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Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen

Susana Prado[1,*], Jesús L. Romalde[1], Jaime Montes[2], Juan L. Barja[1]

[1]Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2]Centro de Investigacións Mariñas, Xunta de Galicia, Aptdo. 13, 36620 Vilanova de Arousa, Spain

ABSTRACT: Shellfish hatcheries are often affected by disease outbreaks. Three such episodes were investigated in different Galician hatcheries in order to establish the relationship between present microbiota and mortalities. Isolates were obtained from various parts of the hatcheries. Experimental tests for pathogenicity were carried out in microscale experiments using selected strains on *Ostrea edulis* larvae. The pathogenicity of 1 strain from each outbreak was demonstrated and shown to cause high mortalities (ranging from 98.5 to 100%) in 72 to 96 h after inoculation of larval cultures. All 3 strains belong to the genus *Vibrio*. One of the strains was identified as *Vibrio neptunius* and is the first description of this species as a molluscan pathogen. The other 2 strains showed low similarity with the *Vibrio* species analysed and may constitute new species within this genus.

KEY WORDS: Flat oyster · *Vibrio* · *Vibrio neptunius* · Pathogenicity test · Shellfish hatchery

INTRODUCTION

The coast of Galicia (NW Spain) is an exceptional environment for shellfish aquaculture, which thus represents an economically relevant sector for the region. Among other molluscs, flat oyster *Ostrea edulis* and various clam species (*Ruditapes decussatus*, *R. philippinarum*, *Venerupis pullastra*) are widely cultured because of their high value and rapid growth in this area. The decline of natural beds has resulted in the need to establish hatcheries to provide farms with juveniles (Montes et al. 1992). Shellfish hatcheries in Galicia rear several species in the same facilities, usually combining flat oyster and different species of clams. However, they are not able to supply enough spat of these bivalves to all shellfish farmers. Juveniles are therefore imported from different countries, a practice that can facilitate entry of non-indigenous pathogens. Although culture techniques for efficient husbandry have been developed, hatcheries suffer epizootic episodes, often causing the complete loss of batches. As a result, the inability to ensure constant production makes the development of oyster and clam farming difficult.

Optimal conditions for growth and development of bivalve larvae in hatcheries (densities, temperature, load of organic matter, etc.) enhance the growth and multiplication of bacteria and the accumulation of their metabolites (Brown & Tettelbach 1988, Araya et al. 1999). It is well known that bacteria are associated with decreased growth and mortalities in the larvae and juveniles of many bivalve molluscs. Walne (1958) reported the relationship between bacteria and growth problems of oyster larvae. In 1959, Guillard published a study about specific bacteria causing mortalities in *Mercenaria mercenaria* larvae. Tubiash et al. (1965) established the term 'bacillar necrosis' to name a characteristic disease caused by *Vibrio* spp. in different bivalve larvae. Further work on this subject confirmed the link between bacteria and disease in several mollusc species, including *Ostrea edulis*, *Crassostrea virginica*, *C. gigas*, *M. mercenaria*, *Mytilus galloprovincialis* and species of pectinids (Tubiash et al. 1970, Helm & Smith 1971, Brown & Losee 1978, DiSalvo et al. 1978, Elston & Leibovitz 1980, Brown 1981, Elston et al. 1982, Jeffries 1982, Brown 1983, Bolinches et al. 1986, Tubiash & Otto 1986, Lodeiros et al. 1987, Brown &

*Email: sprado@usc.es

Tettelbach 1988, Lodeiros et al. 1992, Riquelme et al. 1995, Nicolas et al. 1996, Sáinz et al. 1998, Sugumar et al. 1998, Araya et al. 1999, Elston et al. 1999, Lacoste et al. 2001, Anguiano-Beltrán et al. 2004, Estes et al. 2004, Gay et al. 2004).

In this paper, we present the results of bacteriological studies performed during 3 episodes of severe mortalities in 3 shellfish hatcheries in Galicia. The pathogenicity of isolated bacterial strains was tested in order to identify the aetiological agents. In addition, the first evidence of *Vibrio neptunius* as mollusc pathogen is presented.

