 to 10 g. A total of 40 fish were analysed. Twenty fish (10 fish from each sampling) were used for microbiological examinations, 10 fish for histological examination and 10 fish for parasitological examination. Samples for microbiological examination were obtained aseptically (10 fish

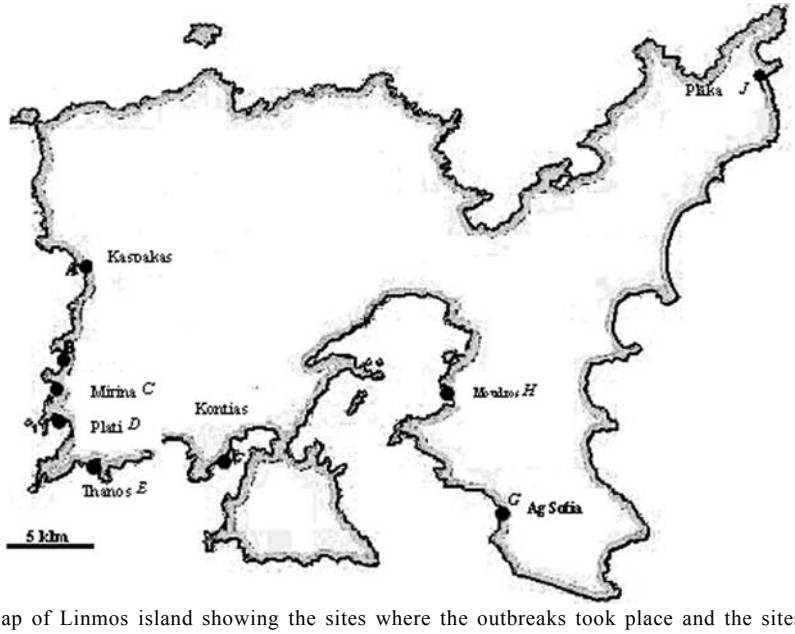

Figure 1. Map of Linmos island showing the sites where the outbreaks took place and the sites where the various samples were taken.

from each sampling) from head kidney and brain and were subsequently inoculated on tryptic soy agar (TSA, Oxoid), tryptic soy broth (TSB, Oxoid) supplemented with 2% NaCl and thiosulphate-citrate-bile salts-sucrose agar (TCBS Oxoid). The cultures were incubated at 22^{0}C for 24-48 h. Identification of the isolates obtained was performed using the commercial miniaturized API 20E system (Biomerieux) modified for marine isolates (Biosca et al., 1993). The confirmation of the diagnose *V. anguillarum* was performed by the slide agglutination test using Mono-Va kit, Bionor (Romalde et al., 1995). Antibiotic sensitivity test was performed on Muller Hinton agar by the diffusion method (Woods & Washington, 1995). The following antibiotics were used: oxytetracycline (30µg), oxolinic acid (2µg), amoxicillin (10µg), ampicillin (10µg), furazolidone (50µg), trimethoprim and sulfamethoxazole (1.25 and 23.75 µg), flumequine (30µg). The inhibition zone was estimated, by measuring the diameter in mm, across the center of the antibiotic impregnated disc to the point where the pathogen could be seen growing.

Parasitological examination of fresh smears and the histological examination of sections of organs was carried out according to the procedures described by Noga (2000). Tissue samples were fixed in 10% neutral buffered formalin, processed and embedded in paraffin and sections were stained with haematoxylin & eosin and Giemsa, according to the methods described by Bullock (1989).

Experimental infection

The pathogenicity of one of the isolated strains was studied. Two hundred and forty healthy unvaccinated sea bass (*Dicentrarchus labrax*), weighing about 1 g, were obtained from a commercial farm. The fish were divided into 6 groups of 40 each and were held in 25 l tanks supplied with re-circulated seawater, (temperature 21-22^{0}C and salinity 38 ‰). Twenty-four-hour old colonies of *V. anguillarum* isolated from the outbreak in Limnos, grown on TSA (Oxoid) supplemented with 2% NaCl, were collected into sterile phosphate buffered saline (PBS) and centrifuged at 5000g for 10 min. The resulting pellet was re-suspended in sterile PBS. This bacterial suspension was used for the experimental infection. The infection was performed by 10 min. bath exposure, using 6x10^7 CFU of the bacterium/ml of sterile PBS, in three of the six tanks. In the remaining three tanks (control tanks) the fish were immersed for 10 min in sterile PBS without bacteria. The fish were daily monitored for a period of 15 days and the dead fish were collected and examined bacteriologically. Bacteria re-isolated from the kidney of moribund or dead fish on TSA supplemented with 2% NaCl at 22^{0}C, were identified as *Vibrio anguillarum* by biochemical and immunological tests as previously.

