Vol. 55: 109–115, 2003 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published July 8

Development of a PCR-based method for the detection of *Listonella anguillarum* in fish tissues and blood samples

Santiago F. Gonzalez*, Carlos R. Osorio, Ysabel Santos

Department of Microbiology and Parasitology, Faculty of Biology, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

ABSTRACT: A PCR assay for detection and identification of the fish pathogen *Listonella anguillarum* was developed. Primers amplifying a 519 bp internal fragment of the *L. anguillarum rpoN* gene, which codes for the factor σ^{54}, were utilized. The detection limit of the PCR using *L. anguillarum* pure cultures was approximately 1 to 10 bacterial cells per reaction. For tissue or blood samples of infected turbot *Scophthalmus maximus*, the detection limit was 10 to 100 *L. anguillarum* cells per reaction, which corresponds to 2×10^3 to 2×10^4 cells g^{-1} fish tissue. Our results suggest that this PCR protocol is a sensitive and specific molecular method for the detection of the fish pathogen *L. anguillarum*.

KEY WORDS: PCR · *rpoN* gene · *Listonella anguillarum* · Detection

INTRODUCTION

Vibriosis is one of the most important and the oldest recognized fish disease in marine aquaculture worldwide. The causative agent, *Listonella anguillarum*, was initially isolated by Canestrini (1893). Since the first identification, vibriosis has been described in anadromous and catadromous species (Toranzo & Barja 1990, Austin & Austin 1999, Pedersen et al. 1999a) and is reported to produce disease in more than 48 fish species (Austin & Austin 1999).

Among the 23 different O-serogroups described for *Listonella anguillarum* (Pedersen et al. 1999b), only Serogroups O1, O2 and O3 are important as the causative agent of mortalities in farmed and feral fishes, with the remaining serogroups considered to comprise mainly environmental strains isolated from water and sediment (Sorensen & Larsen 1986, Austin et al. 1995).

Diagnosis of vibriosis is mainly based on the study of the phenotypical traits of the isolated bacteria followed by serological confirmation of the identification. Immunological assays such as dot-blot (Cipriano et al. 1985) and ELISA (Adone et al. 1996) have also been developed for the detection of bacteria in fish tissues. However, the use of these methods has been limited by their poor detection levels and the existence of cross-reactions with the close relative *Vibrio ordalii* species. Thus, there is a need for fast, sensitive and specific molecular methods to detect *Listonella anguillarum* in field samples.

Hirono et al. (1996), cloned a hemolysin gene of *Listonella anguillarum*, and used the sequence to design a DNA probe. However, this hemolysin gene is not present in all the *L. anguillarum* isolates, and its use as a diagnosis tool does not guarantee the detection of all isolates of this fish pathogen.

Sequencing of the 5S rRNA and 16S rRNA gene sequences in *Listonella anguillarum* (MacDonell & Colwell 1985, Valle et al. 1990) and in other vibrio species (Kita-Tsukamoto et al. 1993, Ruimy et al. 1994) has facilitated the design of oligonucleotide probes. Rehnstam et al. (1989) and Ito et al. (1995) developed probes for the identification of *L. anguillarum* by RNA-DNA colony hybridization targeted to the 16S rRNA and 5S rRNA gene sequences, respectively. Later, Martínez-Picado et al. (1996) published a method for detection of *L. anguillarum* based on the combined use of the selec-

