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16S rRNA Gene Sequence Analysis of *Photobacterium damselae* and Nested PCR Method for Rapid Detection of the Causative Agent of Fish Pasteurellosis

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The causative agent of fish pasteurellosis, the organism formerly known as *Pasteurella piscicida*, has been reclassified as *Photobacterium damselae* subsp. *piscicida* on the basis of 16S rRNA gene sequence comparisons and chromosomal DNA-DNA hybridization data; thus, this organism belongs to the same species as *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damselae*). Since reassignment of *P. damselae* subsp. *piscicida* was based on only two strains, one objective of the present work was to confirm the taxonomic position of this fish pathogen by sequencing the 16S rRNA genes of 26 strains having different geographic and host origins. In addition, a nested PCR protocol for detection of *P. damselae* based on 16S rRNA was developed. This PCR protocol was validated by testing 35 target and 24 nontarget pure cultures, and the detection limits obtained ranged from 1 pg to 10 fg of DNA (200 to 20 cells). A similar level of sensitivity was observed when the PCR protocol was applied to fish tissues spiked with bacteria. The PCR approach described in this paper allows detection of the pathogen in mixed plate cultures obtained from asymptomatic fish suspected to be carriers of *P. damselae* subsp. *piscicida*, in which growth of this bacterium cannot be visualized. Our results indicate that the selective primers which we designed represent a powerful tool for sensitive and specific detection of fish pasteurellosis.

Pasteurellosis or pseudotuberculosis is one of the most important fish diseases in marine aquaculture, causing substantial economic losses especially in yellowtail (*Seriola quinqueradiata*), gilthead seabream (*Sparus aurata*), and seabass (*Dicentrarchus labrax*) cultures worldwide (for reviews see references 16 and 19). The causative agent of fish pasteurellosis was initially isolated from natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963 during a massive epizootic in the Chesapeake Bay (20). Janssen and Surgalla (12) proposed the name *Pasteurella piscicida* for this bacterium. Since then, this pathogen has been extensively characterized, and strains of the bacterium have been shown to be biochemically and serologically homogeneous (14, 16, 19), although minor variability has been detected by genetic methods, such as ribotyping analysis (17). Recently, *P. piscicida* has been reclassified as *Photobacterium damselae* subsp. *piscicida* (6, 21); thus, this organism belongs to the same species as *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damsela*). The two subspecies differ in important biochemical and physiological traits, such as motility, gas production from glucose, nitrate reduction, urease, lipase, amylase, and hemolysin production, and ranges of temperature and salinity for growth, as well as host specificity (3, 4, 16). Moreover, in contrast to *P. damselae* subsp. *piscicida*, which is serologically homogeneous, at least four serotypes are recognized within *P. damselae* subsp. *damselae* (3). Despite having marked phenotypic differences, the two subspecies of *P. damselae* exhibit a high degree of overall DNA base sequence similarity, as revealed by chromosomal DNA-DNA pairing (6). Similarly, the two subspecies reportedly have almost identical 16S rRNA gene sequences, although sequence comparisons have been performed with only three strains.

In recent years there has been much interest in the development of specific PCR protocols for detecting 16S rRNA genes of several bacterial fish pathogens, such as *Vibrio anguillarum*, *Aeromonas salmonicida*, *Renibacterium salmoninarum*, *Flexibacter maritimus*, and *Flavobacterium* species (for a review see reference 8). Previously, several studies have described the design of molecular aids for detection of *P. damselae* subsp. *piscicida* as the causative agent of pseutotuberculosis in cultured fish (1, 22, 23). However, these methods were based on nonconserved DNA regions (plasmid sequences and a gene library fragment of unknown function). Additionally, despite the wide distribution of this bacterium in the Northern Hemisphere, only Japanese isolates have been used to test the DNA-based diagnostic methods designed up to now.

