Quantitative detection of *Aeromonas salmonicida* in fish tissue by real-time PCR using self-quenched, fluorogenic primers

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In this study a real-time PCR assay using self-quenched primers labelled with a single fluorophore for the detection of *Aeromonas salmonicida* was developed. Probe specificity was confirmed by amplification of 16 *A. salmonicida* strain templates and by the lack of a PCR product with 26 non-*A. salmonicida* strains. With a pure culture of *A. salmonicida*, the assay was linear over a range of 0.5 pg to 50 ng and was able to detect 16 c.f.u. per reaction. A similar sensitivity was observed in DNA extracted from a mixture of *A. salmonicida* and fish tissue. Results using artificially inoculated tissues and diseased fish from outbreaks indicated that the assay can provide sensitive species-specific detection and quantification of *A. salmonicida* in fish tissue.

INTRODUCTION

Aeromonas salmonicida is the aetiological agent of furunculosis, a disease that affects many species of fish and is a significant cause of economic losses in the aquaculture of trout and salmon (McCarthy & Roberts, 1980; O'Brien *et al.*, 1994). The disease is characterized by the presence of general septicaemia, lack of appetite, lethargy and haemorrhagic and necrotic lesions in gills, gut and muscle (Hiney & Olivier, 1999). This bacterium has also been associated with ulcer disease in carp and marine flatfish and erythrodermatitis in carp (Austin & Austin, 1999).

Methods currently used to identify *A. salmonicida* within infected fish can be time-consuming, labour-intensive and hard to implement because of difficulties in distinguishing the bacterium from other bacterial species given the wide variety of existing biochemical profiles, the absence of an efficient selective medium and the poor plating efficiency of the bacterium in mixed cultures. For this reason, attention has focused on the application of different serological techniques (Adams & Thompson, 1990; Gilroy & Smith, 2003), and DNA sequence-based methods employing PCR have been developed (Gustafson *et al.*, 1992; Hiney *et al.*, 1992; Miyata *et al.*, 1996).

Real-time PCR technology may be able to provide an innovative method of detecting and enumerating several

organisms in different environments (Sharkey *et al.*, 2004). With this method, PCR product accumulation is continuously monitored during cycle progression by means of fluorescent detection. In addition, this technique is characterized by a large dynamic range of quantification, sensitivity and throughput capacity and requires no post-amplification manipulation, thus avoiding possible carry-over contamination.

A fluorescence-based PCR technique that uses a fluorogenic primer labelled with a single fluorophore was recently developed as a cost-effective alternative to other fluorescence-based PCR techniques (Nazarenko *et al.*, 2002a, b). Light Upon eXtension (LUX) primers (Invitrogen) are designed with the fluorophore attached near the 3' end and a short complementary sequence at the 5' end that creates a hairpin structure. This structure effectively quenches the fluorophore so that a separate quenching moiety is not required. When the primer is incorporated into a double-stranded PCR product and extended by DNA polymerase, the fluorophore is dequenched, resulting in a significant increase in fluorescent signal (Lowe *et al.*, 2003).

The most frequent target of species-specific *A. salmonicida* DNA probes and PCR assays is a 6.4 kb cryptic plasmid known to occur in 90 % of *A. salmonicida* subsp. *salmonicida* (Hiney *et al.*, 1992; Sørum *et al.*, 1993; O'Brien *et al.*, 1994; Mooney *et al.*, 1995; Byers *et al.*, 2002). This locus is also found in all *A. salmonicida* subsp. *achromogenes* strains tested and some *A. salmonicida* subsp. *masoucida* strains. No reaction has been found with over 60 related aeromonads and aquatic bacteria tested to date (Mooney *et al.*, 1995).

Abbreviations: C_t, cycle threshold; FAM, 6-carboxyfluorescein; LUX, Light Upon eXtension.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the *A. salmonicida* strains determined in this study are AM296501–AM296510.