*Email: xanti@usc.es

tive *Vibrio anguillarum* medium (VAM: Alsina et al. 1994) and a nonradioactively labelled oligodeoxynucleotide sequence complementary to 16S rRNA (Martínez-Picado et al. 1994). Although these nucleic acid hybridization techniques are useful for *L. anguillarum* identification, they are hampered by the need for previous isolation of the pathogen. The same difficulty in the detection of other bacterial species was solved by PCR-based methods (Hiney & Smith 1998). There are many DNA target sequences that can be used for amplification in the PCR reaction. Sequencing of the 16S rRNA gene in virtually all the bacterial species described to date has made this gene one of the most important tools in the design of PCR-based diagnostic methods (Hiney & Smith 1998, Osorio et al. 1999, Osorio & Toranzo 2002). However, extensive database comparisons demonstrate that differences in the 16S gene sequence between *L. anguillarum* and closely related species are insufficient to warrant the design of species-specific PCR primers using that gene as a target. Among the currently available *L. anguillarum* gene sequences in the EMBL database, we selected *rpoN* gene coding for the cellular sigma factor σ^{54} as a PCR target. As a housekeeping gene, *rpoN* is expected to be present in all *L. anguillarum* isolates. Moreover, regions of high sequence variability are found in this gene compared to the homologous genes in other *Listonella* and *Vibrio* species. Therefore, the aim of the present study was to design 2 specific primers for *L. anguillarum* based on the *rpoN* gene sequence and validate a PCR method for the identification of this pathogen in different kinds of samples.

MATERIALS AND METHODS

Bacterial strains. A total of 57 strains from 22 bacterial species were used in the study. The geographical origin and host species are given in Table 1. The identity of bacterial strains was confirmed, when necessary, by standard biochemical procedures (Santos et al. 1993) or by slide agglutination assays (Toranzo et al. 1987).

Bacteria were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland), American Type Culture Collection (ATCC, Manassas, Virginia, USA), laboratories in different countries and our own collection. The strains were routinely cultured on tryptic soy agar (TSA; Difco), supplemented with 1% (w/v) NaCl at 22°C for 24 to 48 h. Stock cultures of the strains were stored at −80°C in tryptic soy broth (TSC, Difco) supplemented with 15% (v/v) glycerol.

Primer design. The *rpoN* gene of *Listonella anguillarum*, which codes for the cellular factor σ^{54}, was chosen for primer design. The *rpoN* sequence of a Seroype O1 strain was recently cloned and sequenced by O'Toole et al. (1997). The sequence (listed in the EMBL database under Accession No. U86585) was compared with other *rpoN* gene sequences described for the genus *Vibrio* using the MegaAlign program (DNAstar by Lasergene). On the basis of the alignment, 2 variable regions were chosen and a set of primers was designed. A forward primer, *rpoN*-ang5′ (5′-GTTC-ATAGCATCAATGAGGAG-3′, Positions 181 to 201, in the *L. anguillarum rpoN* gene), and a reverse primer, *rpoN*-ang3′ (5′-GAGCAGACAATATGTTG-GATG-3′, Positions 693 to 714 in the *L. anguillarum rpoN* gene) were developed. These primers flank a 519 bp internal fragment of the *rpoN* gene.

Preparation of samples from pure cultures, infected tissues and blood. Samples of DNA were obtained from pure bacterial cultures or tissues and blood from healthy or infected turbot *Scophthalmus maximus*. Bacterial cell suspensions were adjusted to contain 10^9 cells ml^{-1} (McFarland Scale 4) in sterile phosphate-buffered saline, PBS (8 g l^{-1} NaCl; 0.3 g l^{-1} ClK; 0.73 g l^{-1} PO_4HNa_2; 0.2 g l^{-1} PO_4H_2K), and serially diluted from 1×10^9 to 1×10^1 cells ml^{-1}. Samples of spleen and kidney from turbot free of vibriosis were homogenized with PBS to achieve 50% (w/v) suspension. Aliquots of fish tissue homogenate were seeded with an equal volume of the bacterial suspension. The final dilution of the tissue was 25% (w/v). Blood samples from healthy turbot were collected by vein puncture using a heparinized syringe and mixed with an equal volume (0.5 ml) of the bacterial suspensions. Samples of spleen and kidney from turbot suffering vibriosis, as confirmed by isolation and conventional identification of *Listonella anguillarum*, were also taken. These samples were homogenized with PBS by the procedure described above. In all cases CFU were verified by plating dilutions onto TSA-1 and counting the bacterial colonies produced.

DNA extraction. DNA was extracted using 2 different commercial systems: InstaGene matrix (BioRad) for the pure bacterial cultures, and Dynabeads DNA DIRECT™ (Dynal) for fish tissue homogenates and blood samples. In all cases, DNA purification was performed as recommended by the manufacturer.