There were two objectives of the present study. First, we wanted to perform a comparative 16S rRNA gene sequencing analysis with *P. damselae* strains from geographically diverse sources and a variety of homiotherm and poiquilotherm hosts in order to study a good cross-section of the natural diversity of this species. Second, we wanted to utilize the sequence information generated to develop a nested PCR protocol for rapid and specific diagnosis of pseudotuberculosis in fish. Development of such a PCR-based test for *P. damselae* is particularly important, since diagnosis of pasteurellosis may be hampered by the slow growth of the organism in laboratory media (16), which is easily obscured by the growth of other fast-growing bacteria, as well as by the fact that this fish pathogen has the capacity to rapidly form viable but nonculturable cells in the

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TABLE 1. *P. damselae* strains used in this study

Strain[a]	Origin	Country
P. damselae subsp. *piscicida* strains		
DI 21	*Sparus aurata*	Spain
DI 91	*Sparus aurata*	Spain
B 21	*Dicentrarchus labrax*	Spain
B 51	*Dicentrarchus labrax*	Spain
C.1	*Sparus aurata*	Spain
C.2	*Sparus aurata*	Spain
R 46	*Sparus aurata*	Spain
DS 11	*Sparus aurata*	Spain
619.1	*Sparus aurata*	Portugal
666.1	*Dicentrarchus labrax*	Portugal
693.2	*Sparus aurata*	Portugal
10831	*Dicentrarchus labrax*	France
IT-1	*Sparus aurata*	Italy
IT-2	*Sparus aurata*	Italy
O69 A	*Sparus aurata*	Greece
O69 E	*Sparus aurata*	Greece
ATLIT 2	*Morone* sp.	Israel
2101	*Morone* sp.	Israel
MP-7801	*Seriola quinqueradiata*	Japan
EPOY-8803-II	*Epinephelus akaara*	Japan
P3333	*Seriola quinqueradiata*	Japan
MZS 8001	*Seriola quinqueradiata*	Japan
P3335	*Seriola quinqueradiata*	Japan
ATCC 29690	*Seriola quinqueradiata*	Japan
ATCC 17911	*Roccus americanus*	United States
P. damselae subsp. *damselae* strains		
RG 91	*Scophthalmus maximus*	Spain
RG 153	*Scophthalmus maximus*	Spain
RM 71	*Scophthalmus maximus*	Spain
CDC 2227-81	Human wound infection	United States
ATCC 33539	*Chromis punctipinnis*	United States
ATCC 35083	*Carcharhinus plumbeus*	United States
192	*Tursiops truncatus*	United States
PG-801	*Penaeus monodon*	Taiwan
158	*Anguilla anguilla*	Belgium
162	*Anguilla anguilla*	Belgium

[a] CDC, Centers for Diseases Control; ATCC, American Type Culture Collection.

TABLE 2. Organisms used in the 16S rRNA gene sequence comparisons and included in the PCR analysis as negative controls

Organism	Strain[a]	Accession no.
Photobacterium angustum	ATCC 25915^T	X74685
Photobacterium damselae	ATCC 33539^T	X74700
Photobacterium histaminum	JCM 8968^T	D25308
Photobacterium leiognathi	ATCC 25521^T	X74686
Photobacterium phosphoreum	ATCC 11040^T	X74687
Vibrio alginolyticus	ATCC 17749^T	X74690
Vibrio anguillarum	ATCC 12964^T	X16895
Vibrio campbelli	ATCC 25920^T	X74692
Vibrio fischeri	ATCC 7744^T	X74702
Vibrio fluvialis	NCTC 11327^T	X76335
Vibrio furnissii	ATCC 35016^T	X74704
Vibrio harveyi	ATCC 14126^T	X74706
Vibrio hollisae	ATCC 33564^T	X74707
Vibrio logei	ATCC 15832^T	X74708
Vibrio metschnikovii	NCTC 11170^T	X74712
Vibrio mimicus	ATCC 33653^T	X74713
Vibrio natriegens	ATCC 14048^T	X74714
Vibrio nereis	ATCC 25917^T	X74716
Vibrio nigripulchritudo	ATCC 27043^T	X74717
Vibrio orientalis	ATCC 33934^T	X74719
Vibrio parahaemolyticus	ATCC 17802^T	X74721
Vibrio pelagius	ATCC 25916^T	X74722
Vibrio proteolyticus	ATCC 15338^T	X74723
Vibrio splendidus	ATCC 33125^T	X74724
Vibrio tubiashi	ATCC 19109^T	X74725
Vibrio vulnificus	ATCC 29307^T	X76334

[a] ATCC, American Type Culture Collection; JCM, Japan Collection of Microorganisms; NCTC, National Collection of Type Cultures.

natural environment (15). In addition, a great number of fish harbor the bacterium in an asymptomatic carrier state, and fish that survive disease outbreaks remain covertly infected (13).