MATERIALS AND METHODS

Description of facilities and bacterial isolation. The facilities where mortalities occurred are situated at different locations along the Galician coast in NW Spain (Fig. 1). Their management practices vary: one is a commercial hatchery beside a molluscan depuration facility (hatchery A); the second is a plant where molluscs and fishes are cultured (hatchery B); and the third is exclusively dedicated to molluscs (hatchery C). Two of the facilities (B and C) suffered mortalities in oyster larval stages and one (A) in post-larvae.

Samples were taken in the different areas of the hatcheries: larvae, spat, broodstock, tank surfaces, phytoplankton and water. Larvae, spat and broodstock gonad (pieces excised aseptically) were washed, ground and homogenized in sterile seawater (SSW). Appropriate dilutions were made with these suspensions as well as with samples of water and phytoplankton used as larval feed. They were spread on Marine Agar (MA, Pronadisa) and Thiosulphate Citrate Bile Sucrose (TCBS, Oxoid). Samples taken directly from inner surfaces of the tanks containing the oysters were spread on the above mentioned media with sterile swabs.

Plates were incubated at 22°C and selected colonies were isolated after 24 h (TCBS) or 7 d (MA) on MA, and further restreaked to purity. Pure cultures of strains were frozen at −80°C in Marine Broth with glycerol (15% v/v).

Pathogenicity tests. Pathogenicity assays were carried out in 6-well sterile plastic cell-culture microplates (Nunc). Oyster larvae (10 to 15 d old) obtained from the 3 different hatcheries were placed in each well with 10 ml of SSW at an average density of 11 larvae ml^{-1}. Microplates were incubated on an orbital shaker (100 rpm) at room temperature (22°C). No food was added during the experimental period.

Nineteen isolates obtained from the outbreaks were cultured on MA plates for 24 h at 22°C. Suspensions of bacterial cultures of each isolate were prepared in SSW, adjusted by optical density of McFarland scale (tube 3). Bacteria were added to wells to achieve final concentrations of 10^5 to 10^6 colony forming units per millilitre (cfu ml^{-1}). Wells without added bacteria served as negative controls for all experiments. Bioassays were performed in duplicate.

Larvae were observed under light microscopy (4× to 10×) and the effects of the bacteria recorded as follows:

(1) *Live larvae*, including swimming larvae and larvae with valves closed but showing internal movement;

(2) *Dead larvae*, or closed larvae without internal movement.

Pathogenicity results were expressed as mean percentages of mortality, and standard deviations were calculated (Sokal & Rohlf 1995). Isolates showing mortalities lower than 10% were considered non-pathogenic.

Identification of pathogenic strains. Isolates showing pathogenic properties were examined for their phenotypic characteristics as described by Romalde et al. (1990). Cell morphology and motility were determined by phase-contrast microscopy. Routine oxidase, catalase and Gram tests were conducted. Growth in TCBS was observed. The oxidation-fermentation of glucose was performed in ZOF (ZoBell-based oxidation-fermentation) (Lemos et al. 1985). Aminoacid decarboxylation was determined in Moeller's medium, and Thornley's arginine-dihydrolase test was also performed. Indol production, Voges-Proskauer, nitrate reduction and Simmon's citrate commercial media were prepared with the addition of 1.0% NaCl. The extracellular enzymes produced were examined for starch, gelatine, lipase (Tween 80) and esculine.

The NaCl requirement was determined by plating isolates on basal medium (4.0 g l^{-1} peptone + 1.0 g l^{-1} yeast extract + 15 g l^{-1} agar) containing 0, 0.5, 3, 6, 8 and 10% NaCl and incubated for up to 1 wk. The tem-

