Vol. 35: 77–80, 1999 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published January 7

NOTE

Isolation and characterization of *Vibrio parahaemolyticus* causing infection in Iberian toothcarp *Aphanius iberus*

Elena Alcaide*, Carmen Amaro, Rafael Todolí, Rafael Oltra

Departamento de Microbiología y Ecología, Facultad de Ciencias Biológicas, Universitat de València, Dr. Moliner 50, E-46100 Valencia, Spain

ABSTRACT: High mortality among laboratory cultured Iberian toothcarp *Aphanius iberus* occurred in February 1997 in Valencia (Spain). The main signs of the disease were external haemorrhage and tail rot. Bacteria isolated from internal organs of infected fish were biochemically homogeneous and identified as *Vibrio parahaemolyticus*. The bacteria were haemolytic against erythrocytes from eel *Anguilla anguilla*, amberjack *Seriola dumerili*, toothcarp *A. iberus* and humans, and were Kanagawa-phenomenon-negative. Infectivity tests showed that the virulence for *A. iberus* was dependent on salinity. Finally, all strains were virulent for amberjack and eel.

KEY WORDS: *Vibrio parahaemolyticus* · Vibriosis · Fish disease · *Aphanius iberus*

Vibrio species such as *V. anguillarum*, *V. alginolyticus*, *V. ordalii*, *V. salmonicida* and *V. vulnificus* biotype 2 (serovar E) are pathogens for marine species of fish (Hjeltnes & Roberts 1993). These species cause haemorrhagic septicaemia in marine fish and may lead to substantial mortalities in cultured populations (Wong et al. 1990). Among the genus *Vibrio*, the species *V. parahaemolyticus* is a marine bacterium which causes acute gastroenteritis and food poisoning in humans who consume raw or improperly cooked seafood (Hlady & Klontz 1996, Pan et al. 1997).

The Iberian toothcarp *Aphanius iberus* is a cyprinodontid native to the Iberian peninsula. This fish is euryhaline and found in salt marshes and coastal lagoons (Sanz 1985). It is considered an endangered species (Blanco & González 1992), with decreasing numbers attributed to pollution and destruction of its habitats, as well as competition from allochthonous species such as eastern mosquitofish *Gambusia holbrooki*. In 1996 work began on the reproduction of Iberian toothcarp at different salinities. During February-March 1997, an infectious disease occurred in the fish maintained in laboratory culture tanks. Diseased fish first appeared in tanks with 30‰ salinity, and later in the tanks maintained at 5 and 15‰ salinities. The affected fish showed tail rot, red spots on the head and a swollen intestine containing ascitic fluid. Fish held in tanks at 45 and 60‰ salinities were not affected. Mortality was high and all fish in the affected tanks died within 7 d. Pure cultures of a bacterium identified as *V. parahaemolyticus* were isolated from internal organs of all moribund fish. In this paper the authors report on the isolation and characterization of *V. parahaemolyticus* from diseased Iberian toothcarp for the first time.

Material and methods. ***Microbiological analysis:*** Diseased fish were 3 mo old and the water temperature was above 25°C when the outbreak was registered. Samples from the surface and liver of moribund fish were analysed. Each sample was streaked on thiosulphate citrate bile salt agar and tryptone soy agar supplemented with 3% NaCl (TCBS-3 and TSA-3 respectively). All plates were incubated at 25°C for 24 to 48 h. Isolates were characterized by morphological tests and API 20E strips (BioMérieux). Similar analysis was made of the water from the tanks in which the fish were held as well as the food supply. Further characterization of the isolates was carried out by additional biochemical tests (Table 1) and type strain *Vibrio parahaemolyticus* ATCC 17802 was included as a control.