The present study employed this locus to develop a quantitative real-time PCR assay using fluorogenic primers. Assay sensitivity and specificity were examined in pure or mixed cultures, and enumeration of *A. salmonicida* by real-time PCR in tissue samples from rainbow trout (*Oncorhynchus mykiss*) was carried out.

METHODS

Bacterial strains and culture conditions. A total of 40 bacterial strains comprising *A. salmonicida* ($n=16$) and non-*A. salmonicida* strains ($n=26$) was employed to test the specificity of the PCR assay (Table 1). Strains were obtained from the following sources: ATCC (American Type Culture Collection, Manassas, VA, USA), NCIMB (National Collections of Industrial and Marine Bacteria, Aberdeen, UK) and LMG (Laboratory for Microbiology, Ghent University, Belgium), as indicated in Table 1; strains CLFP 501–505, isolated from rainbow trout during a furunculosis outbreak at fish farms, from the Autonomous Community of Aragon, Spain; strains 2320, 793 and 4814 from Dr D. Colquhoun (Department for Fish Health, National Veterinary Institute, Oslo, Norway); strains 265/87, MT-004, F-661-2/89, Fin 3, 870626-1/1C, S-228/91, S-123-91-1, Vib 293, Vib 305, Vib 281 and M 147/92 from Dr B. K. Gudmundsdóttir (Institute for Experimental Pathology, University of Iceland, Reykjavík, Iceland); strains 146 and 147 from Dr José Guijarro (Department of Functional Biology, University of Oviedo, Spain); strains CECT 4342, CECT 4224, CECT 4245, CECT 4227, CECT 4257, CECT 4199, CECT 5761 and CECT 4237 from Dr María José Figueras (Biology and Microbiology Research Unit, Rovira and Virgili University, Spain); and strains Rs 146 and Rs 147 from Dr Eva Jansson (Department of Wild Life, Fish and Environment, National Veterinary Institute, Sweden). All strains were grown in brain heart infusion broth (Scharlau Chemie) overnight at 22 °C, with the exception of *Flavobacterium psychrophilum*, which was grown in Anackel–Ordal broth (0.5 % tryptone, 0.05 % yeast extract, 0.02 % sodium acetate, 0.02 % beef extract, adjusted to pH 7.4) for 2 days at 15 °C with agitation, and *Renibacterium salmoninarum*, which was grown in modified KDM2 broth (1 % bactopeptone, 0.05 % yeast extract, 0.05 % L-cysteine, adjusted to pH 6.5) for 6 days at 15 °C with agitation.

16S rRNA gene sequencing. In order to ensure that strains were *A. salmonicida*, the 16S rRNA gene sequence was PCR amplified in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler, as described by Suzuki *et al.* (1996). The PCR products were sequenced directly using the prokaryotic 16S rRNA gene universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3') on a MegaBACE 500 sequencer following the manufacturer's protocols (Amersham Biosciences). Sequences were corrected manually and aligned with 16S rRNA gene sequences obtained from the GenBank database using the BLAST algorithm (Altschul *et al.*, 1990). Subsequent multiple alignments were carried out using CLUSTAL W (Thompson *et al.*, 1994).

DNA extractions. Bacterial cultures (1.0 ml) were homogenized in 200 µl TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA], centrifuged at 12 000 g for 1 min and pellets were extracted using InstaGene Matrix (Bio-Rad) following the manufacturer's instructions. DNA yield and purity were determined spectrophotometrically by measuring $A_{260}:A_{280}$ ratios (Gene Quant pro RNA–DNA calculator; Amersham Pharmacia Biotech). Concentrated DNA was stored at –20 °C.