MATERIALS AND METHODS

Bacterial cultures and DNA extraction. A total of 35 *P. damselae* strains, including representatives of both subspecies, were used in this study. The sources of isolation and geographic origins of these strains are listed in Table 1. Other *Photobacterium* and *Vibrio* species used to design and validate the PCR primers are shown in Table 2. All of the strains were grown aerobically on brain heart infusion agar (Pronadisa, Madrid, Spain) supplemented with 1% NaCl at 25°C. DNA was isolated by using the Instagene matrix (Bio-Rad) as recommended by the manufacturer.

In addition, broodstock gilthead seabream (*S. aurata*) that were suspected carriers of pasteurellosis, as indicated by sensitive serological methods (13), were sacrificed, and then samples from kidneys and spleens were immediately directly streaked onto plates containing brain heart infusion agar supplemented with 1% NaCl, which were incubated at 25°C for 48 h. After this, at least one representative of each of the different morphological colony types was subcultured, subjected to standard biochemical and physiological tests (7), and identified by using the schemes of Holt et al. (10). Simultaneously, the total bacterial growth was scraped off the plates and resuspended in a saline solution, and a sample was used to extract the DNA of the mixed culture by the procedure described above.

Since healthy gilthead seabream may not necessarily be free of *P. damselae* subsp. *piscicida*, kidney tissues from fish species that are not susceptible to pasterellosis, such as trout and salmon, were employed as negative controls; these tissues were processed as described above for carrier gilthhead seabream.

Unless otherwise specified, between 1 and 10 μl of a DNA suspension was routinely used for each PCR.

Sequencing of 16S rRNA genes. 16S rRNA genes of 18 strains of *P. damselae* subsp. *piscicida* and eight strains of *P. damselae* subsp. *damselae* were amplified by PCR with universal primers pA and pH (11). The PCR were performed with a DNA thermal cycler (Biometra). A typical reaction mixture (100 μl) consisted of 0.5 μg of each specific primer, 2 U of *Taq* polymerase (Perkin-Elmer), 10 μl of 10× *Taq* polymerase buffer (Perkin-Elmer), 4 μl of a 50 mM $MgCl_2$ solution, and each deoxynucleoside triphosphate at a concentration of 200 μM. The reaction mixtures were each overlaid with 30 μl of mineral oil (Sigma) and subjected to amplification at 95°C for 4 min, followed by 30 cycles consisting of 95°C for 1 min, 55°C for 1 min, and 72°C for 90 s. A final extension step consisting of 5 min at 72°C was carried out. The PCR products were cleaned by using a QIAquick PCR purification kit (Quiagen).

Primers (5) corresponding to internal conserved regions of the gene were used for sequencing reactions performed with a GeneAmp PCR System 9600 instrument (Perkin-Elmer); a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) was used, and the program (35 cycles) consisted of denaturation at 96°C for 15 s, annealing at 48°C for 1 s, and elongation at 60°C for 4 min. The sequencing products were analyzed with a model 373A automatic DNA sequencer (Applied Biosystems).

Primers and DNA probe design. 16S rRNA gene sequences of 25 species belonging to the genera *Photobacterium* and *Vibrio* (Table 2) representing the closest relatives of *P. damselae* were retrieved from the GenBank database and compared with the sequences obtained in the present work. These sequences were aligned by using the program PILEUP (2). On the basis of the alignment, two regions of the 16S rRNA gene were chosen as highly variable regions within the family *Vibrionaceae*, and this allowed us to design three primers for an heminested PCR assay, as well as a short confirmatory DNA probe.

