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PCR detection and PFGE DNA macrorestriction analyses of clinical isolates of *Pseudomonas anguilliseptica* from winter disease outbreaks in sea bream *Sparus aurata*

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ABSTRACT: A PCR-based detection system for *Pseudomonas anguilliseptica* was evaluated. The primer combination PAF-PAR (forward primer PAF = 5′-GACCTCGCCATTA-3′, reverse primer PAR = 5′-CTCAGCAGTTTTGAAAG-3′) gave a unique and specific amplification product of 439 bp at an annealing temperature of 46°C with all the *P. anguilliseptica* isolates and strains (n = 56) but no amplification products were observed with any other *Pseudomonas* species or phylogenetically related bacteria tested. The PCR assay had a detection limit of 170 to 200 cells per PCR tube, which was improved 8-fold when the PCR amplification product was used as a nonradioactive probe in blotting hybridization experiments. The PCR assay allowed the specific and reliable detection of *P. anguilliseptica* within 8 h, compared with up to 10 d required for its isolation and further characterization by conventional microbiological approaches. Clinical isolates of *P. anguilliseptica* recovered from several winter disease (WD) outbreaks diagnosed in sea bream *Sparus aurata* in Spain and Portugal between 1996 and 2001 were characterized by pulse field-gel electrophoresis (PFGE) macrorestriction analysis. The 54 clinical isolates analyzed were included in 4 different pulsotypes. Pulsotypes B and C represented 54 and 25% of the isolates, respectively, and were responsible for most of the WD outbreaks diagnosed in Spain between 1996 and 2001. The implication of asymptomatic infected carriers in the dissemination and spread of WD is discussed.

KEY WORDS: *Pseudomonas anguilliseptica* · PCR detection · PFGE characterization · Winter disease

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INTRODUCTION

Pseudomonas anguilliseptica is an emerging fish pathogen that has gained clinical significance in being responsible for mortality outbreaks in different fish species. It was first isolated from cultured Japanese eels *Anguilla japonica* affected by red spot disease (Wakabayasi & Egusa 1972). Subsequently it has been isolated from different fish species in several countries (Nakai & Muroga 1982, Wiklund & Bylund 1990, Lönnström et al. 1994, Wiklund & Lönnström 1994, Berthe et al. 1995, Doménech et al. 1997, 1999, Al-Marzouk 1999, Haenen & Davidse 2001). Recent microbiological studies have associated infection by *P. anguilliseptica* with the etiology of winter disease (WD) in sea bream *Sparus aurata* (Doménech et al. 1997, 1999). WD is a multifactorial syndrome associated with the nutritional and immune status of fish and stressful environmental conditions (Cinquina et al. 1998, Tort et al. 1998). It is characterized by a septicemic infection in which the most evident clinical signs are an abdominal distension because of the abundant ascitic fluid, pale liver, haemorrhagic kidney and congestive intestine with fibrinous yellowish exudate in the affected fish. During the past few years, several WD outbreaks in sea bream have been

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reported in different countries (Bovo et al. 1995, Doimi 1996, Doménech et al. 1997, 1999, Tort et al. 1998, Sarusic 1999). The severe mortality that can be observed during the WD outbreaks (Domenech et al. 1997), together with the fact that sea bream is one of the main marine non-salmonid fish species cultured (Caggiano 2000), make *P. anguilliseptica* a fish pathogen of emerging clinical significance.

The diagnosis of *Pseudomonas anguilliseptica* infections is made difficult by the slow growth rate and weak reactivity of this microorganism in most biochemical tests (Michel et al. 1992, Doménech et al. 1997, 1999). The detection and identification of fastidious microorganisms, like *P. anguilliseptica*, can be significantly improved by using molecular techniques. In this sense, PCR assays, based on primers designed to amplify variable regions of the 16S rRNA gene, have been described for many bacterial fish pathogens (Martínez-Picado et al. 1994, Saulnier & Kinkelin 1997, Urdaci et al. 1998, Zlotkin et al. 1998a, Gibello et al. 1999).

Despite the clinical and commercial significance of *Pseudomonas anguilliseptica* infections, there is a lack of information about the epidemiological relationships between strains of *P. anguilliseptica* isolated from different disease outbreaks, as well as the source of infection and the ways in which *P. anguilliseptica* is transmitted. For these purposes, DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) has proved to be a reliable and highly discriminatory technique that has been successfully applied to the molecular characterization of different fish pathogens (Hanninen & Hirvela-Koski 1999, Vela et al. 2000).

