

Simple and rapid detection of *Photobacterium damsela* ssp. *piscicida* by a PCR technique and plating method

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ABSTRACT

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Aims: To detect *Photobacterium damsela* ssp. *piscicida* using the PCR technique and plating method.

Methods and Results: Two strains of *P. damsela* ssp. *piscicida* were isolated from cultured cobia (*Rachycentron canadum*) at two different fish farms in Taiwan. A pair of primers was designed to detect the capsular polysaccharide gene of *P. damsela* ssp. *piscicida* by PCR. Reference strains of different genus and different clinical strains were used for this study. The expected product (410 bp) was obtained from both *P. damsela* ssp. *piscicida* and *P. damsela* ssp. *damsela*, and they were differentiated by culturing on thiosulphate citrate bile salts–sucrose agar (TCBS-1). *Photobacterium damsela* ssp. *damsela* grew on TCBS-1 producing green colonies whereas *P. damsela* ssp. *piscicida* did not grow.

Conclusions: The methods used are cost and labour effective when compared with the other methods and commercially available kits.

Significance and Impact of the Study: This work provides an integrated set of methods to identify the species *P. damsela* and to differentiate *P. damsela* ssp. *piscicida* from *P. damsela* ssp. *damsela*.

Keywords: aquaculture, bacterial disease diagnosis, cobia, marine fish, *P. damsela* ssp. *piscicida*, *Photobacterium*, quick screen by PCR and plating, TCBS plating.

INTRODUCTION

Photobacterium damsela ssp. *piscicida* (Gauthier *et al.* 1995) is a Gram-negative, nonmotile, bipolar coccobacillus (Snieszko *et al.* 1964), which was previously known as *Pasteurella piscicida* (Snieszko *et al.* 1964). It is the causative agent of the fish disease photobacteriosis, also known as pasteurellosis or pseudotuberculosis. High mortalities of *P. piscicida* infection were first observed in natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in 1963 in Chesapeake Bay (USA) (Snieszko *et al.* 1964), and it has caused economic loss to the fish farming industries in Japan (Kusuda and Yamaoka 1972). Several countries in the Mediterranean area (Toranzo

et al. 1991; Bakopoulos *et al.* 1995, 1997; Baptista *et al.* 1996; Candan *et al.* 1996; Topic Popovic *et al.* 2001) have encountered similar high mortalities in cultured sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). The pathogen has also been isolated and caused high mortalities in farmed cobia (*Rachycentron canadum*) in Taiwan 2001 (Lopez *et al.* 2002).

Photobacterium damsela ssp. *damsela* (Smith *et al.* 1991; Truper and De Clari 1997) which was formerly classified as *Vibrio damsela* is a halophilic bacterium causing skin ulcers in warm and cold water fish (Love *et al.* 1981; Sakata *et al.* 1989; Fouz *et al.* 1992a,b).

Rapid diagnosis of disease outbreaks is essential for proper management and effective control. Current microbiological methods of culture and biochemical characterization (e.g. use of the API 20E and API CH50 rapid identification system, bioMerieux, Marcy l'Etoile, France) are time consuming and

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labour intensive. Diagnostic methods using immunological techniques such as agglutination or the ELISA are comparatively quicker (Janssen and Surgalla 1968; Toranzo *et al.* 1987; Jung *et al.* 2001). PCR is not only more sensitive, but also faster (Eisenstein 1990), cost and labour effective when compared with biochemical tests and immunoassays.

The aim of the present study was to develop a convinced PCR-based test for *P. damsela* ssp. *piscicida*, which belongs to the same species as *P. damsela* ssp. *damsela*. The two subspecies have an identical 16S rRNA gene sequences (Osorio *et al.* 1999) but they differ in their biochemical and physiological characteristics (Fouz *et al.* 1992b, 1998; Magariños *et al.* 1996). A pair of primers was designed to detect *P. damsela* ssp. *piscicida* by PCR. Also, thiosulphate citrate bile salts–sucrose agar (TCBS) was used to differentiate *P. damsela* ssp. *piscicida* from *P. damsela* ssp. *damsela*.