For the first round of PCR, forward primer Car1 (18-mer; 5′-GCTTGAAGA GATTCGAGT-3′; positions 1016 to 1033 in the *Escherichia coli* 16S rRNA gene) and reverse primer Car2 (18-mer; 5′-CACCTCGCGGTCTTGCTG-3′; positions 1266 to 1283) were developed; these primers flanked a 267-bp fragment of the 16S rRNA gene. For the second round of the heminested PCR, primer Car1 was used as the forward primer in combination with reverse primer Nestcar1 (17-mer; 5′-GGTCTTGCTGCCCTCTG-3′; positions 1259 to 1275) flanking a 259-bp fragment. An internal fragment designated CARSOND (18-mer; 5′-TACAATGGCATATACAGA-3′; positions 1245 to 1262) was labelled with digoxigenin (Pharmacia Biotech) and used as a DNA probe to assess the specificity of the fragment amplified in the nested PCR.

Sensitivity of the primers. In order to test the sensitivity of primers Car1, Car2, and Nestcar1, spectrophotometrically quantified purified DNA of *P. damselae* subsp. *piscicida* ATCC 17911 was used. DNA dilutions containing from 400 ng to 10 fg of pure DNA were prepared as templates for PCR amplification.

In addition, 50-mg pieces of kidneys from rainbow trout were spiked with different dilutions in phosphate-buffered saline (10^8 to 10 cells/ml) of a culture suspension of *P. damselae* subsp. *piscicida* DI 21 and homogenized for 30 s. Tissue lysis and total DNA purification were performed with an EZNA Mollusc DNA kit (Omega Biotek) as recommended by the manufacturer. In this case, 10 μl of the extracted DNA solution was used in each PCR.

Nested PCR detection and Southern blotting. Primers Car1, Car2, and Nestcar1 were used under the PCR conditions described above for 16S rRNA gene amplification, except that the annealing temperatures ranged from 55 to 65°C and the elongation cycle was 20 s long. A 0.5-μl portion of the first-round PCR product (obtained with primers Car1 and Car2) was used as the DNA template for the second-round or heminested PCR, in which primers Car1 and Nestcar1 were used. Amplification products were analyzed on 1% (wt/vol) agarose gels with TAE (0.04 M Tris-acetate, 1 mM EDTA) electrophoresis buffer and were visualized with a UV transilluminator after staining with ethidium bromide. A 1-kb DNA ladder (Bio-Rad) was included as a molecular weight marker.

The presence of the internal DNA CARSOND sequence was determined as follows. PCR products obtained from an amplification reaction in which primers Car1 and Car2 were used were separated in a 0.8% agarose gel. Transfer to nylon membranes, hybridization, and development of the labelling were carried out as described previously (17). In this analysis, 5 μl of probe solution (0.1 μg/μl) was used, and the preparation was hybridized for 8 h at 50°C.

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA gene of *P. damselae* subsp. *piscicida* ATCC 29690 has been deposited in the GenBank database under accession no. Y18496.

RESULTS

16S rRNA gene sequences. Almost complete (>1,400-nucleotide) 16S rRNA gene sequences were obtained for 18 strains of *P. damselae* subsp. *piscicida* and for eight strains of *P. damselae* subsp. *damselae*. All of the strains examined exhibited 100% sequence identity, which confirmed the close phylogenetic relationship of the two subspecies and showed that the 16S rRNA gene was homogeneous regardless of the geographic origins, pathogenic properties, and host sources of the strains.

Experimental validation of the primers with pure cultures. The specificity of primers Car1 and Car2 in a single-step PCR was tested experimentally by using 26 pure cultures of *P. damselae* subsp. *piscicida* and 10 pure cultures of *P. damselae* subsp. *damselae*, as well as 24 pure cultures of *Vibrio* and *Photobacterium* reference species listed in Table 2. PCR were performed at annealing temperatures of 55, 60, and 65°C.