Enzymatic and haemolytic activities: Enzymatic and haemolytic activities of isolates were analysed by spot inoculations on TSA-3 supplemented with skimmed milk (2% wt/vol), Tween 80 (1% wt/vol), egg yolk emulsion (2% wt/vol), fibrinogen (0.28% wt/vol)

*E-mail: elena.alcaide@uv.es

Table 1 *Vibrio parahaemolyticus*. Biochemical and morphological characterization of isolates from tooth-carp *Aphanius iberus* and *V. parahaemolyticus* ATCC 17802. TCBS: thiosulphate citrate bile salt agar; ONPG: ortho-nitrophenyl-galactoside; VP: Voges-Proskauer test

Test	Strains (n = 12)	*V. parahaemolyticus* ATCC 17802
Growth on TCBS	G[a]	G
Gram stain	–	–
Cytochrome oxidase	+	+
O/129 sensitivity:		
10 µM	+	+
150 µM	–	–
Growth at % NaCl:		
0%	–	–
3%	+	+
6%	+	+
8%	+	+
10%	+	+
Growth at:		
4°C	–	–
25°C	+	+
37°C	+	+
40°C	+	+
β-Galactosidase (ONPG test)	–	–
Arginine dihydrolase	–	–
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
Citrate	–	–
Production of H_2S	–	–
Urease	–	+
Tryptophan deaminase	–	–
Indol	+	+
VP test	–	–
Gelatin liquefaction	+	+
Acid from:		
Glucose	+	+
Mannitol	+	+
Inositol	–	–
Sorbitol	–	–
Rhamnose	–	–
Sucrose	–	–
Melibiose	–	–
Amygdalin	–	–
Arabinose	+	+
Lactose	–	–
Cellobiose	+	+

Test	Strains (n = 12)	*V. parahaemolyticus* ATCC 17802
Sole carbon source:		
DL-Maltose	–	–
L-Rhamnose	+	+
D-Mannose	(+)[b]	(+)[b]
Cellobiose	–	–
Sucrose	+	+
D-Galactose	+	+
L-Arabinose	+	+
D-Xylose	+	(+)[b]
Citrate	–	–
Propionate	+	+
D-Gluconate	+	+
D-Sorbitol	(+)[b]	(+)[b]
DL-Glycerate	+	+
D-Glucuronate	–	–
p-Hidroxibenzoate	+	+
γ-Aminobutyrate	+	+
L-Manite	+	+
Myo-inositol	+	+
L-Serine	+	+
L-Tyrosine	+	+
L-Alanine	+	+
L-Asparagine	+	+
L-Leucine	+	+
L-Histidine	–	–
Citrulline	+	+
Hydrolysis of:		
Casein	+	+
Starch	+	+
Tween 80	+	+
Fibrinogen	+	+
Lipase (egg yolk)	+	–
DNase	+	+
Mucine	–	–
Elastin	–	–
Collagen	–	–
Haemolysis of:		
Eel erythrocytes	+	+
Yellowtail erythrocytes	+	+
Kanagawa test	–	–

[a]Green colonies on TCBS agar; [b]weakly positive

and elastin (0.1% wt/vol), respectively, as previously described (Amaro et al. 1992). DNase production was assayed on DNase agar (Oxoid). Haemolysis of toothcarp *Aphanius iberus*, eel *Anguilla anguilla*, amberjack *Seriola dumerili* and human blood was tested on TSA-3 supplemented with 1% freshly obtained erythrocytes (Amaro et al. 1992). Plates were incubated at 25 or 37°C for testing haemolysis of fish or human erythrocytes respectively. The Kanagawa test was performed using Wagatsuma agar (Nishibushi et al. 1989) supplemented with human 5% erythrocytes. Haemolysis and Kanagawa plates were streaked in duplicate, plates were spot inoculated with 10 µl of overnight cultures on basal medium (BM) and BM supplemented with 5 mM taurocholic acid (Sigma) as described by Osawa & Yamai (1996).

Infectivity tests: The 50% lethal dose (LD_{50}) test, with batches of 8 fish per dose, was conducted by intraperitoneal (i.p.) injection as previously described (Amaro et al. 1992). Briefly, Iberian toothcarp (mean weight 2 g $fish^{-1}$) were injected with 0.05 ml of a bacterial suspension containing 10^8 to 10^4 cfu ml^{-1} (determined by optical density at 600 nm), in phosphate-buffered saline (PBS). Sterile PBS was injected

i.p. into fish as control. Experimental infections were carried out at salinities of 5, 15, 30 and 45‰, at 20°C, in duplicate. Mortalities were recorded daily for 7 d and were only considered infected if *Vibrio parahaemolyticus* was recovered from assayed fish. The LD_{50} was calculated by the method of Reed & Muench (1938). The assays for pathogenicity were also made on elvers (5 to 8 g $fish^{-1}$) and amberjack (100 g $fish^{-1}$) at 15 and 30‰ salinity respectively.