The aim of this study was to develop a PCR-based detection system for *Pseudomonas anguilliseptica*, as well as to characterize, by PFGE DNA macrorestriction analysis, the *P. anguilliseptica* clinical isolates recovered from several WD outbreaks diagnosed in sea bream in Spain and Portugal between 1996 and 2001.

MATERIALS AND METHODS

Bacterial strains. The study included 54 clinical isolates of *Pseudomonas anguilliseptica* (Table 1) obtained from sea bream affected by WD in Spain and Portugal between 1996 and 2001. *P. anguilliseptica* isolates were isolated and identified as described previously (Doménech et al. 1997), and were frozen at –80°C until further use. All clinical isolates were used for PCR and PFGE studies. Other *Pseudomonas* species and phylogenetically related taxa used for the specificity studies of the PCR assay are listed in Table 1.

Isolation of bacterial DNAs. Bacterial chromosomal DNA was extracted by the method of Lawson et al. (1989). Purified DNA was dissolved in 50 µl of distilled water and then stored at –20°C until use.

Primer design and PCR amplification. The forward primer PAF (5′-GACCTCGCGCCATTA-3′) and the reverse primer PAR (5′-CTCAGCAGTTTTGAAAG-3′) were designed from non-conserved regions of the *Pseudomonas anguilliseptica* 16S rRNA gene sequence (Accession No. X99540) from Positions 202 to 216 and 624 to 640, respectively, and were synthesized by ISOGEN (Bioscience BV). The apparent specificity of each oligonucleotide used as primer was determined according to Maidak et al. (1994). Primers were tested for PCR amplification at 2 different annealing temperatures (43 and 46°C), with DNA from selected strains of *P. anguilliseptica*, other *Pseudomonas* species and other taxa included in Table 1.

The PCR amplifications were performed in 100 µl reaction volumes containing DNA template (50 to 70 ng of chromosomal bacterial DNA, 20 µl of boiled cells, or 10 µl of DNA extracted from fish tissue, later subsection), 1 mM of each primer (PAF and PAR), 2 mM of each deoxynucleotide triphosphate, and 2.5 U of Amplitaq gold DNA polymerase (PE Biosystems) in 1 × reaction buffer. After an additional denaturation step of 95°C for 7 min and 35 cycles of 95°C for 1 min, annealing at the selected temperature for 1 min and extension at 72°C for 1 min 30 s were performed, followed by a final extension step of 72°C for 5 min. Negative (no template DNA) and positive (50 ng of purified DNA from *Pseudomonas anguilliseptica* Strain CECT 899) controls were included in each batch of PCR reactions. The amplifications were carried out in a PT-100 thermal cycler (MJ Research). PCR-generated products were detected by electrophoresis of 15 µl of each amplification mixture in 1% agarose gels in 1% tris-acetate-EDTA buffer. Gels were stained with 0.5 mg ml^{-1} ethidium bromide.

Specificity and sensitivity of the PCR. The specificity of the PCR assay was tested by using, as template, the extracted DNA of all *Pseudomonas* strains and the clinical isolates obtained between 1996 and 1998 and other bacteria listed in Table 1. For screening the clinical isolates of *P. anguilliseptica* isolated between 1999 and 2001, 1 bacterial colony was suspended in 0.2 ml of sterile water and boiled for 10 min, and 20 µl of this suspension was added directly to 80 µl of PCR mixture.

To determine the detection limit of the PCR, a suspension of *Pseudomonas anguilliseptica* Strain CECT 899 containing 1.3×10^6 $cells^{-1}$ ml was serially diluted 2-fold in 0.9% saline buffer to 2.5×10^3 cells ml^{-1}. Twenty microliters of each dilution was boiled for 10 min and added directly to the PCR mixture. The bacterial concentration was determined by plating 0.1 ml of each dilution onto MacConkey agar plates (bioMérieux España S.A.).