At the three different temperatures evaluated, all 36 *P. damselae* strains produced a unique and clear PCR band of the expected size (267 bp). However, at both 55 and 60°C most of the non-*P. damselae* strains produced nonspecific amplification bands (data not shown). Most of the PCR bands produced by the non-*P. damselae* strains were eliminated at an annealing temperature of 65°C; the only exception was a nonspecific higher-molecular-weight band produced by *Vibrio splendidus*. Consequently, 65°C was used for additional PCR experiments. Figure 1A shows the results of PCR analyses performed with primers Car1 and Car2 and with 19 pure cultures of *P. damselae* (including members of both subspecies) and 12 pure cultures of other *Photobacterium* and *Vibrio* species at an annealing temperature of 65°C. After Southern blotting and hybridization of the PCR products with the internal DNA probe CARSOND, all of the *P. damselae* amplification products exhibited a positive reaction, which confirmed the identities of the PCR products (Fig. 1B).

Sensitivity of primers. When primers Car1 and Car2 were tested for amplification with purified DNA of *P. damselae* subsp. *piscicida*, 267-bp PCR products were obtained with samples containing from 400 ng to 1 pg of DNA (Fig. 2). A second round of PCR (nested PCR) did not result in any further improvement in sensitivity, although the intensity of the bands increased substantially (data not shown). Application of this PCR protocol to DNA obtained from experimentally infected kidney tissue allowed us to detect as few as 100 bacterial cells.