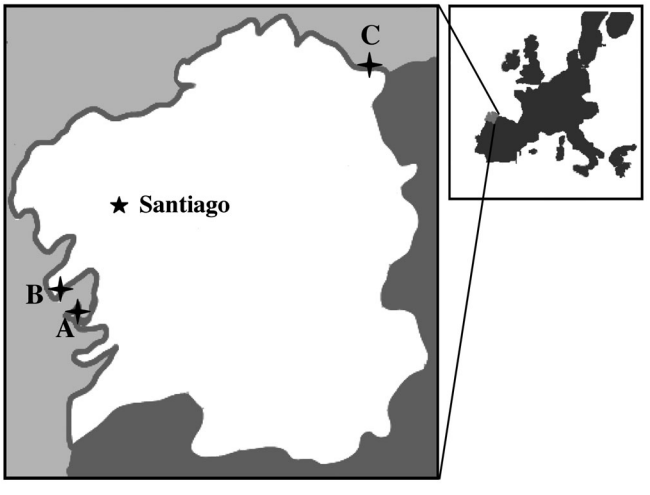

Fig. 1. Location of the hatcheries (A, B, and C) in NW Spain

perature range of growth was determined by plating isolates on MA plates and incubating them at 6, 15, 25, 35 and 43°C for up to 2 wk. Antibiograms were carried out by disc diffusion methodology, using antibiotic-impregnated discs (Oxoid or BBL) with: amoxicillin 25 µg (AML25), cephalexin 30 µg (CL30), enrofloxacine 5 µg (ENR5), erythromicine 15 µg (E15), florfenicol 30 µg (FFC30), flumequine 30 µg (UB30), nalidixic acid 30 µg (NA30), nitrofurantoin 300 µg (F300), oxytetracycline 30 µg (OT30), sulphamethoxazole/trimethoprim 25 µg (SXT25) or tetracycline 30 µg (TE30). The vibriostatic agent, pteridine (O/129 150 µg), was also included. The inhibition zone was determined on MA after 24 to 48 h at 25°C.

Additional phenotypic analyses were performed using the API20E system (bioMérieux) according to the manufacturer's instructions, except that saline solution (NaCl 0.85 %) was used to prepare the inocula.

Genetic characterization by sequencing of the 16S rRNA gene was also carried out. Isolation of DNA from pure cultures was performed using the 'Instagene' matrix (Bio-Rad), following the manufacturer's recommendations. 16S rRNA genes were amplified by PCR with universal primers pA and pH (Hutson et al. 1993). Primers (Funke et al. 1995) corresponding to internal conserved regions of this gene were used for the sequencing reactions in a GeneAmp PCR System 9600 (Perkin Elmer), using a *Taq* dye-Deoxy terminator cycle sequencing kit (Applied Biosystems). Sequencing products were analysed using an Automatic DNA Sequencer (model 373A, Applied Biosystems). Comparative sequence analyses were conducted using FASTA3 program. Sequences of the closest relatives were retrieved from Gene bank/EMBL, and the alignments and phylogenetic analysis were performed by the neighbour-joining method using the ClustalW (Thompson et al. 1994). Bootstraps were calculated with 1000 replications, and *Vibrio cholerae* (X74695) was used as the outgroup sequence. The phylogenetic tree was drawn using the software NJPlot (Perrière & Gouy 1996).

RESULTS

Isolation of bacteria

In the 3 hatcheries, samples were taken after the appearance of disease indicators, including growth depression, reduction of motility, abnormal swimming, velum deformation in the larval stages or the clearance of the spat mass in the bottom of the bins at postlarval stages.

In hatchery A, with continuous mortalities of young spat (<500 µm in size), bacteriological samples revealed that vibrios were the predominant bacteria, with approximately 10^3 cfu spat^{-1} on both TCBS and MA media. A batch with early signs of disease showed a similar level of vibrios (≈10^3 cfu spat^{-1}), while lower numbers (<10^1 cfu spat^{-1}) were observed in a healthy batch analysed in parallel. Analysis of seawater used in the hatchery showed the absence of growth in TCBS. Samples obtained directly from tank surfaces also showed a predominance of vibrios. A total of 5 isolates corresponding to the predominant bacterial phenotypes observed were selected from this hatchery (Table 1).