Table 1. Bacterial species assayed in the PCR experiments. CECT: Colección Española de Cultivos Tipo (Spanish Type Culture Collection), Valencia, Spain

Species	Strain	Source and/or geographic origin
***Pseudomonas* spp.**		
P. anguilliseptica		
Collection strains	CECT 899^T	Japanese eel, Japan
	CECT 900	European eel, Scotland
Clinical isolates		
	A/1168, A/1169	Sea bream, Portugal
	A/1123, A/1203, A/1204, A/1379, A/1380, A/1413-A/1419, A/1442, A/1443, A/1445, A/1748-A/1754, A/1763-A/1766, 99/4422, 99/4472, 00/4854, 00/4862, 00/4891, 00/4895, 00/4897, 00/4900, 00/4923, 00/4964, 01/5237, 01/5246, 01/5254, 01/5255, 01/5263, 01/5296, 01/5297, 01/5305, 01/5311, 01/5328, 01/5330, 01/5331, 01/5348, 01/5357, 01/5386, 01/5421	Sea bream, Spain
P. aeruginosa	CECT 110^T	
	CECT 118	Human, UK
	CECT 4001	Human clinical isolate, UK
P. mendocina	CECT 320^T	Soil, Argentina
P. oleovorans	CECT 4079^T	Industrial lubricant
P. fluorescens	CECT 378^T	Pre-filter water tanks, UK
P. alcaligenes	CECT 929^T	Swimming pool water
P. putida	CECT 324^T	Soil, USA
P. stutzeri	CECT 930^T	Human spinal fluid
P. pseudoalcaligenes	CECT 318^T	Human sinus discharge
P. tolaasii	CECT 4472^T	Diseased mushrooms, UK
Pseudomonas sp.	S99/104	Chinese softshell, Spain
Other genera		
Burkholderia cepacia	CECT 322	Forest soil, Trinidad
Burkholderia caryophylli	CECT 117	*D. caryophilus*, Denmark
Ralstonia picketti	CECT 330^T	Human clinical isolate, USA
Brevundimonas diminuta	CECT 317^T	Fresh water stream, USA
Comamonas acidovorans	CECT 311^T	Soil, The Netherlands
Serratia marcescens	CECT 846	Pond water, Czech Republic

^{T}: the strain is the type strain for the species

Preparation of homogenates of artificially contaminated sea bream tissue. Samples of liver, kidney and spleen from 25 sea bream were obtained aseptically. These organs were weighed and blended with the appropiate volume of 0.9% saline buffer to obtain a 1/10 dilution of each organ. The absence of *Pseudomonas anguilliseptica* in the tissue homogenates was determined by plating 0.1 ml onto MacConkey agar plates which were incubated at 22°C for 7 d. Samples (1 ml) of tissue homogenate were inoculated with *P. anguilliseptica* CECT 899 to obtain a final concentration of 8×10^6 bacteria g^{-1}. The enumeration of *P. anguilliseptica* in the contaminated tissues was determined by plating 0.1 ml of appropriate 10-fold dilutions of these homogenates onto MacConkey agar plates incubated for 7 d at 22°C. Non-inoculated tissue homogenates were used as controls.

DNA extraction from tissues. Two different methods were evaluated for the DNA extraction from tissue homogenates: the method described by Lawson et al. (1989), which includes the use of phenol/chloroform (PhC method), and the technique described by Casas et al. (1995), which involves a single extraction using a guanidinium thiocyanate acid buffer (GuSCN method). Fifty microlitres of tissue homogenate were processed by both methods. The total extracted DNA was dissolved in 10 µl of sterile distilled water and used for PCR experiments.

Slot blot hybridization. Two hundred microlitres of *Pseudomonas anguilliseptica* suspensions containing 3×10^8 to 3×10^0 cells ml^{-1} were transferred to a nylon membrane (Roche Laboratories) by a slot blot procedure using a Bio-Dot microfiltration apparatus (Bio-Rad). Bacterial DNA was extracted by alkaline lysis as

described by Sambrook et al. (1989) and DNA was fixed to the membrane by UV crosslinker in a Spectrolinker XL-1000 (Spectronic Corporation). Then, the blot was assayed against the 439 bp biotin-labelled product obtained from the strain *P. anguilliseptica* CECT 899 DNA PCR amplification, with the biotin primers PAF and PAR labelled at the 5′-end. Prehybridization and hybridization were performed at 65°C for 3 h in a solution of 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS). The labelled probe was used at a concentration of 20 ng ml^{-1}. Washes under high-stringency conditions and hybridized DNA detection using CDP-Star procedure (Roche Molecular Biochemicals) were carried out as previously described (Gibello et al. 1999).