MATERIALS AND METHODS

Bacterial strains

The origin of strains used in this study is listed in Table 1. The reference strains were obtained from the Culture Collection and Research Center (CCRC, Hsinchu, Taiwan) and most of them were originated from ATCC. Clinical

samples (*Vibrio* spp. and *P. damsela* ssp. *piscicida*) (Carmen *et al.* 2002; Rajan *et al.* 2001) were isolated in our laboratory from infected sea farmed cobia (*Rachycentron canadum*) collected from Penghu (Taiwan). One strain of *P. damsela* ssp. *piscicida* was obtained from a laboratory in Pingtung (Taiwan). All strains except *P. damsela* ssp. *piscicida* were cultured in tryptic soya broth (TSB-1-5) and tryptic soya agar (TSA-1-5) (Difco, Detroit, MI, USA) supplemented with 1.5% (w/v) NaCl. Also, *P. damsela* ssp. *piscicida* was cultured in brain–heart infusion broth (BHIB-1) and agar (BHIA-1) (Difco) supplemented with 1% (w/v) NaCl.

DNA primers

A pair of primers was selected to amplify a 410-bp fragment of capsular polysaccharide gene derived from *P. damsela* ssp. *piscicida* (Gene Bank accession number AB074290). The forward primer, CPSF, was 20 nucleotides long (5'-AGGGGATCCGATTATTACTG-3') corresponding to positions 531–550 of the *P. damsela* ssp. *piscicida* gene for capsular polysaccharide (Fig. 1) and the reverse primer, CPSR, was 20 nucleotides long (5'-TCCCATGAGAAGATTTGAT-3') corresponding to positions 921–940. Primers were synthesized by mission biotech (MB) (Taipei, Taiwan).

Table 1 Bacterial strains used in this study

Species	Strain	Source and origin
<i>Photobacterium damsela</i> ssp. <i>piscicida</i>	Lab isolate	Spleen of cobia (Taiwan)
<i>P. damsela</i> ssp. <i>piscicida</i>	Kaohsiung lab	Cobia (Taiwan)
<i>P. damsela</i> ssp. <i>piscicida</i>	CCRC 17065; ATCC 29687	Spleen of young yellow tail (Japan)
<i>P. damsela</i> ssp. <i>damsela</i>	CCRC 13854; ATCC 33536	Seawater (USA)
<i>P. damsela</i> ssp. <i>damsela</i>	CCRC 15429	Tiger shrimp (Taiwan)
<i>P. phosphoreum</i>	CCRC 13804; ATCC 35082	Type strain – B1
<i>P. angustum</i>	CCRC 13805; ATCC 25915	Seawater
<i>P. leiognathi</i>	CCRC 13806; ATCC 25521	Light organ of <i>Leiognathus equula</i> (Singapore)
<i>Vibrio harveyi</i>	CCRC 12907; ATCC 14126	Dead luminescing amphipod (USA)
<i>V. alginolyticus</i>	Lab isolate	Skin lesion of cobia (Taiwan)
<i>V. parahaemolyticus</i>	Lab isolate	Skin lesion of cobia (Taiwan)
<i>V. vulnificus</i>	Lab isolate	Skin lesion of cobia (Taiwan)
<i>V. alginolyticus</i>	CCRC 12829; ATCC 17749	Spoiled horse mackerel (Japan)
<i>V. parahaemolyticus</i>	CCRC 12959; ATCC 17802	Shrimp
<i>V. vulnificus</i>	CCRC 15431	Diseased eel (Taiwan)
<i>Listonella anguillarum</i>	CCRC 12908; ATCC 19264	Ulcerous lesion in cod (Norwehlan)
<i>V. salmonicida</i>	CCRC 12844; ATCC 43839	Diseased farmed Atlantic salmon (Norway)
<i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	CCRC 14163; ATCC 33658	Salmon kill in River Cletter
<i>Aer. hydrophila</i>	CCRC 13922	Cuttle fish pickles (Taiwan)
<i>Pseudomonas putida</i>	CCRC 10459; ATCC 11172	Biotype A
<i>Streptococcus imiae</i>	CCRC 14744; ATCC 29177	Amazon freshwater dolphin (<i>Inia geoffrensis</i>)