Results. ***Microbiological analysis:*** Pure cultures were obtained from liver and ascitic fluid of diseased fish. Colonies were 3 to 4 mm and opaque on TSA-3 and green on TCBS. The strains were Gram-negative straight rods, motile, oxidase- and catalase-positive, sensitive to the vibriostatic O/129 at 150 µM and fermentative. Isolates were first characterized by API 20E (BioMérieux) strips, which gave profiles typical of *Vibrio parahaemolyticus*. On the basis of these results and those listed in Table 1, the isolates were identified as *V. parahaemolyticus*. *V. parahaemolyticus* strains were also recovered from the water of the tank and the skin of the fish, but analysis of food supply gave a negative result.

Enzymatic and haemolytic activities: All isolates showed identical enzymatic and haemolytic activity (Table 1). Isolates tested, including the type strain, were haemolytic against *Aphanius iberus*, eel, amberjack and human erythrocytes. Cells grown in a medium containing taurocholic acid produced prominent haemolysis on blood agar compared with cells grown on BM, which confirms previously reported data on the enhancement of virulence factors production in bile salt media (Osawa & Yamai 1996). The Kanagawa test was negative in all isolates independent of whether the strains had previously been grown on bile salt medium.

Infectivity tests: In infectivity assays, salinities between 5 and 30‰ gave similiar LD_{50} results, since lethal doses were around 10^6 cfu $fish^{-1}$ in both cases (Table 2). At 45‰ salinity, mortalities were only achieved when more than 10^9 cells were injected in fish; therefore, these strains were classified as avirulent at the higher salinity (Santos et al. 1988). This agrees with the fact that the infection occurred at salinities between 5 and 30‰ and fish held at 45 and 60‰ salinity were not affected. Pure cultures of *Vibrio parahaemolyticus* were reisolated from liver and skin of moribund fish. No mortality was detected in the controls. External signs, which included haemorrhage, exophthalmia and occasionally ulceration, but no tail rot, appeared 1 d after i.p. injection and mortalities began 2 to 7 d post-challenge. LD_{50} was assessed in amberjack and eel by i.p. infections and was 5×10^3 cfu $fish^{-1}$ and 6.2×10^5 cfu $fish^{-1}$ respectively (Table 2).

Table 2. *Vibrio parahemolyticus*. Virulence (cfu $fish^{-1}$) at different salinities. ND: not determined

Salinity (‰)	Virulence[a] Toothcarp	Elver	Amberjack
5	+ (6.13×10^6)	ND	ND
15	+ (5.3×10^6)	+ (6.2×10^5)	ND
30	+ (3.1×10^6)	ND	+ (5×10^3)
45	- (>10^9)	ND	ND

[a]No. of bacteria needed to kill 50% of the inoculated animals in a 7 d period

Discussion. We have isolated *Vibrio parahaemolyticus* as the causative agent of infection in Iberian toothcarp *Aphanius iberus* and have reproduced the same gross signs observed in the outbreak after bacterial challenge. All isolates were haemolytic on Iberian toothcarp, eel, amberjack and human erythrocytes, and Kanagawa-phenomenon-negative. The Kanagawa phenomenon has been related to thermostable direct haemolysin (TDH) production, and a strong association between TDH and pathogenenicity has been established (Osawa & Yamai 1996). However, outbreaks of gastroenteritis caused by Kanagawa-negative strains have been reported (Honda et al. 1988). These Kanagawa-negative strains produce a TDH-related haemolysin, which is immunologically similar to TDH, but different in physico-chemical and haemolytic activities (Osawa et al. 1996).