PFGE. *Pseudomonas anguilliseptica* strains were grown on Columbia blood agar plates at 22°C for 5 d. Cells were harvested with a loop and resuspended in 2 ml of SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5), to obtain a final absorbance of 0.6 at 600 nm. Cells were harvested by centrifugation at 3500 rpm for 10 min (821 × *g*) at 4°C, washed twice with 2 ml of SE buffer, and resuspended in 3 ml of SE buffer. The cell suspension was mixed with an equal volume of 2% (w/v) low-melting-point agarose. When solidified, blocks were incubated in lysis buffer (50 mM Tris-HCl, 50 mM EDTA and 1% n-lauroyl-sarcosine sodium salt [pH 9.5], 5 μg^{-1} ml lysozyme) for 24 h at 37°C. The lysis buffer was replaced with 2 ml lysis buffer containing 2.5 μg^{-1} ml of Proteinase K, and the blocks were incubated at 56°C for 48 h. Blocks were washed 3 times with tris-EDTA buffer for 1 h at 4°C. *Spe*I and *Xba*I (Promega), were used for restriction endonuclease digestion according to the manufacturer's instructions. *Spe* I and *Xba* I have been previously used in PFGE analysis of *P. aeruginosa* (Hla et al. 1996, Speijer et al. 1999). DNA fragments were resolved by pulsed field-gel electrophoresis (PFGE) using a CHEF-DR III System (Bio-Rad Laboratories). Electrophoresis of digested samples was performed through 1% (w/v) electrophoresis-grade agarose (Roche Molecular Biochemicals) under the following conditions: running time 19 h, temperature 14°C, voltage gradient 200 V, initial pulse time 0.1 s, final pulse time, 15 s, included angle 120º. Gels were stained with 0.5 mg of ethidium bromide l^{-1} and DNA was visualized by UV transillumination. Lambda ladder PFGE Marker 1 (Boehringer Mannheim, Roche) was used for molecular weight size determination.

Macrorestriction patterns were analyzed both visually and by computer-aided methods. The visual genetic relationship among isolates was evaluated following the criteria of Tenover et al. (1995). PFGE patterns that differed by more than 3 bands were recorded as types and are identified with a capital letter. Patterns that differed by 1 to 3 bands were recorded as different subtypes and are identified with a capital letter followed by an arabic numeral. The similarities between restriction endonuclease digestion profiles were expressed as the Jaccard similarity index with a TAXAN program and cluster analysis by the unweighted pair group method using arithmetic averages.

RESULTS AND DISCUSSION

PCR assay

The primer combination PAF-PAR gave a unique and specific amplification product of 439 bp length at an annealing temperature of 46°C with *Pseudomonas anguilliseptica* isolates only. At an annealing temperature of 43°C, amplification products were observed with *P. fluorescens* and *P. aeruginosa* (data not shown). Identical amplification products were obtained when the PCR was performed with whole bacterial cells boiled for 10 min instead of extracted DNA. The annealing temperature of 46°C was used for all subsequent PCR assays. All clinical *P. anguilliseptica* isolates had a 439 bp amplification product, but no amplification products were observed with any of the other *Pseudomonas* species or phylogenetically related bacteria listed in Table 1 (Fig. 1). Therefore, the primer combination PAF and PAR at this annealing temperature proved to be specific for *P. anguilliseptica*. The PCR assay had a detection limit of 1×10^4 cells ml^{-1}, which equates to 200 cells per PCR reaction mixture (Fig. 2), assuming that the lysis procedure was complete (no viable cells were detected after the boiling treatment). The level of detection increased 8-fold, when the PCR amplification product was used as a nonradioactive probe in blotting hybridization experiments, where the minimum amount of *P. anguilliseptica* detected was 24 bacterial cells (data not shown).

Initial experiments with artificially contaminated tissue homogenates of 5 sea bream showed that the observed yield of the PCR products was greater with the GuSCN DNA extraction method than with the PhC method (data not shown). In addition, the former procedure was less time-consuming (1 h) compared with the 4 h needed for the PhC method. This result indicated that, as previously reported for viral DNA (Casas et al. 1995), the GuSCN method is also a simple, reliable and useful procedure for *Pseudomonas anguilliseptica* DNA extraction from tissues. Thus, the GuSCN method was further routinely used for DNA extraction from the homogenates of artificially contaminated sea bream tissue.