1	ATGGGGCAGT	TAAGTCGACA	AATTAAAGAA	AAATTGCGTC	AAGTAATATG
51	CCGATTTTCGC	TCCCAGCCTG	TTTTATCTGC	AGAGATACAA	AGTAAAGGCC
101	AAAAAGCTTT	ACTTGAATTG	AGAAAAAAC	AGCCGTTATC	TGAACAGGAA
151	AACAAGGTTA	TTTGGATGTA	TTGGGACTCG	GGTCTCGATA	GAGCACCTGA
201	AGTTGTTTCGC	CTGAGTCATC	AAAGTTGGTG	TCAATTTAAT	CCAGATTATC
251	AAGTTGTTTT	ACTGAGTAAT	GCAAATATAG	AACAATATTT	AGATTTTTGAT
301	TTTAATGATA	TTTTTCCTAT	GTTGTCAGTA	AAGTTAGGGG	CTGCGGGTAA
351	GAGTGATTTG	TTACGCTTAT	ATCTGCTTAA	TCGCTATGGT	GGTGTATGGG
401	CAGATGCAAC	AACGTTTTGT	GTCAAGCCAC	TTTCACAATG	GCTAGATCTC
451	TCTCAAACATA	GCTTCTTCTG	CTTCAGAGAA	AAGCATTCTA	ATGATCGTAA
				CPSF	
501	TTTAGTGTCT	TGGTTTTTAG	CCGCAAAGC	<u>AGGGGATCCG</u>	<u>ATTATTACTG</u>
551	ACCTTCTTAA	TGCGTCGCTA	GATTACTTAA	<u>TTCAGCCAAG</u>	<u>GCCTTATCGG</u>
601	TTAGATATTG	TTGGCTTAAA	AACTACATTA	CAATTAGCGC	AAGGAACACA
651	ATTTATATCA	AAGCATCACT	CTGGCTTTGC	TTTATTAAAT	TATCTTGAAA
701	GTAAATATGC	GGCCCCTTAT	TTTTGGCTAT	TTTATTTATT	TAATGAGGTT
751	GTTAAGTTGC	CTCATAATCA	GTTGGCTTGG	CAATATATCG	AGCAGCAACA
801	TAATTTATGC	GCAGAAATGT	ATGAAGAGAT	AACAAGCTTT	AAATCGTCTT
851	ATGTTGCGAA	ACAAACGTAT	CGAGAAAAAT	ACATCAGTGG	TACGTTATAT
901	CAGCAAAGAG	CAGAATACAT	<u>ATCAAATCTT</u>	<u>CTCAATGGGA</u>	<u>AAAGCTAG</u>
				CPSR	

Fig. 1 Nucleotide sequences of *Photobacterium damsela* ssp. *piscicida* capsular polysaccharide gene (GeneBank accession number AB074290). Underlined nucleotides (CPSF, CPSR) correspond to the pair of primers used to amplify DNA in PCR assays

Preparation of samples and the PCR reaction

Pure cultures grown on BHIA-1/TSA-1.5 were used for PCR amplification. For each bacterial isolate, a single colony of the bacteria was suspended in 1 ml of sterile distilled water and 1.5% (w/v) saline separately. The samples were not boiled. Each bacterial suspension (10 μ l) was used for the PCR amplification performed with Hybaid, MBS 0.2G using the PCR kit (New England BioLabs, Cambridge, MA, USA). A mixed sample was also prepared by incorporating 10 μ l of each bacterial suspension in 1 ml of distilled water and 1.5% (w/v) saline separately and was used for PCR amplification. PCR reaction mixture containing 10 μ l of the bacterial suspension, 5 μ l of 10x PCR buffer (100 mM Tris-HCl, 20 mM MgCl₂, 500 mM KCl pH 8.3), 0.2 mM of each dNTP, 1 μ M of each primer, 1.25 U of *Taq* DNA polymerase and double-distilled water up to a final volume of 50 μ l. DNA denaturation was carried out at 94°C for 5 min and then a total of 35 PCR cycles were run under the following conditions: DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and DNA polymerization at 72°C for 1 min. After the final cycle, reactions were terminated at 72°C for 7 min.

Photobacterium damsela ssp. *damsela* grows on TCBS-1 and produces a green colour (Love *et al.* 1981; Thyssen

et al. 1998) whereas *P. damsela* ssp. *piscicida* does not grow (Toranzo *et al.* 1991; Zorrilla *et al.* 1999). Hence, to differentiate *P. damsela* ssp. *piscicida* from *P. damsela* ssp. *damsela*, pure bacterial isolates were plated on TCBS-1 (Difco) supplemented with 1% (w/v) NaCl.

Determination of PCR sensitivity

In order to test the sensitivity of the primer CPSF and CPSR, *P. damsela* ssp. *piscicida* cells resuspended in different dilutions in sterile distilled water (10^8 – 10^1 cells ml⁻¹) were used for PCR amplification.