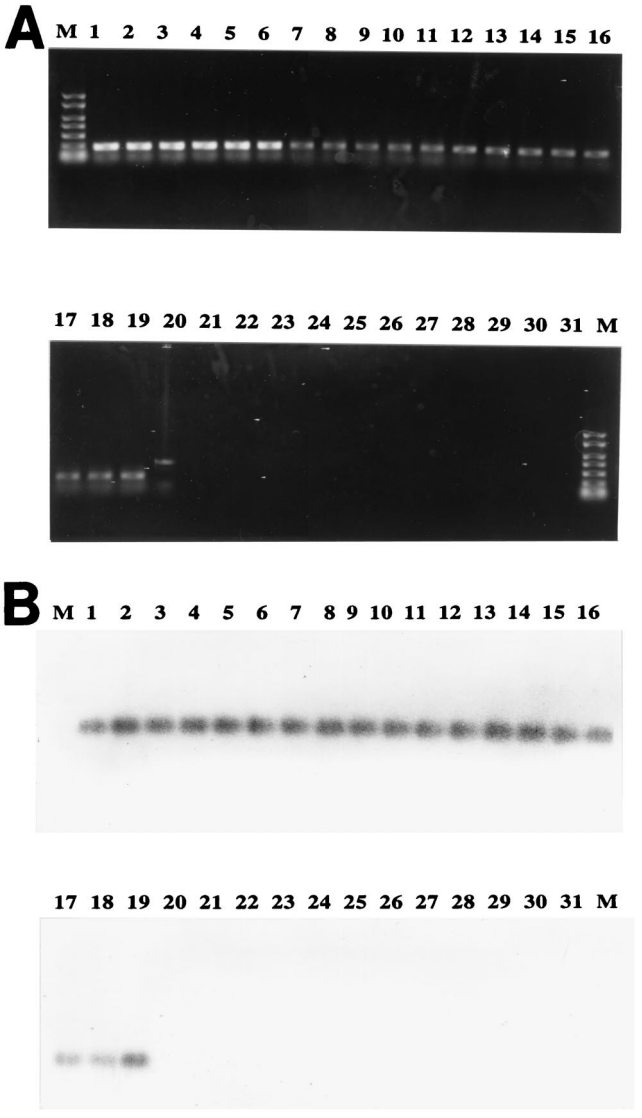

FIG. 1. Specific PCR products (A) and hybridization of the products with digoxigenin-labelled probe CARSOND (B) obtained with pure cultures of 19 *P. damselae* strains and 13 isolates of related species. Lanes M, 1-kb molecular weight marker; lanes 1 to 13, *P. damselae* subsp. *piscicida* DI 21, C.1, R46, DS 11, ATCC 17911, ATCC 29690, 619.1, 10831, IT1, 069E, ATLIT-2, EPOY 8803, and MZS 8001, respectively; lanes 14 to 19, *P. damselae* subsp. *damselae* RG 91, RG 153, RM 71, CDC 2227-81, ATCC 33539, and ATCC 35083, respectively; lane 20, *V. splendidus*; lane 21, *V. fischeri*; lane 22, *V. anguillarum*; lane 23, *V. pelagius*; lane 24, *V. harveyi*; lane 25, *V. proteolyticus*; lane 26, *V. alginolyticus*; lane 27, *P. leiognathi*; lane 28, *P. phosphoreum*; lane 29, *V. metschnikovii*; lane 30, *V. nereis*; lane 31, *V. campbelli*.

Detection of *P. damselae* subsp. *piscicida* in mixed cultures. A bacteriological analysis of the different predominant colonies present in the plate cultures obtained from tissues of gilthead seabream individuals suspected to be carriers of *P. damselae* subsp. *piscicida* revealed a mixed bacterial population composed of *V. splendidus* and *Vibrio pelagius*, as well as other unidentified *Vibrio* and *Pseudomonas* species. DNA extracted from these mixed plate cultures were used as templates for a heminested PCR assay (as described above). A first PCR

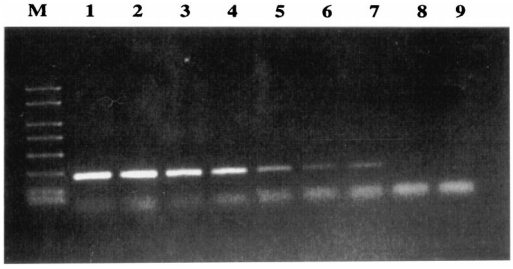

FIG. 2. Detection limit of the nested PCR designed in this work, as determined with different dilutions of purified DNA from strain DI 21 of *P. damselae* subsp. *piscicida*. Lane M, 1-kb molecular weight marker; lanes 1 to 8, 400 ng, 100 ng, 10 ng, 1 ng, 10 pg, 1 pg, 10 fg, and 1 fg of DNA, respectively; lane 9, no DNA.

round performed with primers Car1 and Car2 generated multiple amplification products that ranged in size from 0.2 to 1 kb (Fig. 3A). These PCR products were used as templates for a second round of PCR in which primers Car1 and Nestcar1 were used. This heminested PCR approach yielded the specific 259-bp amplification product (Fig. 3B) which, as expected, hybridized with the internal DNA probe CARSOND (data not shown). DNA extracted from bacterial colonies obtained from fish used as negative controls did not yield any amplification product (Fig. 3A, lane 12). These results validated the assay used for specific and sensitive detection of the pathogen in plate cultures in which typical colonies were not visually evident due to overgrowth of other saprophytic microbiota.