Results

Gross examination

Dark coloring and white spots at the base of the dorsal fins and/or the tail were observed in all big scale sand smelt sampled during the outbreak. In some cases, dermal hemorrhages were also observed. Examination of the gills using light microscopy revealed the presence of myxobacteria-like organisms. No ecto-parasites were observed in the gills. Examination of the smears prepared from the guts revealed the presence of

Gram stain	Negative
Morphology	Bacillus
Motility	+
Oxidase	+
Growth with NaCl (0%)	-
Growth with NaCl (2%)	+
Growth on TCBS	+ (Yellow colonies)
β-galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate utilization	+
H2S production	-
Urease	-
Tryptophan deaminase	-
Indole	+
Voges-Proskauer	+
Gelatin hydrolysis	+
Use of:	
Glucose	+
Manitol	+
Inositol	-
Sorbitol	+
Rhamnose	-
Sucrose	+
Melibiose	-
Amygdalin	-
Arabinose	-
Resistance to: O129 10μg	+
Resistance to: O129 150μg	-
Resistance to: Ampicillin 10μg	+
Swarming	-

Table 1. Cultural and biochemical characteristics of *Vibrio anguillarum* isolated from sand smelt in Greece.

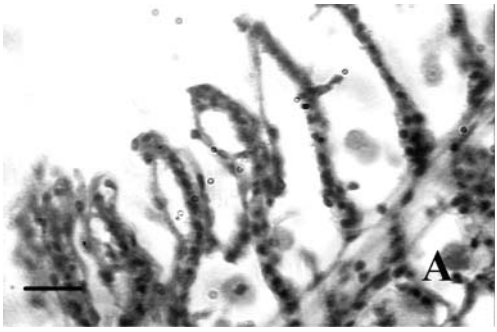

Figure 2A. Gills, extensive separation of the epithelium, H.E, bar = 10 μm.

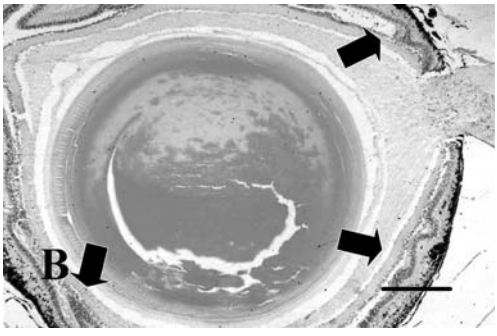

Figure 2B. Eye, corrugation of the retina (arrows), H.E., bar = 500 μm.

platyhelminthes.

Bacteriological examination

Twenty bacterial isolates were characterized and identified from the twenty examined sand smelts. Another forty five bacterial isolates were characterized and identified from challenged sea bass. All sixty five isolates were identical morphologically and biochemically and all were identified as *V. anguillarum*. The morphological, biochemical and cultural characteristics of *V. anguillarum* isolated from fish are shown in Table 1. Antibiotic sensitivity results showed that flumequin, oxytetracycline, oxolinic acid and sulfamethoxazole -trimethoprim inhibited the growth of the bacterium.

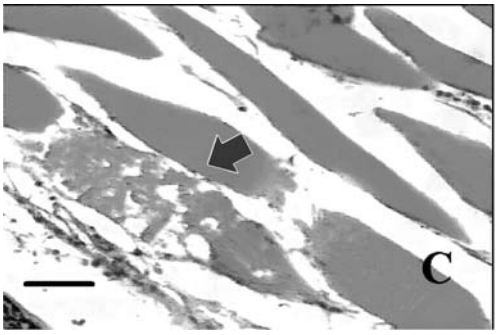

Figure 2C. Muscles, myophagia, H.E. bar= 100 µm.

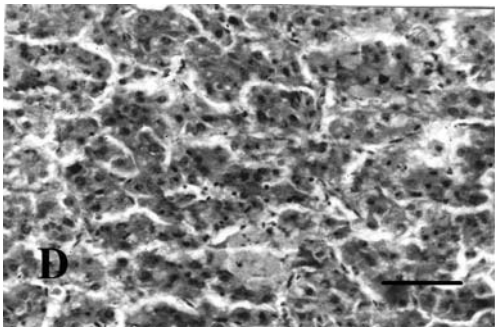

Figure 2D. Liver, vacuolation and necrosis, H.E. bar= 100 µm.