In hatcheries B and C larval cultures were affected. The first sign was growth depression and shortly before settlement larvae began to die. Mortalities were higher in small individuals (≈150 µm) than in large ones (>200 µm). In hatchery B, 2 samples were collected, the first when the first signs of disease were observed and the second when mortalities appeared. A change in bacterial populations was recorded, with no vibrios detected in the first sample, and a dominant yellow colony recovered on TCBS in the second (Table 1). Oyster broodstock, kindly provided in this case by the farmer, allowed us to investigate the possibility of vertical transmission of the pathogenic bacteria. However, bacterial phenotypes observed in these samples did not correspond with those observed in diseased larvae (Table 1). A total of 9 isolates was recovered from diseased larvae and culture water in hatchery C (Table 1).

Table 1. Strains associated with disease outbreaks isolated in this study. TCBS = Thiosulphate Citrate Bile Sucrose; MA = Marine Agar

Hatchery	Strain	Sample	Medium of isolation
A	156	Diseased spat	TCBS
	157	Diseased spat	TCBS
	160	Diseased spat	TCBS
	182	Inner tank surface	TCBS
	203	Inner tank surface	MA
B	143.98	Diseased larvae	MA
	145.98	Diseased larvae	TCBS
	151.98	Diseased larvae	MA
	318.98	Broodstock	MA
	319.98	Broodstock	MA
C	632	Water[a]	TCBS
	633	Water[a]	TCBS
	634	Water[b]	TCBS
	636	Water[b]	TCBS
	637	Water[b]	TCBS
	638	Water[b]	TCBS
	650	Diseased larvae	MA
	651	Diseased larvae	MA
	652	Diseased larvae	MA

[a]Water from cultures with high mortalities
[b]Water from cultures with first signs of disease

Pathogenicity tests

Pathogenicity assays were conducted with the isolates obtained from the different hatcheries to determine their pathogenicity to flat oyster larvae. In each of 3 disease outbreaks, it was possible to demonstrate the association of one of the selected isolates with high mortalities in the experimental challenges (Table 2).

Strains PP-203 (hatchery A), PP-145.98 (hatchery B) and PP-638 (hatchery C) were able to cause high mortalities in 72 to 96 h (98.5, 100 and 100%, respectively). The 'spotting' phenomenon, defined as an accumulation of larvae agglutinated in the bottom of the well, was evident after this period. Microscopical examination showed that, in all cases, the first sign of disease was reduction of motility, followed by an abnormal circular pattern of swimming or the inability to swim. In addition, we detected larvae with closed valves but internal movement and, to a lesser extent, with velum abnormalities or even detached portions of velum, which continued to move. Moreover, at the peak of the infections, bacteria swarming inside and around dead and moribund larvae were observed (Fig. 2). On the other hand, larval survival was higher than 94% for all the other isolates tested. Mortality in the negative controls ranged from 0 to 3.7%.

Table 2. Mortality rates (%) of oyster larvae obtained in experimental challenges using the bacterial strains isolated from mollusc culture systems. Inoculated doses ranged from 10^5 to 10^6 cfu ml^{-1}. C = negative control

Origin	Strain	Time after inoculation (h)			
		24	48	72	96
Hatchery A	C	2.6	3.4	3.7	–
	156	1.5	2.7	3.2	–
	157	3.8	4.1	4.5	–
	160	1.9	1.9	2.7	–
	182	0.8	1.6	1.6	–
	203	86.4	98.5	98.5	–
Hatchery B	C	0.0	0.0	–	0.0
	143.98	0.0	0.0	–	0.0
	145.98	76.8	98.6	–	100.0
	151.98	0.0	0.0	–	0.0
	318.98	0.0	0.8	–	1.8
	319.98	0.0	0.3	–	5.8
Hatchery C	C	–	0.1	–	0.1
	632	–	0.0	–	0.0
	633	–	0.0	–	0.0
	634	–	0.3	–	0.3
	636	–	0.0	–	0.0
	637	–	0.0	–	0.0
	638	–	100.0	–	100.0
	650	–	0.0	–	0.0
	651	–	1.2	–	1.2
	652	–	0.5	–	42.1

Identification of pathogenic strains

The 3 pathogenic strains were Gram-negative, oxidase and catalase-positive, motile rods. They were fermentative in ZOF and OF medium, susceptible to vibriostatic agent and grew in TCBS. These characteristics indicate that they belong to the genus *Vibrio*.