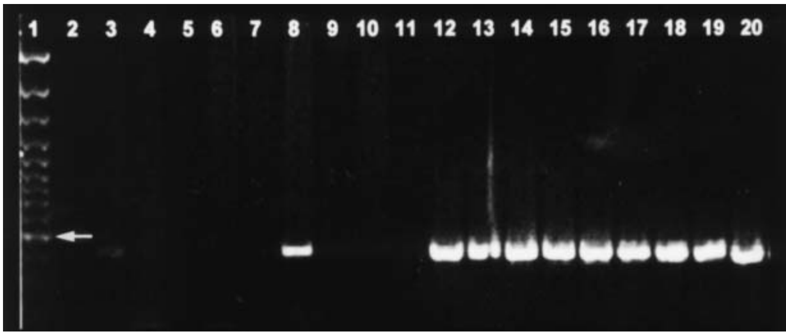

Fig. 1. Specificity of the PCR assay *Pseudomonas anguilliseptica*: ethidium-bromide-stained gel with the specific amplification product of 439 bp obtained by extracted DNA PCR at 46°C annealing temperature with primers PAF-PAR from different bacterial species. Lane 1: Gene Ruler 100 bp DNA ladder plus (MBI Fermentas) (arrow indicates 500 bp marker); Lane 2: negative control; Lane 3: *Pseudomonas anguilliseptica* CECT 900; Lanes 4 to 7: *P. aeruginosa* CECT 110^T, *P. fluorescens* CECT 378^T, *P. putida* CECT 324^T and *P. pseudoalcaligenes* CECT 318^T, respectively; Lane 8: *Pseudomonas anguilliseptica* CECT 899^T; Lanes 9 to 11: *Burkholderia cepacia* CECT 322, *Ralstonia picketti* CECT 330^T, and *Serratia marcescens* CECT 846, respectively; Lanes 12 to 17: *Pseudomonas anguilliseptica* A-1123, A-1765, 99/4422, 00/4862, 00/4900, and 01/5246, respectively; Lanes 18 to 20: spleen, liver, and kidney tissue homogenates, artificially contaminated with *Pseudomonas anguilliseptica* CECT 899, respectively

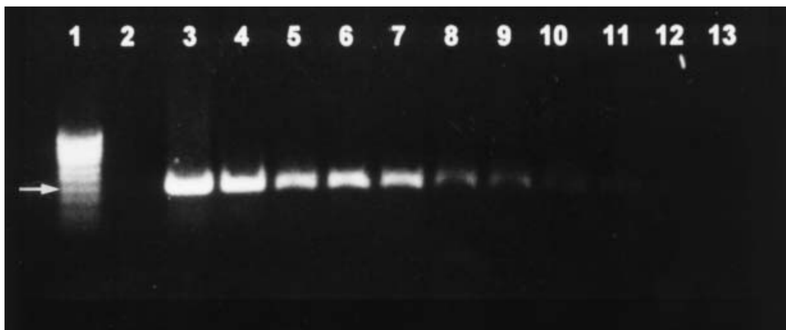

Fig. 2. *Pseudomonas anguilliseptica.* Sensitivity of detection by PCR. Lane 1: 1000 bp ladder (Biotools) (arrow indicates 400 bp marker); Lane 2: negative control; Lane 3: *Pseudomonas anguilliseptica* CECT 899^T; Lanes 4 to 13: 2-fold decreasing boiled cell concentrations ranging from 2.6×10^4 to 50 per PCR reaction mixture, respectively

When the PCR assay was used for the detection of *Pseudomonas anguilliseptica* in experimentally inoculated tissues, the 439 bp amplification product was detected from all tissue homogenates (Fig. 1, Lanes 18 to 20), regardless of the type of tissue. The detection limit of the PCR assay in tissues was 170 cells per reaction mixture, which equates to 3.4×10^4 cells g^{-1} tissue (data not shown). This detection limit is similar to those described in PCR assays with other bacterial fish pathogens (McIntosh et al. 1996, Urdaci et al. 1998, Gibello et al. 1999). Although the presence of host DNA or inhibitory substances in tissue extracts has been reported to reduce the sensitivity of the PCR assays (Coleman et al. 1996, McIntosh et al. 1996), the sensitivity of the PCR assay with tissue extracts was very similar to that obtained with cell suspensions. The absence of inhibitory effects may be due to the 10-fold dilution step of the tissues (Gibello et al. 1999) and/or the DNA extraction procedure used, which would reduce or eliminate the possible inhibitory substances present in the tissue extracts (Altinok et al. 2001).