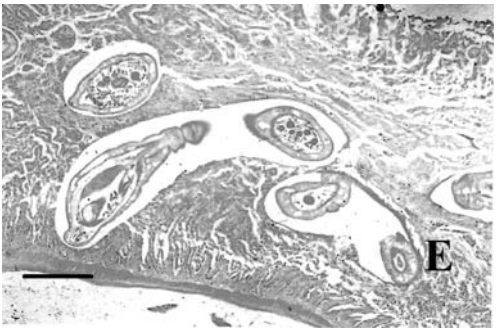

Figure 2E. Intestine, heavy parasitic infestation, H.E. bar= 500 µm.

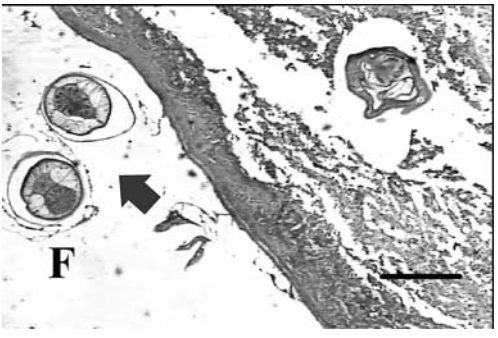

Figure 2F. Intestine, parasites encysted around the intestine, H.E. bar= 500 µm.

Histological examination

In general, focal or more extensive necrosis was found in almost all organs, in all fish examined from the outbreak. In the gills, extensive separation of the epithelial cells was observed and as a result, secondary lamellae appeared quite thin (Figure 2A). The retina of the eyes appeared corrugated (Figure 2B, arrows). Focal degenerative and inflammatory changes in the skeletal muscles were noted in five fish. These changes included hyaline degeneration, lysis of sarcoplasm, infiltration with macrophages and in some cases, myophagia (Figure 2C). In the liver, swelling, vacuolation and in many cases necrotic cells were observed in all fish (Figure 2D). In the alimentary canal, a heavy parasitic infestation by platyhelminthes and sloughing of the intestinal mucosa were observed in all fish examined (Figure 2E,F). In addition, encysted larval stages were detected around the gut (Figure 2F, black arrow). In the spleen,

Group	Tank 1	Tank 2	Tank 3
Control	40	40	40
Total moribund/dead	0	0	0
% cumulative mortality	0	0	0
	Tank 4	**Tank 5**	**Tank 6**
Challenged	40	40	40
Total moribund/dead	39	38	39
% cumulative mortality	97.5	95	97.5

Table 2. Number of fish for the experimental infection.

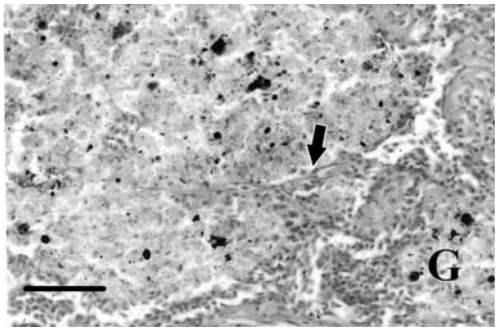

Figure 2G. Spleen, necrosis surrounded by epithelioid cells (arrow), H.E. bar= 100 µm.

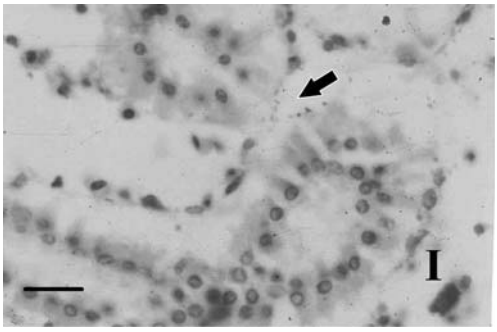

Figure 2I. Kidney, depletion of the haemopoietic tissue and bacteria (arrow), Giemsa, bar= 20 µm.

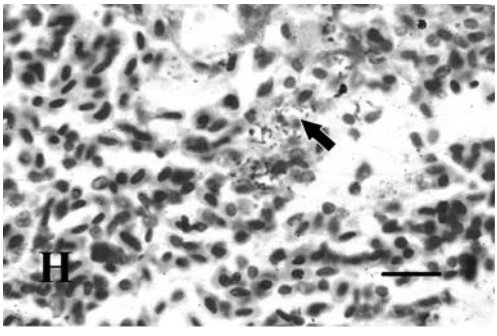

Figure 2H. Spleen, bacteria (arrow), Giemsa, bar= 20 µm.