All 3 strains were positive for acid production from glucose, amylase, gelatinase, lipase and for growth with 3 to 6% NaCl and at 15 to 25°C. They were negative for gas production from glucose, lysine- and ornitine-decarboxylases, Simmon's citrate (with and without NaCl addition), hydrolysis of esculine, H_2S production, urease and for growth without NaCl as well as at 4 to 6°C or 43°C. Antibiotic susceptibility was observed to E15, FFC30, SXT25, UB30; and resistance to TE30, NA30 and OT30. All of them fermented glucose, but not inositol, sorbitol, rhamnose or arabinose.

Differential characteristics are illustrated in Table 3. Strain PP-145.98 was positive for arginine-dihydrolase and growth in 8% NaCl and at 35°C, and formed yellow colonies in TCBS agar. It was negative for indole production and nitrate reduction. Strain PP-203 was negative for arginine-dihydrolase, indole production, nitrate reduction, growth in 8% NaCl and at 35°C, and formed green colonies in TCBS. Strain PP-638 was positive for arginine-dihydrolase, indole production, nitrate reduction, growth in 0.5% NaCl and formation of yellow colonies in TCBS. It was negative for growth in 8% NaCl and at 35°C.

The 16S rDNA sequences of the pathogenic isolates were determined and were allocated to the genus *Vibrio* using the FASTA program. Clustering obtained by the Clustal method indicated that PP-145.98 belongs to

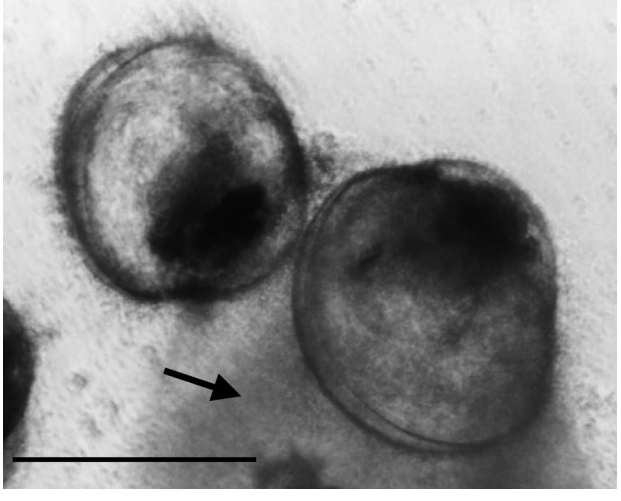

Fig. 2. Disease signs of oyster larvae in the experimental challenges: bacterial swarming around spotted larvae. Arrow indicates accumulation of bacterial cells. Scale bar = 200 µm

Table 3. Differential phenotypic characteristics of the pathogenic strains. R = resistant; S = susceptible

Test	PP-145.98	PP-203	PP-638
Growth on TCBS	+ (yellow)	+ (green)	+ (yellow)
Arginine-dihydrolase	+	–	+
Nitrate reduction	–	–	+
Indole production	–	–	+
Voges-Proskauer	–	–	+
ONPG (β-galactosidase)	–	–	+
Fermentation of:			
manose	–	–	+
sucrose	+	–	+
melibiose	–	–	+
amigdaline	–	–	+
Growth at:			
35°C	+	–	–
0.5% NaCl	–	–	+
8% NaCl	(+)	–	–
Amoxicillin	R	R	S
Cephalexin	S	R	S
Enrofloxacine	R	S	R
Nitrofurantoin	S	R	S

Vibrio neptunius, with a similarity of 99.47%. Strains PP-203 and PP-638 showed low values of similarity (≤97%) with the other *Vibrio* species included in the analysis. In the case of PP-203, the closest phylogenetic relatives were *V. vulnificus* (97.38%), *V. aestuarianus* (97.22%), *V. splendidus* biovar II (97.15%), *V. parahaemolyticus* (97.07%) and *V. chagassi* (97.07%), while PP-638 was more similar to *V. orientalis* (96.93%), *V. campbellii* (96.66%), *V. mytili* (96.54%) and *V. tubiashii* (96.51%). Fig. 3 shows the phylogenetic tree obtained after the neighbour-joining analysis.