RESULTS

PCR

The pair of primers, CPSF and CPSR, designed from the capsular polysaccharide gene successfully amplified an expected 410-bp DNA fragment (Fig. 2) of all *P. damsela* ssp. *piscicida* (Fig. 2, lanes 1–3) and *P. damsela* (Fig. 2, lanes 4 and 5) strains tested. However, PCR product was not obtained when *Photobacterium* spp., such as *P. phosphoreum*, *P. angustum*, *P. leiognathi* and *V. harveyi* (formerly *P. harveyi*) cells were used (Fig. 2, lane 6–9).

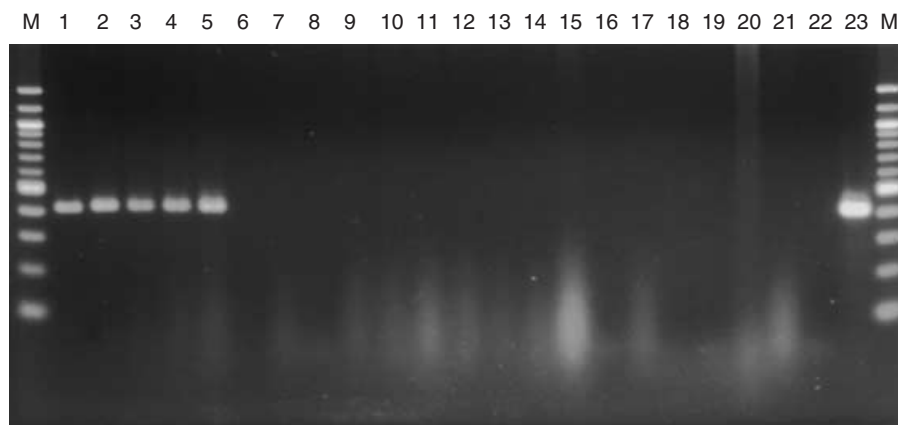


Fig. 2 Agarose gel electrophoresis of PCR amplified product using CPSF and CPSR primers. Arrow indicates the expected PCR product (410 bp). Lane M, DNA molecular mass marker (100-bp ladder); lane 1, *Photobacterium damsela ssp. piscicida* (cobia isolate); lane 2, *P. damsela ssp. piscicida* (Taiwan isolate); lane 3, *P. damsela ssp. piscicida* (CCRC 17065); lane 4, *P. damsela ssp. damsela* (CCRC 15429); lane 5, *P. damsela ssp. damsela* (CCRC 13854); lane 6, *P. phosphoreum* (CCRC 13804); lane 7, *P. angustum* (CCRC 13805); lane 8, *P. leiognathi* (CCRC 13806); lane 9, *V. harveyi* (CCRC 12907); lane 10, *V. alginolyticus* (cobia isolate); lane 11, *V. parahaemolyticus* (cobia isolate); lane 12, *V. vulnificus* (cobia isolate); lane 13, *V. alginolyticus* (CCRC 12829); lane 14, *V. parahaemolyticus* (CCRC 12959); lane 15, *V. vulnificus* (CCRC 15431); lane 16, *V. anguillarum* (CCRC 12908); lane 17, *V. salmonicida* (CCRC 12844); lane 18, *Aer. salmonicida ssp. salmonicida* (CCRC 14163); lane 19, *Aeromonas hydrophila* (CCRC 13922); lane 20, *Ps. putida* (CCRC 10459); lane 21, *Strep. iniae* (CCRC 14744); lane 22, Mixture of all bacteria used in this study except *P. damsela ssp. piscicida* and *P. damsela ssp. damsela*; lane 23, mixture of one *P. damsela ssp. piscicida* and all other bacteria used in this study except other *P. damsela ssp. piscicida* and *P. damsela ssp. damsela*.

In addition, other Gram-negative bacteria such as *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* (Fig. 2, lanes 10–12) isolated from infected cobia, *Vibrio* spp. including *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *L. anguillarum*, *V. salmonicida* (Fig. 2, lanes 13–17), and other pathogenic bacteria, i.e. *Aeromonas salmonicida ssp. salmonicida*, *Aer. hydrophila*, *Pseudomonas putida* and *Streptococcus iniae* (Fig. 2, lanes 18–21) did not produce the PCR product. A mixture containing one *P. damsela ssp. piscicida* (cobia isolate) and all the bacteria used in this study except *P. damsela ssp. damsela* amplified the expected product (Fig. 2, lane 23) whereas product was not obtained from the mixture containing all the other bacteria except *P. damsela ssp. piscicida* and *P. damsela ssp. damsela* (Fig. 2, lane 22). PCR product was not obtained in the bacteria resuspended in saline (data not given).