Pseudomonas anguilliseptica is a very slow growing organism that is difficult to identify by standard biochemical tests due to its slow reactive metabolism (Doménech et al. 1997). Hence, the PCR assay developed here allows the specific and reliable detection of *P. anguilliseptica* within 8 h of receiving a sample compared with up to 10 d required for its isolation and further identification by conventional microbiological approaches (Doménech et al. 1997). The data presented here point out the efficacy of the PCR assay developed for a rapid and accurate identification of *P. anguilliseptica*.

The detection limit using artificially contaminated tissue homogenates of the PCR assay was lower than the bacterial counts that *Pseudomonas anguilliseptica* can reach in target organs of experimentally infected fish (Haenen & Davidse 2001). Thus, it is likely that the PCR assay could be also useful for the rapid and specific diagnosis of natural infections by *P. anguilliseptica*, including WD.

PFGE analysis

PFGE of *Pseudomonas anguilliseptica* chromosomal DNA digested with *Xba*I yielded 13 to 16 fragments in the 48 to 194 kb size range, while *Spe*I digested DNA yielded patterns of 20 to 25 fragments of 30 to 291 kb (Fig. 3). The PFGE patterns of the same isolate generated for each of the 2 restriction endonucleases were found to be stable and reproducible in at least 3 separate trials on different gels. With the *Xba*I digestion, 2 restriction patterns were identified through visual comparison of gels. One restriction pattern included 46 clinical isolates, the remaining isolates (01/5255, 01/5263, 01/5297, 01/5305, 01/5311,

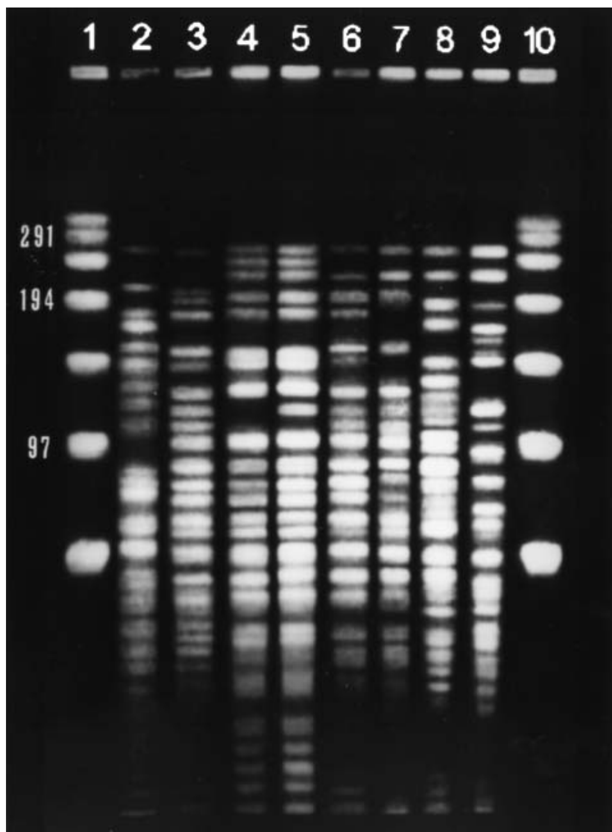

Fig. 3. *Pseudomonas anguilliseptica.* Pulsed-field gel electrophoresis patterns (PFGE) of *Spe*I digested DNA of clinical isolates. Lanes 1 and 10: Roche molecular weight marker (48.5 to 1000 kbp); Lane 2: Pulsotype F (Strain CECT 900); Lane 3: Pulsotype B (Isolate A/1380); Lane 4: Pulsotype D2 (Isolate A/1168); Lane 5: Pulsotype D1 (Isolate A/1123); Lane 6: Pulsotype C1 (Isolate 99/4422); Lane 7: Pulsotype C2 (Isolate 01/5254); Lane 8: Pulsotype E (Isolate 01/5263); Lane 9: Pulsotype A (Strain CECT 899)