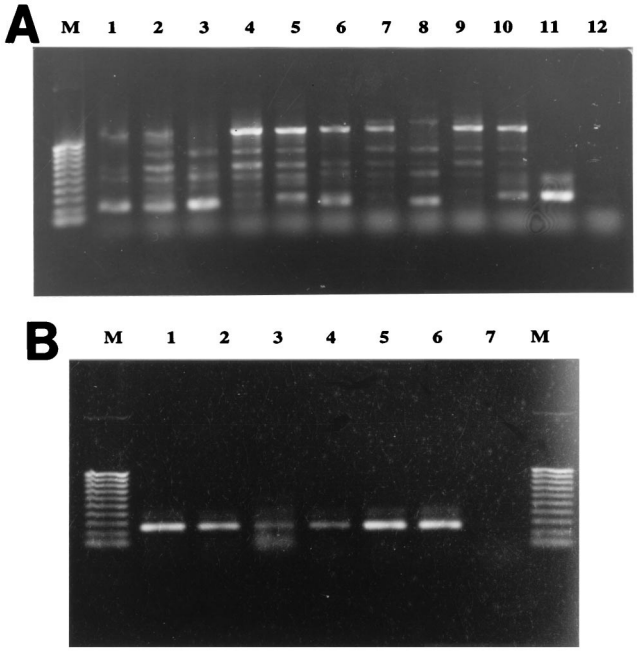

FIG. 3. Detection of *P. damselae* in mixed cultures obtained from asymptomatic carrier gilthead seabream by the PCR (A) and nested PCR (B) procedures developed in this study. (A) Lane M, 1-kb molecular weight marker; lanes 1 to 5, seabream 1 to seabream 5 (100 ng of DNA extracted from mixed bacterial culture); lanes 6 to 10, seabream 1 to seabream 5 (200 ng of DNA extracted from mixed bacterial culture); lane 11, positive control (pure DNA from strain ATCC 29690); lane 12, DNA from negative control. (B) Lanes M, 1-kb molecular weight marker; lanes 1 to 5, 0.1-μl portions of samples 1 to 5 from panel A; lane 6, positive control; lane 7, no DNA.

DISCUSSION

Our 16S rRNA gene sequence analyses confirmed unambiguously that the causative agent of fish pasteurellosis, which was formerly designated *P. piscicida*, clearly belongs to the genus *Photobacterium*, as reported previously (6). *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* strains, irrespective of the host or geographic source, exhibited 100% 16S rRNA gene sequence identity. In a sequencing study that included one strain of *P. damselae* subsp. *piscicida* and two strains of *P. damselae* subsp. *damselae*, Gauthier et al. (6) found a single nucleotide difference between the two subspecies. In the present study, all 26 strains of *P. damselae* examined had the same 16S rRNA gene sequence.

Due to the difficulty of rapidly diagnosing pasteurellosis because of the slow growth of the causative agent and the persistence of the bacterium in asymptomatic fish, there is an urgent need to develop a sensitive and reliable method for fast detection of this pathogen, not only in clinically infected animals but also in egg stocks, in carrier fish, and in the environment, where it rapidly enters a viable yet nonculturable, state (15, 16). PCR and DNA probe-based detection procedures have previously been designed for *P. damselae* subsp. *piscicida* based on amplification of DNA fragments obtained from a genomic library of this bacterium (22) and on amplification of plasmid sequences (1, 23). However, these methods were based on nonconserved regions of the genome, and they have been tested exclusively with Japanese and American isolates of this fish pathogen, whose plasmid contents differ from the plasmid contents of European isolates (14).

Recently, a serological method for rapidly detecting fish pasteurellosis based on a magnetic bead enzyme immunoassay (Bionor Aquaeia-Pp kit) was evaluated in our laboratory, and a limit of detection of 10^4 cells was obtained (13). In this study, we developed a rapid procedure for detecting *P. damselae* by nested PCR. Our experiments to determine minimum DNA detection levels showed that between 10 fg and 1 pg of *P. damselae* DNA, corresponding to approximately 20 to 200 cells (9), can be accurately amplified during the first round of PCR. Interestingly, when the PCR protocol was used with DNA extracted from experimentally inoculated fish tissues (to simulate the expected field conditions), similar sensitivities were obtained. Therefore, we concluded that the PCR-based protocol which we describe here is at least 100 times more sensitive than the serological approach, which indicates that the method is suitable for fast, precise, and sensitive detection of this fish pathogen not only in diseased fish but also in asymptomatic carriers, in which bacteria can persist in low numbers and below the detection limits of serological techniques. Since we were able to detect *P. damselae* in experimentally infected tissues by this procedure (18), the accuracy of the nested PCR assay is currently being investigated with samples from different fish farms in order to evaluate its efficacy under field conditions.

Finally, it is pertinent that *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* have identical 16S rRNA genes, and, therefore, the assay which we developed should detect both subspecies of *P. damselae*. However, since the two subspecies have different ecological habitats and distinct host specificities, our protocol can be used not only for rapid diagnosis of fish pasteurellosis but also for detection of *P. damselae* subsp. *damselae* in clinical samples. Additional studies will focus on the search for subspecies-specific genes which can be used as targets for discrimination of *P. damselae* strains at the subspecies level.

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