Vibrio parahaemolyticus is considered as a human pathogen more than a fish pathogen. To our knowledge, there are only 2 reports linking it to fish infections (Wong et al. 1990, Yii et al. 1997). In both papers this species is described as the causative agent of vibriosis in groupers *Epinephelus coioides* in Taiwan. This is the first description of a bacterial pathogen affecting Iberian toothcarp. Moreover, this *Vibrio* species has not been previously reported as fish pathogen in the Mediterranean area. In order to assess the virulence of the isolates to other fish, lethal dose for amberjack and eel was also established. These fish are representative of the species cultured along the Mediterranean coast of Spain. Amberjack are extensively cultured in seawater, and eels are cultured in water at low salinity (0.2%). The results confirm the bacteria as virulent for other fish at salinities approximating that of seawater.

Acknowledgements. This study was supported by grant GV-D-AG-02-138-96, from the Conselleria de Cultura, Educació i Ciència, Generalitat de València (Spain). We thank Conselleria de Medi Ambient, Generalitat de València, for supplying the fish.

LITERATURE CITED

Amaro C, Biosca EG, Esteve C, Fouz B, Toranzo AE (1992) Comparative study of phenotypic and virulence properties in *Vibrio vulnificus* biotypes 1 and 2 obtained from an European eel farm experiencing mortalities. Dis Aquat Org 13:29–35

Blanco JC, González JL (1992) Libro rojo de los vertebrados de España. ICONA-CSIC, Serie Técnica, Madrid

Hjeltnes B, Roberts RJ (1993) Vibriosis. In: Inglis V, Roberts RJ, Bromage N (eds) Bacterial diseases of fish. Blackwell Scientific Publications, London, p 109–121

Hlady WG, Klontz KC (1996) The epidemiology of *Vibrio* infections in Florida, 1981–1993. J Infect Dis 173:1176–1183

Honda T, Ni Y, Miwatani T (1988) Purification and characterization of a hemolysin produced by clinical isolates of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* related to the thermostable direct hemolysin. Infect Immun 56:961–965

Nishibushi M, Taniguchi T, Misawa T, Khaemanee-iam V, Honda T, Miwatani T (1989) Cloning and nucleotide sequence of gene *trh* encoding the haemolysin related to the thermostable direct haemolysin of *Vibrio parahaemolyticus*. Infect Immun 57:2691–2697

Osawa R, Yamai S (1996) Production of thermostable direct hemolysin by *Vibrio parahaemolyticus* enhanced by conjugated bile acids. Appl Environ Microbiol 62: 3023–3025

Osawa R, Okitsu T, Morozumi H, Yamai S (1996) Occurrence of urease-positive *Vibrio parahaemolyticus* in Kanagawa, Japan, with specific reference to presence of thermostable direct hemolysin (TDH) and the TDH-related-hemolysin genes. Appl Environ Microbiol 62:725–727

Pan TM, Wang TK, Lee CL, Chien SW, Horng CB (1997) Food-borne disease outbreaks due to bacteria in Taiwan, 1986 to 1995. J Clin Microbiol 35:1260–1262

Reed MJ, Muench H (1938) A simple method for estimating fifty per cent endpoints. Am J Hyg 27:493–497

Santos Y, Toranzo AE, Barja JL, Nieto TP, Villa TG (1988) Virulence properties and enterotoxin production of *Aeromonas* strains from fish culture systems. Infect Immun 56:2658–2665

Sanz A (1985) Límites de hiperhalinidad de los ciprinodóntidos ibéricos. Doñana: Acta Vertebrata 12:166–170

Wong SY, Leong TS (1990) A comparative study of *Vibrio* infections in healthy and diseased marine finfishes cultured in floating cages near Penang, Malaysia. Asian Fish Sci 3:353–359

Yii KC, Yang TI, Lee KK (1997) Isolation and characterization of *Vibrio carchariae*, a causative agent of gastroenteritis in the groupers, *Epinephelus coioides*. Curr Microbiol 35: 109–115

Editorial responsibility: David Bruno, Aberdeen, Scotland, UK

Submitted: June 8, 1998; Accepted: October 22, 1998
Proofs received from author(s): November 23, 1998