01/5328, 01/5331 and 01/5357) displayed indistinguible restriction patterns with a genetic similarity of 70% with the predominant clone (data not shown). The *Spe*I enzyme was found to be more discriminatory than *Xba*I. The 46 clinical isolates that were indistinguishable with *Xba*I digestion were distributed in 3 pulsotypes, identified as B, C and D after *Spe*I digestion (Fig. 4, Table 2). Most of the isolates (53.8%) were included in Pulsotype B. Pulsotype C (25% of the strains) was discriminated by PFGE band analysis into 2 subtypes with more than 85% similarity; Subtype C1 was the most prevalent (11 isolates). Pulsotype D (9.6% of the isolates) included a majority subtype, Subtype D1, with most of the clinical isolates from the first outbreaks of WD detected in 1996 in Spain (Doménech et al. 1997), and Subtype D2, with only the Strain A/1168. The levels of similarity of Pulsotypes C and D to Pulsotype B according to the Jaccard coefficient were 75 and 55%, respectively. The isolates 01/5255, 01/5263, 01/5297, 01/5305, 01/5311, 01/5328, 01/5331 and 01/5357 displayed a different restriction pattern (Pulsotype E) (Fig. 4, Table 2). The Collection Strains *P. anguilliseptica* CECT 899 and CECT 900 displayed different restriction profiles, designated Pulsotypes A and F, respectively.

Pseudomonas anguilliseptica has been isolated from different farmed fish species in different countries (Wiklund & Bylund 1990, Berthe et al. 1995, Al-Marzouk 1999, Haenen et al. 2001), suggesting a widespread distribution of this microorganism. Although there are no previously reported molecular characterization studies about the genetic and epidemiological relationships of its clinical isolates, *P. anguilliseptica* could be expected to have, like some other widely distributed microorganisms (Chomarat et al. 1998, Hanninen & Hirvela-Koski 1999), a great genetic heterogeneity. However, the clinical isolates of *P. anguilliseptica* studied, with only 4 different pulsotypes, displayed low genetic diversity. This result could be related to the fact that 52 of the 54 isolates examined were recovered from the same host fish species affected by WD, and a somewhat limited geographic area. Pulsotypes B and C were responsible for

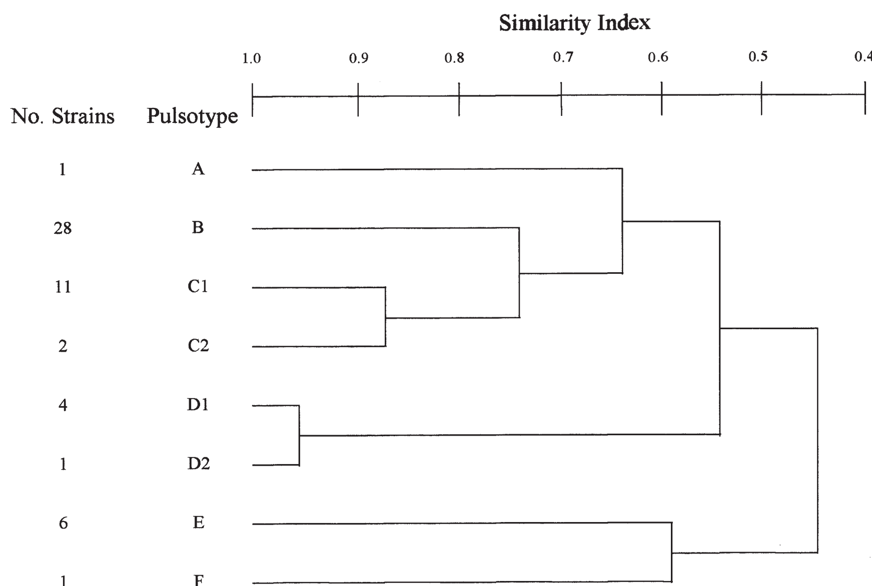

Fig. 4. *Pseudomonas anguilliseptica.* Dendogram of isolates based on UPGMA cluster analysis of the 6 different pulsotypes observed in this study following *Spe*I endonuclease DNA digestion

Table 2. Pulsotype characterization of the *Pseudomonas anguilliseptica* isolates by PFGE obtained after DNA digestion with the *Spe* I restriction enzyme. CAT: Cataluña (north-east Spain); LEV: Levante (east Spain); AND: Andalucía (south Spain); LIS SET: Lisboa and Setubal, respectively (south-west Portugal). The different numbers follwing the same geographic origin indicate different fish farms