Polymerase chain reaction (PCR). For the amplification, the Ready-to-go™ PCR beads (Amersham Pharmacia Biotech) system was used. Each bead contains all necessary reagents for the PCR reactions, except specific primers and template; 150 pmol of primers and 0.5 µl of DNA extracted from bacterial suspensions, fish tissue homogenates and blood were added.

Samples were subjected to 30 amplification cycles in a DNA thermal cycler (Mastercycler personal: Eppendorf). In order to optimize the thermal conditions for

Table 1. Bacterial species and strains used in this study. ATCC: American Type Culture Collection; NCIMB: National Collection of Marine and Industrial Bacteria; JCM: Japan Collection of Type Cultures

Organism	Strain	Origin
Listonella anguillarum	ATCC 43305 (O1)	*Onchorhynchus mykiss*, Denmark
	R-82 (O1)	*Scophthalmus maximus*, Spain
	96-F (O1)	*Morone saxatilis*, USA
	775 (O1)	*Oncorhynchus kisuch*, USA
	TM-14 (O1)	*O. mykiss*, Spain
	NCIMB 571 (O1)	*O. mykiss*, Japan
	RG-111 (O2α)	*S. maximus*, Spain
	ATCC 14181 (O2α)	*Salmo trutta*, USA
	ATCC 43306 (O2α)	*Gadus morhua*, Denmark
	43-F (O2β)	*Dicentrarchus labrax*, USA
	RV-22 (O2β)	*S. maximus*, Spain
	ATCC 43307 (O3*A*)	*O. mykiss*, Denmark
	13 A5 (O3*A*)	Seawater, Spain
	PT 493 (O3*A*)	*Plecoglosus altivelis*, Japan
	11008 (O3*A*)	*Sparus aurata*, France
	ET-208 (O3*B*)	*Anguilla japonica*, Japan
	ATCC 43308 (O4)	*G. morhua*, Denmark
	ATCC 43310 (O6)	*G. morhua*, Denmark
	ATCC 43311 (O7)	*A. anguilla*, Denmark
	ATCC 43312 (O8)	*G. morhua*, Denmark
	ATCC 43313 (O9)	*G. morhua*, Denmark
	ATCC 43314 (O10)	*G. morhua*, Denmark
Listonella pelagia	ATCC 25916	Seawater, Spain
	RG-165	*S. maximus*, Spain
	ST-11	*Salmo salar*, Spain
	NF-182	*O. mykiss*, Spain
	RPM-87.1	*S. maximus*, Spain
	RPM-58.1	*S. maximus*, Spain
	NCIMB 1900	Seawater, USA
Vibrio splendidus	ATCC 33125	Marine fish
	ATCC 25914	Seawater, USA
	RG 52	*S. maximus*, Spain
Vibrio ordalii	NCIMB 2167	*O. kisutch.*, USA
Vibrio tubiashii	EX 1	*Crassostrea gigas*, Spain
Vibrio fischeri	ATCC 7744	Unknown
Vibrio harveyi	ATCC 14126	*Talorchestia* sp., USA
Vibrio vulnificus	ATCC 29307	Human
Vibrio proteolyticus	ATCC 15338	*Limnoria tripunctata*
Vibrio aestuarianus	ATCC 35048	Oyster, USA
Vibrio natriegens	ATCC 14048	Salt-marsh mud, USA
Vibrio metschnikovii	ATCC 11170	Unknown
Vibrio nereis	ATCC 25917	Seawater, USA
Vibrio campbellii	ATCC 25920	Seawater
Photobacterium phosphoreum	ATCC 11040	Unknown
Photobacterium leiognathi	ATCC 25521	*Leiognathus equula*, Thailand
Photobacterium histaminum	JCM 8968	*Labracoglassa argentiventris*, Japan
Photobacterium damselae ssp. *damselae*	RG-91	*S. maximus*, Spain
	RM-71	*S. maximus*, Spain
Photobacterium damselae ssp. *piscicida*	DI-21	*S. aurata*, Spain
	ATCC-17911	*M. americanus*, USA
Aeromonas hydrophila	80A1	*O. mykiss*, Spain
	B-32	*O. mykiss*, Spain
	B-35	*O. mykiss*, Spain
Aeromonas caviae	1.25	Human, USA
Aeromonas salmonicida ssp. *salmonicida*	ATCC 35658	*S. salar*, USA
	MT- 004	*S. salar*, Scotland
	SEG-9.1	*S. salar*, Spain