In order to identify *P. damsela ssp. piscicida*, bacterial colonies of the bacteria that gave a PCR product were plated on TCBS-1. *Photobacterium damsela ssp. piscicida* could not grow on TCBS-1 whereas *P. damsela ssp. damsela* did so (Table 2).

The sensitivity of the PCR was evaluated by serial dilution of *P. damsela ssp. piscicida* cell suspension as detailed elsewhere (Cascon *et al.* 1996). Amplification resulted in detectable levels of PCR product when a minimum of 10 CFU of *P. damsela ssp. piscicida* was used.

Table 2 Growth of bacterial strains on brain–heart infusion agar (BHIA) and thiosulphate citrate bile salts–sucrose agar (TCBS-1) plates

Bacterial strains	Growth on BHIA	Growth on TCBS-1
<i>P. damsela ssp. piscicida</i> (lab isolate)	+	–
<i>P. damsela ssp. piscicida</i> (Taiwan)	+	–
<i>P. damsela ssp. piscicida</i> (CCRC 17065)	+	–
<i>P. damsela ssp. damsela</i> (CCRC 13854)	+	G
<i>P. damsela ssp. damsela</i> (CCRC 15429)	+	G

G, green colonies.

DISCUSSION

The use of nucleic acid amplification by PCR has application in many fields, especially in the rapid identification of bacteria. Amplification of specific regions of bacterial DNA with synthetic oligonucleotide primers, using PCR, has enormous potential of sensitivity and specificity in detecting disease in fish. The technique has already been developed for some important fish pathogens: *Aer. salmonicida ssp. salmonicida* (Miyata *et al.* 1996), *Cytophaga psychrophila* (Toyama *et al.* 1994), *Edwardsiella tarda* (Aoki and Hirono 1995), *Pasteurella piscicida* (Aoki *et al.* 1995), *Renibacterium salmoninarum* (Brown *et al.* 1994; Leon *et al.* 1994) and *Yersinia ruckeri* (Temprano *et al.* 2001; Yugueros *et al.*

2001). Osorio *et al.* (1999) have developed PCR detection technique using 16S rRNA for *P. damsela* ssp. *piscicida*.

In this study we designed primers for the rapid identification of *P. damsela* ssp. *piscicida*. It was found that the primers showed specificity for *P. damsela* ssp. *piscicida* and *P. damsela* ssp. *damsela* when tested against a range of bacteria, either alone or in mixture. This could be easily explained as the two subspecies differed only by a single nucleotide (Gauthier *et al.* 1995). Osorio *et al.* (1999) reported that the two subspecies have the same 16S rRNA gene sequence and have used the nested PCR to differentiate *P. damsela* ssp. *piscicida* from *P. damsela* ssp. *damsela*. Toranzo *et al.* (1991) and Zorrilla *et al.* (1999) reported that *P. damsela* ssp. *piscicida* did not grow on TCBS. Conversely, it was reported that *P. damsela* ssp. *damsela* could grow on TCBS (Love *et al.* 1981; Thyssen *et al.* 1998). Hence in our study, we plated the PCR positive bacterial colonies on TCBS-1 to differentiate *P. damsela* ssp. *piscicida* from *P. damsela* ssp. *damsela*. The sensitivity of the PCR agrees with that described for various bacteria, i.e. a detection limit of 1–20 CFU (Cascon *et al.* 1996, 1997; Gibello *et al.* 1999; Temprano *et al.* 2001; Yugueros *et al.* 2001). In previous reports, bacteria grown on BHIA were scraped off from the plates, resuspended in saline to extract DNA of the mixed cultures and were then used for PCR (Osorio *et al.* 1999). In this study, all bacteria were resuspended in sterile distilled water and were used for PCR amplification. Bacteria resuspended in saline could not be amplified by PCR, possibly because of the salt present. Moreover, in previous studies (Temprano *et al.* 2001), the samples were boiled for 10 min. But in the present study the samples were not boiled, thus simplifying the process.

The techniques (PCR and TCBS-1 plating) followed in this study are faster than the conventional API 20E and API CH50 used for the biochemical characterization of the *P. damsela* ssp. *piscicida*. The cost and labour involved in this method is less when compared with previous reports and commercially available kits and hence is suitable for use in all laboratories.

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