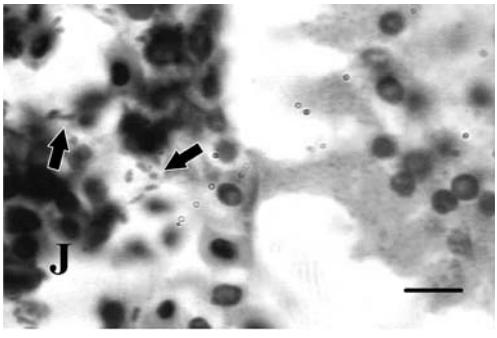

Figure 2J. Kidney, bacteria (arrow), Giemsa, bar= 10 µm.

the necroses observed were focal, surrounded by inflammatory and epitheloid cells (Figure 2G, arrow). Finally, the kidneys of all the fish examined were characterized by an almost complete depletion of the haematopoietic tissue (Figure 2I) associated with some tubular necrosis. When tissue sections were stained with Giemsa, many small rod-shaped bacteria were observed throughout the spleen and kidney tissue of almost all fish examined from the outbreak (Figure 2H, I, J).

Experimental infection

In the experimental infection, 97% (mean mortality) of the fish died over a period of 15 days post-challenge (Table 2). *Vibrio anguillarum* was re-isolated from the kidneys of all dead fish. Clinically, the affected fish showed darkening of the body and hemorrhages on the base of the fins. No mortality was observed in the control fish during the experiment.

Discussion

Although originally, the term 'salt water furunculosis' has been used to describe *Vibrio* infections, today 'vibriosis' is the prevailing name. In farmed fish, vibriosis usually occurs in warm weather, particularly when stocking densities are high and when salinities and organic loads are high. In the case we report, the temperature was however low, 11-12^{0}C. Maeda et al. (2003) have also reported occurrence of vibriosis at low temperatures.

Possible factors contributing to the occurrence of bacterial diseases may be water quality, virulence of bacterial strains and other factors inducing stress to fish (Actis et al., 1999). In the present study, a possible factor contributing to the outbreak of the disease was the presence of parasites (Platyhelminthes) in the intestine. Gastric parasites (Platyhelminthes) were found, sometimes in high number in populations of *Atherina boyeri,* as Alessio et al. reported (1990). It has also been reported that debilitating attacks by these helminthes may be accompanied by invasions of viruses, bacteria or fungi. (Thorney & Hargis, 1991).

The histological findings suggest extensive septicemia and systemic infection especially in iron-rich filtering organs, such as spleen and kidney and this is characteristic in infections of the species *V. anguillarum.* Muscle lesions were also observed and this may be the result of proteases induced by the bacterium (Norqvist et al, 1990). No bacteria were however observed in those lesions. On the other hand, the role of myxobacteria found in the fresh preparations of the gills, and their relation with the gill lesions are not clear.

Vibrio anguillarum is serologically diverse and up to ten "O"antigen serotypes have been described (Sørensen & Larsen, 1986). Most of the vibriosis outbreaks throughout the world are caused by only 2 serotypes: O1 and O2 (European serotype designation) (Toranzo & Barja, 1990). Strains belonging to serotypes O3 to O10 have been mainly isolated from marine environmental samples, including water, sediment, phytoplankton and zooplankton. In the present study we used Bionor kit to serologically confirm the species. This kit does not distinguish the different serotypes.

Although we have isolated some strains of *V. anguillarum* that were arabinose positive, the majority of our isolates, from farmed fish, have been arabinose negative, as the strains isolated in the present study. Håstein & Smith (1977), found all isolates of *V. anguillarum,* from farmed salmonids, arabinose positive but those from wild fish, more commonly appeared arabinose negative.

Concerning the source of the pathogen, it was suggested that the sand smelts were infected via contact to other infected or carrier fish, farmed or wild. This has also been suggested by other authors (Muroga & Egusa, 1988; Kanno et al., 1989). In addition, Hoff (1989) has shown that *V. anguillarum* is able to survive in seawater for more than 50 months.

Interestingly, even though in most cases of vibriosis, cultured fish are mainly affected, in the present study no simultaneous infection at other fish farms in the vicinity were reported. However, since there are disease interactions between wild and cultured fish, infected wild fish, as reported in the present study, can act as reservoir of the pathogen.

In this study we report for the first time an infection of wild sand smelt *Atherina boyeri* by *V. anguillarum*. In the appearance of the disease a contributing factor was suggested to be stress due to parasitic infestation and possibly the change in the quality of the water due to preceding rain. These infected fish could act as potential reservoir for the bacterium.

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