specific detection of *Listonella anguillarum*, different annealing temperatures, from 55 to 65°C, were tested. In the PCR program a first denaturation step at 95°C for 3 min was included, followed by 30 amplification cycles consisting of 95°C for 1 min, annealing of primers at 55 to 65°C for 1 min, and 72°C for 40 s. A final extension step of 5 min at 72°C was also included in the PCR program. In all the PCR experiments, DNA from pure cultures of *L. anguillarum* was included as a positive control, whereas non-inoculated tissues, DNA from other bacterial species and PBS were used as negative controls.

Analysis of PCR products. PCR products were analyzed by horizontal 1% (w/v) agarose gel electrophoresis (100 V, 1 h) in TAE 1X (Tris 0.04 M, EDTA 0.001 M, pH 8) electrophoresis buffer, to which 0.5 µg ml^{-1} of ethidium bromide was added. A 1 kb DNA ladder (Bio-Rad) was included as a molecular weight marker. After electrophoretic separation, PCR products were visualized using a UV transilluminator (Fotodyne) and photographed using a polaroid MP-4 camera.

RESULTS

Experimental validation of primers

The specificity of the primers *rpoN*-ang5′ and *rpoN*-ang3′ was tested with 22 pure cultures of *Listonella anguillarum* and 35 pure cultures of other bacterial species (Table 1). PCR reactions were performed at different annealing temperatures ranging from 55 to 65°C. At 58 and 62°C, all 22 *L. anguillarum* strains produced a unique and clear PCR band of the expected 519 bp length. However, at 58°C this amplification band was also produced with *Vibrio ordalii* (NCIMB 2167). This undesirable reaction was eliminated using the annealing temperature of 62°C. When the other non-*L. anguillarum* bacterial strains in Table 1 were tested with the same primers and annealing temperatures, no amplification occurred. The results of PCR analysis performed at annealing temperatures of 62 and 58°C with *L. anguillarum* and other bacterial species tested are shown in Figs. 1 & 2, respectively.

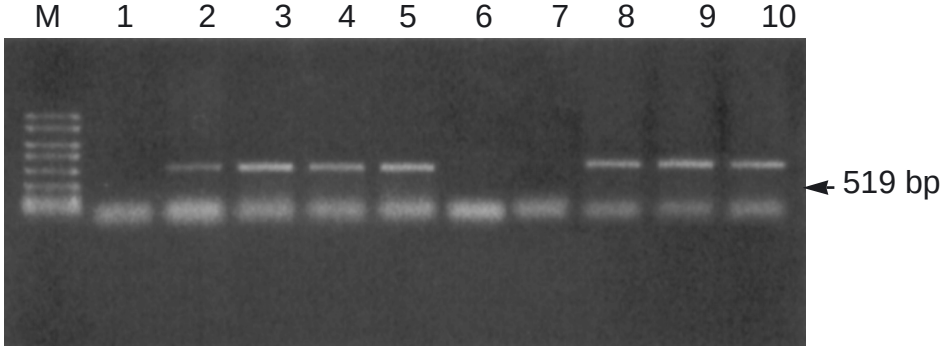

Fig. 1. Specific PCR products obtained with pure cultures of 7 *Listonella anguillarum* strains and other related species at an annealing temperature of 62°C. Lane M: 1 kb molecular weight marker; Lane 1: PCR negative control; Lanes 2 to 5: *L. anguillarum* 775 (Serotype O1), ATCC 43305 (Serotype O1), ATCC 43306 (Serotype O2α) and RV-22 (Serotype O2β), respectively; Lane 6: *Vibrio ordalii*: Lane 7: *V. splendidus*; Lanes 8 to 10: *L. anguillarum* ATCC 43307 (Serotype O3A), ATCC 43308 (Serotype O4) and ATCC 43310 (Serotype O6), respectively