16S rDNA sequences of PP-145.98, PP-203 and PP-638 were deposited in the EMBL database with accession numbers AJ296157, AJ296159 and AY792622, respectively.

DISCUSSION

A large number of works, as detailed in the introduction, have established bacterial implication in outbreaks of disease in molluscan larval cultures. Usual culture conditions, such as high larval density, high temperatures and addition of food, favour the appearance of high concentrations of bacteria in the hatcheries (Walne 1958), many of them with pathogenic potential. Proliferation of opportunistic pathogens may lead to outbreaks of disease. The identification of pathogens affecting cultures in a determined area is the first step towards establishing measures for disease control. For this purpose, different approaches, such as UV-treatment of water or use of chemotherapeutants, are commonly employed. However, these treatments can favour the proliferation of specific bacterial populations able to survive in adverse conditions or resistant to antibiotics (Davies 1994), which may constitute a risk for larvae.

In this study we investigated 3 episodes of mortality that occurred in Galician hatcheries. In all cases, an

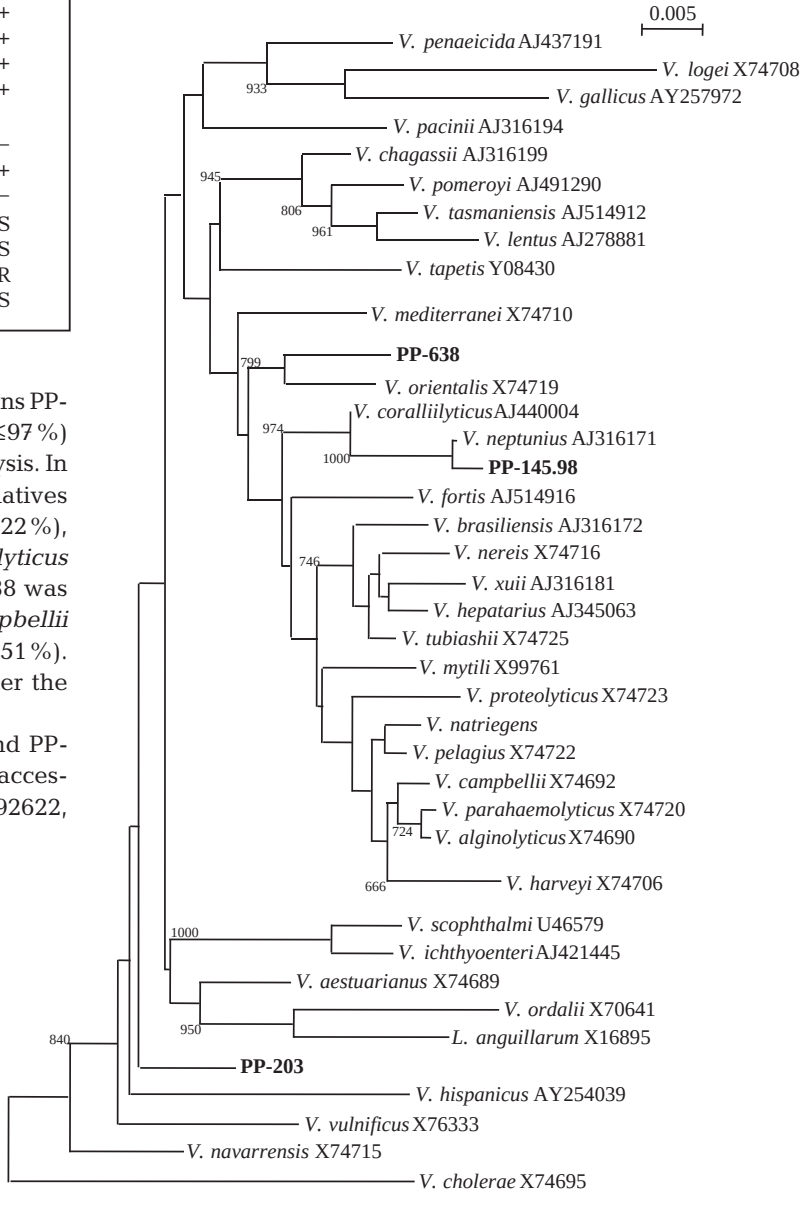

Fig. 3. Phylogenetic tree of 16S rRNA sequences for the pathogenic strains obtained in this study and the type strains of the closest relatives within the genus *Vibrio*. *V. cholerae* (Acc. No. X74695) was employed as outgroup. Horizontal branch lengths are proportional to evolutionary divergence. Significant bootstrap values of 1000 replicates appear next to the corresponding branch

increase in the numbers of bacteria in water, mainly representatives of the genus *Vibrio*, were observed in comparison with bacterial levels in healthy larval cultures. This fact confirms the quick proliferation of opportunistic pathogens naturally present in the culture system when conditions are favourable (DiSalvo 1978, Jeffries 1982). Initially, before invasion of larvae, bacteria may be present in water, but after colonization they multiply rapidly, usually attached to larvae, and are more difficult to detect in water (Brown 1981, Brown & Tettelbach 1988). This could explain why none of the isolates from water of cultures showing high mortalities was able to cause larval death. On the other hand, mortalities were higher in smaller larvae from the same batch. This greater susceptibility to infection seems logical, taking into account that smaller individuals always show slower development, a slight protective shell, and are usually unable to settle (Elston 1984).