Pulsotype	Isolate(s)	Year of isolation	Geographic location of fish farm
A	CECT 899^T	1972	Collection strain
B	A/1379, A/1380	1996	CAT-2
	A/1413-A/1418	1997	LEV-1
	A/1419	1997	LEV-2
	A/1442, A/1443	1997	CAT-2
	A/1748-A/1754	1997	CAT-5
	A/1763-A/1766	1998	CAT-2
	99/4472	1999	CAT-2
	00/4897	2000	CAT-5
	00/4900	2000	LEV-2
	00/4923	2000	CAT-2
	01/5237	2001	CAT-2
	01/5296	2001	LEV-2
C1	A/1445	1997	CAT-4
	99/4422	1999	CAT-4
	00/4854, 00/4862	2000	AND-2
	00/4891, 00/4964	2000	CAT-3
	00/4895	2000	CAT-4
	01/5246, 01/5421	2001	CAT-4
	01/5330	2001	LEV-3
	01/5386	2001	LEV-2
C2	01/5254	2001	CAT-6
	01/5348	2001	CAT-9
D1	A/1123	1996	AND-1
	A/1169	1996	SET
	A/1203, A/1204	1996	CAT-1
D2	A/1168	1996	LIS
E	01/5255	2001	CAT-8
	01/5263	2001	CAT-7
	01/5297, 01/5305, 01/5311 01/5328	2001	CAT-4
	01/5331, 01/5357	2001	CAT-3
F	CECT 900	1981	Collection strain

most of the WD outbreaks studied by us in Spain between 1996 and 2001, which is in agreement with the existence of prevalent clones responsible for a great majority of the clinical cases observed in other fish pathogens (Vela et al. 2000). Experimental pathogenicity studies have suggested that certain strains of *P. anguilliseptica* would be more pathogenic for certain fish species (Haenen & Davidse 2001). Therefore, although the pathogenicity of Pulsotypes B and C was not evaluated, their isolation from most of the WD outbreaks diagnosed by us may be related to a higher pathogenic potential of these pulsotypes for sea bream. In addition, their rapid spread in different geographical areas (Table 2), and their isolation in the same year from fish farms located several hundred kilometers apart, suggest that *P. anguilliseptica* is actually widespread but has only recently emerged as a pathogen as the industry has expanded or environmental conditions have promoted its emergence. The movement of infected fish/asymptomatic carriers into susceptible populations through the trade of livestock is one of the main factors responsible for the dissemination and transmission of fish pathogens (Lönnström et al. 1994, Hanninen & Hirvela-Koski 1999). *P. anguilliseptica* (Pulsotype B) was isolated from asymptomatic sea breams surviving a natural infection, suggesting the existence of a carrier state (A. Rodríguez unpubl. data). Also, the Portuguese sea bream affected by WD in 1996 were imported from Andalucía about 9 mo before the onset of the outbreak, and *P. anguilliseptica* Strains A-1123 and A-1169, with the same Pulsotype D (Subtype D1) was isolated from both Portuguese and Spanish fish farms (Fig. 3, Table 2). These results suggest that asymptomatic carriers may play an important role in the spread of WD between fish farms. This could also be the reason for the detection of Pulsotype E in 4 out of the 8 fish farms with WD outbreaks during 2001. The repeated isolation of Pulsotypes B and C from the same fish farm during several consecutive years (Table 2), indicates that *P. anguilliseptica* is able to persist over time in the same fish farm, which could be related either with the presence of asymptomatic carriers which could act as reservoirs of *P. anguilliseptica* between WD outbreaks, or alternatively with the ability of this microorganism to survive in the water environment (Muroga et al. 1977). Wild fish can be potential reservoirs of bacterial fish pathogens for cultured fish species (Zlotkin et al. 1998b). Moreover, the potential implication of wild fish as reservoirs of *P. anguilliseptica* (Lönnström et al. 1994) for cultured sea bream cannot be ruled out. Regardless of the source of *P. anguilliseptica*, farmed sea bream populations would develop WD under predisposing stressful conditions (Cinquina et al. 1998, Tort et al. 1998).

Our data show that PFGE analysis is a reliable and reproducible molecular technique for discriminating between isolates of *Pseudomonas anguilliseptica* and should facilitate epidemiological studies of this fish pathogen. Clinical isolates of some bacterial fish pathogens isolated from different geographic origins belong to different clones (Skov et al. 1995, Lucangeli et al. 2000, Vela et al. 2000). Although this study provides significant information about the genetic and epidemiological relationship of the *P. anguilliseptica* clinical isolates from WD outbreaks in sea bream in Spain, similar studies should be conducted with clinical isolates from other countries and susceptible fish species for a better understanding of the epizootiology of *P. anguilliseptica* infections.

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