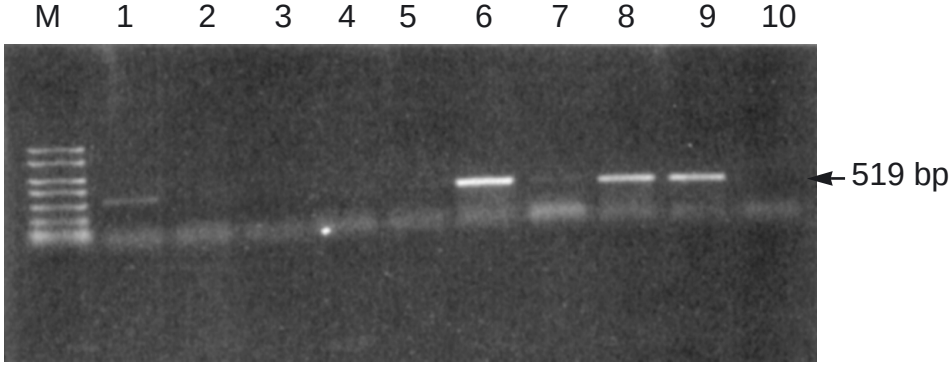

Fig. 2. PCR products obtained with pure cultures of *Listonella anguillarum* strains and other bacterial species at an annealing temperature of 58°C. Lane M: 1 kb molecular weight marker; Lane 1: *Vibrio ordalii*; Lane 2: *Aeromonas caviae*; Lane 3: *A. salmonicida*; Lane 4: *A. hydrophila*; Lane 5: *L. pelagia*; Lane 6: *L. anguillarum* TM-14 (Serotype O1); Lane 7: *V. splendidus*; Lanes 8 and 9: *L. anguillarum* ATCC 43305 (Serotype O1) and ATCC 43306 (Serotype O2α), respectively; Lane 10: PCR-negative control

Sensitivity of PCR system

When the primers *rpoN*-ang5′and *rpoN*-ang3′ were tested for amplification with DNA extracted from the different bacterial suspensions (10^1 to 10^9 cells ml^{-1}) of *Listonella anguillarum*, the expected 519 bp PCR product was obtained with samples containing as few as 1 to 10 bacterial cells per PCR tube (Fig. 3). Moreover, this protocol allowed the detection of 10 to 100 *L. anguillarum* cells per PCR tube (which corresponds to 2×10^3 to 2×10^4 cells g^{-1} fish tissue) using experimentally infected tissues or blood (Fig. 4). The total time for the procedure, including DNA extraction from samples, amplification and gel electrophoresis, was approximately 5 h.

Testing naturally infected fish tissues

With the knowledge that *Listonella anguillarum* could be detected specifically, we tested whether this pathogen could be detected directly in tissues from fish suffering vibriosis. For this purpose, homogenized kidney from naturally infected and healthy fish were used as DNA source for PCR analyses. Application of the *rpoN*-based PCR protocol described above showed that *L. anguillarum* could be detected in the infected fish at a detection level similar to that in experimentally infected fish tissues (Fig. 4) determined from viable counts.

DISCUSSION

To date, nucleic acid-based methods have been used to identify the most important bacterial pathogens in aquaculture (Hill et al. 1991, Corinne et al. 1992, Brown et al. 1994, Toyama et al. 1994, Urdaci et al. 1998, Osorio et al. 1999, Cepeda & Santos 2000). Although molecular diagnosis of vibriosis caused by *Listonella anguillarum* based on the use of DNA probes has been reported (Aoki et al. 1989, Rehnstam et al. 1989, Martínez-Picado et al. 1994, 1996, Ito et al. 1995), all the protocols described to date have required previous culture of the microorganism, necessitating the development of an alternative culture-independent technique for the detection of the pathogen. Moreover, DNA-probes based on *L. anguillarum* 16S rRNA ribosomal gene (as for example that reported by Rehnstam et al. 1989), can hybridize with *Vibrio* species, as evidenced by database comparisons. Also, the