We have been able to demonstrate the ability of at least one isolate from each outbreak to cause severe mortalities in larval cultures. In the experimental challenges, pathogenic isolates caused high mortalities (98 to 100%) in less than 48 h. This fact is of particular importance because water in tanks at hatcheries is renewed every 2 to 3 d and the whole batch could die within this period. Signs observed in the laboratory trials, including reduction of motility and spotting phenomenon, have been related to the action of bacterial toxins (DiSalvo et al. 1978). These findings, together with the fact that bacterial swarming was observed around larvae, indicate that the pathogenic action could correspond to vibriosis type I (Elston & Leibovitz 1980). This hypothesis was confirmed, since all aetiological agents were identified as belonging to the genus *Vibrio*. Characterization of the strain PP-145.98 indicates that it belongs to *Vibrio neptunius* (Thompson et al. 2003). Although it has been reported as normal microbiota in cultures of rotifers and larvae of bivalves and fish (Suantika et al. 2001, Thompson et al. 2003), our results constitute the first evidence of pathogenicity for molluscs of this *Vibrio* species. Strains PP-203 and PP-638, although belonging to the same genus, could not be assigned to any known species.

In hatchery A, the pathogenic strain PP-203 was isolated from surfaces of nursery culture containers. It may be possible that this strain is able to survive the water changes, forming biofilms on the inner tank surfaces (Elston 1984). Protection of bacteria from adverse environmental conditions, including desiccation, has been described as one of the ecological advantages of biofilms (Ophir & Gutnick 1994, Davey & O'Toole 2000). Moreover, it has to be taken into account that the same tanks are used to culture different species (oyster and clams), and preliminary results indicate the pathogenicity of this strain to clam larvae (data not shown). All these facts emphasize that proper cleaning of tanks is critical for control of pathogen persistence in the culture systems (Elston 1984).

In hatcheries B and C growth depression was detected in larval cultures, which caused delays in settlement, followed by death. During settlement and metamorphosis, the most critical stages in larval development, larvae begin a life associated with substrate in high densities and come into contact with the resident microbiota and, hence, with putative pathogens (Sutton & Garrick 1993). All these factors can facilitate the transmission or appearance of diseases.

In summary, the relationship between new bacteria belonging to the genus *Vibrio* and collapses of larval and post-larval cultures of bivalves was established, showing that these opportunistic pathogens are responsible for important losses in Galician hatcheries. Moreover, the pathogenic capacities for molluscs of *Vibrio neptunius* were demonstrated. Further studies are needed with the other pathogenic strains detected in order to characterize them fully and, thus, possibly to describe new *Vibrio* species.

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