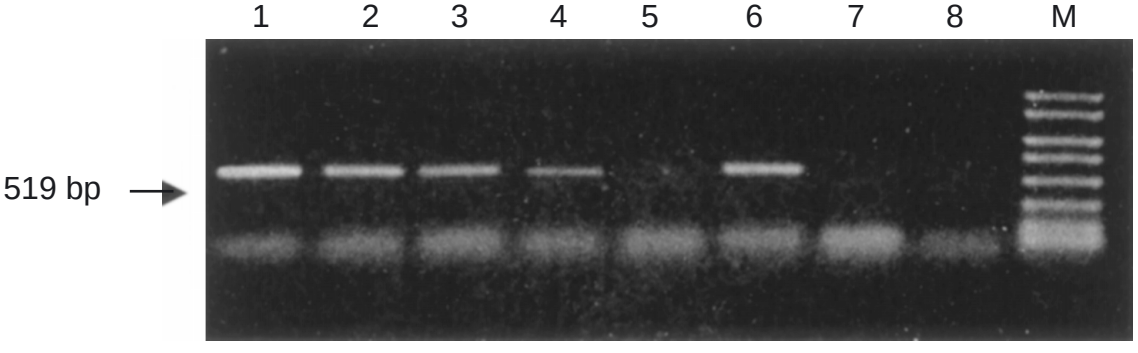

Fig. 3. *Listonella anguillarum*. Detection limit of PCR reaction using different dilutions of purified DNA from Strain R-82. Lane M: 1 kb molecular weight marker; Lanes 1 to 5: PCR products from suspensions of 1×10^4, 1×10^3, 1×10^2, 10 and 1 *L. anguillarum* cells per PCR tube, respectively; Lane 6: positive control; Lanes 7 and 8: negative controls

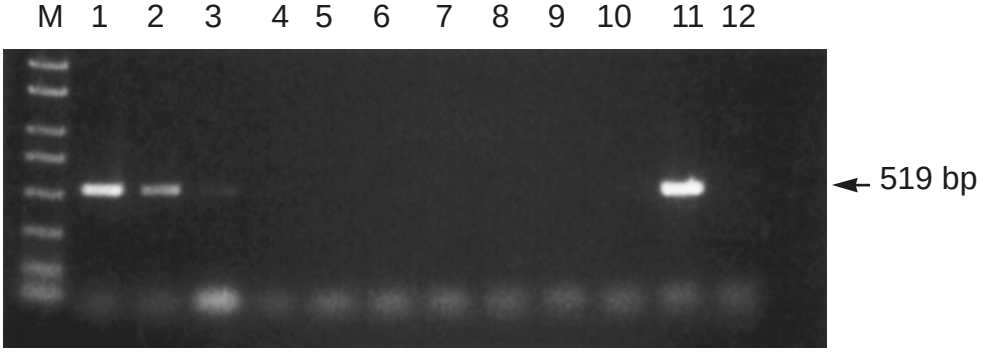

Fig. 4. *Listonella anguillarum*. Detection limit in experimentally infected fish (*Scophthalmus maximus*) tissues. Lane M: 1 kb molecular weight marker; Lanes 1 to 4, 2×10^6, 2×10^5, 2×10^4, 2×10^3, *L. anguillarum* cells g^{-1} of fish tissue, respectively; Lanes 5 to 9: no DNA; Lane 11: positive control; Lanes 10 and 12: negative controls

nucleotide sequence of the DNA probe based on the 16S gene described by Martínez-Picado et al. (1994), shows a 100% identity with the respective 16S gene sequence in related species such as *V. ordalii*, *V. diazotrophicus* and *V. navarrensis*.

Detection of *Listonella anguillarum* by PCR was first reported by Hirono et al. (1996), based on a hemolysin gene of this bacterium. However, this hemolysin gene is not present in all *L. anguillarum* strains, and thus the PCR method described by these authors fails to detect some isolates of this fish pathogen.

Our study has shown that the PCR method is one of the strongest tools for diagnosis, detecting in pure cultures as few as 1 to 10 *Listonella anguillarum* cells per reaction. The results from seeding of kidney, spleen tissues and blood samples also indicate the sensitivity of this method, detecting as few as 2×10^4 cells g^{-1} fish tissue, which corresponds to 100 *L. anguillarum* cells per reaction. Although the detection level with fish tissues and blood samples was lower than that with pure cultures (probably due to the presence of host DNA and undefined inhibitors of the PCR reaction; Wilson 1997), this PCR-based protocol is more sensitive than other molecular or serological methods previously described (Cipriano et al. 1985, Ito et al. 1995, Adone et al. 1996, Martínez-Picado et al. 1996). Blood testing is preferable to tissue analyses because it does not require the sacrifice of the sampled fish and is suitable for monitoring fish on farms. Moreover the high resolution of this DNA-based method makes a nested PCR reaction unnecessary. The PCR assay with the primers *rpoN*-ang5′ and *rpoN*-ang3′ was found to be specific for *L. anguillarum* at an annealing temperature of 62°C. Thus, our PCR protocol represents the first DNA-based molecular tool that allows distinguishing between *L. anguillarum* and *Vibrio ordalii*. By changing the annealing temperature from 62 to 58°C, we can also detect *V. ordalii* by this method, which could be useful when both pathogens are responsible for fish mortalities. This unspecific reaction also confirms the close genetic relationship between these 2 phenotypically separated species.

The PCR assay can be performed in 5 h compared to 48 to 72 h for serological or biochemical analyses. Moreover this method allows detection of the pathogen without previous isolation in culture media. Our results validate its efficacy in the rapid, specific and sensitive detection of this pathogen in field samples as well as in routine identification of the bacteria in mixed or pure cultures.

Acknowledgements. This work was supported by Grant XUGA20008B98 from Xunta de Galicia, Spain, and Grant AGL2000-0492 from Dirección General de Enseñanza Superior e Investigación Científica, Ministerio de Educación y Cultura (MEC), Spain.

LITERATURE CITED

Adone R, Piccininno C, Pistoia C, Bianchi R, Ciuchini F (1996) Detection of *Vibrio anguillarum* by a sandwich enzyme-linked immunosorbent assay performed with monoclonal antibodies. J Vet Med Ser B 43:579–584

Alsina M, Martínez-Picado J, Jofre J, Blanch AR (1994) A medium for presumptive identification of *Vibrio anguillarum*. Appl Environ Microbiol 60:1681–1683

Aoki T, Hirono I, De Castro T, Kitao T (1989) Rapid identification of *Vibrio anguillarum* by colony hybridization. J Appl Ichthyol 5:67–73

Austin B, Austin DA (1999) *Vibrionaceae* representatives: characteristics of the disease. In: Austin B, Austin DA (ed) Bacterial fish pathogens: diseases of farmed and wild fish. Springer Praxis Publ, London, p 29–30

Austin B, Alsina M, Austin DA, Blanch AR and 9 others (1995) Identification and typing of *Vibrio anguillarum*: a comparison of different methods. Syst Appl Microbiol 18:285–302

Brown LL, Iwama GK, Evelyn TPT, Nelson WS, Levine RP (1994) Use of the polymerase chain reaction (PCR) to detect DNA from *Renibacterium salmonilarum* within individual salmonid eggs. Dis Aquat Org 18:165–171

Canestrini G (1893) La malattia dominate delle anguille. Atti Ist Veneto Sci Lett Arti Cl Sci Mat Nat 7:809–814

Cepeda C, Santos Y (2000) Rapid and low-level toxic PCR-based method for routine identification of *Flavobacterium psychrophilum*. Int Microbiol 3:235–238

Cipriano RC, Pyle JB, Starliper CE, Pyle SW (1985) Detection of *Vibrio anguillarum* antigen by the dot blot assay. J Wildl Dis 21:211–218

Corinne EG, Connor JT, Trevor JT (1992) Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene. Appl Environ Microbiol 58:3816–3825

Hill WE, Keasler SP, Trucksess MW, Feng P, Kaysner CA, Lampel KA (1991) Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Appl Environ Microbiol 57:707–711

Hiney M, Smith P (1998) DNA-based diagnostics in aquaculture: can we overcome the problems of interpretation in the field. In: Barnes AC, Davidson GAD, Hiney MP, McIntosh (eds) Methodology in fish research. Fisheries Research Services, Aberdeen, p 143–159

Hirono H, Masuda T, Aoki T (1996) Cloning and detection of the hemolysin gene of *Vibrio anguillarum*. Microb Pathog 21:173–182

Ito H, Ito H, Uchida I, Sekizaki T, Terakado N (1995) A specific oligonucleotide probe based on 5S rRNA sequences for identification of *Vibrio anguillarum* and *Vibrio ordalii*. Vet Microbiol 43:167–171

Kita-Tsukamoto K, Oyaizu H, Nanba K, Shimidu U (1993) Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of the 16S rRNA sequences. Int J Syst Bacteriol 43: 8–19

MacDonell MT, Colwell RR (1985) Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. Syst Appl Microbiol 6:171–182

Martínez-Picado J, Blanch AR, Jofre J (1994) Rapid detection and identification of *Vibrio anguillarum* by using a specific oligonucleotide probe complementary to 16S rRNA. Appl Environ Microbiol 60:723–737

Martínez-Picado J, Alsina M, Blanch AR, Cerdá M, Jofre J (1996) Species-specific detection of *Vibrio anguillarum* in marine aquaculture environments by selective culture and DNA hybrydization. Appl Environ Microbiol 62:443–449

Osorio CR, Toranzo AE (2002) DNA-based diagnosys in seafarming. In: Fingerman M, Nagabhushanam R (eds) Recent advances in marine biotechnology series, Vol 7: Seafood safety and human health. Science Publishers, Enfield, NH, p 253–310

Osorio CR, Collins MD, Toranzo AE, Barja JL, Romalde JL (1999) 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Appl Environ Microbiol 65:2942–2946

O'Toole R, Milton DL, Hörstedt P, Wolf-Watz H (1997) *rpoN* of the fish pathogen *Vibrio* (*Listonella*) *anguillarum* is essential for flagellum production and virulence by the waterborne but not intraperitoneal route of inoculation. Microbiology (Read) 143:3849–3859

Pedersen K, Austin B, Austin DA, Larsen JL (1999a) Vibrios associated with mortality in cultured plaice *Pleuronectes platessa* fry. Acta Vet Scand 40:263–270

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

Rehnstam AS, Norqvist A, Wolf-Watz H, Hagström Å (1989) Identification of *Vibrio anguillarum* in fish by using a partial 16S rRNA oligonucleotide probe. Appl Environ Microbiol 55:1907–1910

Ruimy R, Breittmayer V, Elbaze P, Lafay B, Boussemart O, Gauthier M, Christine R (1994) Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. Int J Syst Bacteriol 44:416–424

Santos Y, Romalde JL, Bandín I, Magariños B, Núñez S, Barja JL, Toranzo AE (1993) Usefulness of the API-20E system for the identification of bacterial fish pathogens. Aquaculture 116:111–120

Sorensen UBS, Larsen JL (1986) Serotyping of *Vibrio anguillarum*. Appl Environ Microbiol 51:593–597

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, Baya AM, Roberson BS, Barja JL, Grimes DJ, Hetrick M (1987) Specificity of slide agglutination test for detecting bacterial fish pathogen. Aquaculture 61: 81–97

Toyama T, Kita-Tsukamoto K, Wakabayashi H (1994) Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. Fish Pathol 29:271–275

Urdaci MC, Chakroun C, Faure D, Bernardet JF (1998) Development of a polymerase chain reaction assay for identification and detection of the fish pathogen *Flavobacterium psychrophilum*. Res Microbiol 149:519–530

Valle O, Dorsch M, Wiik R, Stackebrandt E (1990) Nucleotide sequence of the 16S rRNA from *Vibrio anguillarum*. Syst Appl Microbiol 13:257

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741–3751

Editorial responsibility: Jo-Ann Leong, Kaneohe, Hawaii, USA

Submitted: May 15, 2002; Accepted: March 3, 2003
Proofs received from author(s): June 11, 2003