Fluorogenic real-time PCR. Oligonucleotide sequences were derived from a specific DNA probe for *A. salmonicida* (GenBank accession no. X64214). The probe is specific for a region of the

Table 1. Bacterial strains detected by real-time PCR

Isolate	Detection
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CLFP 501	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CLFP 502	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CLFP 503	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CLFP 504	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> CLFP 505	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> 793	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> MT-004	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> NCIMB 1102	+
<i>A. salmonicida</i> subsp. <i>salmonicida</i> 4814	+
<i>A. salmonicida</i> subsp. <i>achromogenes</i> 2320	+
<i>A. salmonicida</i> subsp. <i>achromogenes</i> NCIMB 1110	+
<i>A. salmonicida</i> subsp. <i>achromogenes</i> 265/87	+
<i>A. salmonicida</i> subsp. <i>achromogenes</i> F-661-2/89	+
<i>A. salmonicida</i> subsp. <i>achromogenes</i> 870626-1/1C	+
<i>A. salmonicida</i> subsp. <i>masoucida</i> Fin 3	+
<i>A. salmonicida</i> subsp. <i>masoucida</i> NCIMB 2020	+
<i>Aeromonas encheleia</i> CECT 4342	–
<i>Aeromonas eucrenophila</i> CECT 4224	–
<i>Aeromonas sobria</i> CECT 4245	–
<i>Aeromonas bestiarum</i> CECT 4227	–
<i>Aeromonas veronii</i> CECT 4257	–
<i>Aeromonas allosaccharophila</i> CECT 4199	–
<i>Aeromonas culicicola</i> CECT 5761	–
<i>Aeromonas media</i> CECT 4237	–
<i>Aeromonas hydrophila</i> S-123-91-1	–
<i>Aeromonas hydrophila</i> S-228/91	–
<i>Vibrio tubiashii</i> LMG 10936	–
<i>Vibrio fluvialis</i> NCIMB 2249	–
<i>Vibrio splendidus</i> NCIMB 2251	–
<i>Vibrio furnissii</i> Vib 293	–
<i>Vibrio pelagius</i> Vib 305	–
<i>Vibrio aestuarianus</i> Vib 281	–
<i>Vibrio anguillarum</i> ATCC 19264	–
<i>Moritella viscosa</i> M 147/92	–
<i>Photobacterium phosphoreum</i> NCIMB 844	–
<i>Yersinia ruckeri</i> ATCC 29473	–
<i>Y. ruckeri</i> 146	–
<i>Y. ruckeri</i> 147	–
<i>Flavobacterium psychrophilum</i> NCIMB 1947	–
<i>F. psychrophilum</i> NCIMB 1826	–
<i>Renibacterium salmoninarum</i> Rs 146	–
<i>R. salmoninarum</i> Rs 147	–

+, Fluorescent signal detected after 15–40 cycles; –, no fluorescent signal detected after 40 cycles.

DNA fragment that has been used previously as a species-specific genetic probe (Hiney *et al.*, 1992). LUX designer software (www.invitrogen.com/lux) was used to design the LUX fluorogenic primers [5'-CGGAACGTAATCTGAATTGTTCTTTTC(FAM)G-3'] and (5'-ATTGCTTATCGAGGAGCCCAAC-3').

For this assay, each 50 µl PCR contained 25 µl Platinum Quantitative PCR SuperMix-UDG (uracil DNA glycosylase) [60 U Platinum *Taq* DNA polymerase ml^{–1}, 40 mM Tris/HCl (pH 8.4), 100 mM KCl,

6 mM MgCl₂, 400 µM each dGTP, dATP and dCTP, 800 µM dUTP, 40 U UDG ml⁻¹ and stabilizers], FAM-labelled LUX primer and corresponding unlabelled primer (200 nM each final concentration), 1 µl ROX reference dye (Invitrogen) and 10 µl template. An ABI PRISM 7000 was programmed as follows: 50 °C for 2 min and denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Data were analysed using ABI PRISM 7000 sequence detection software, version 1.2.3 (Applied Biosystems). The cycle threshold (C_t) was calculated as the cycle number at which the reaction became exponential. The C_t of each sample was then compared with a standard curve and the result was expressed as absolute amounts of genomic DNA. Standard curves for quantification were plotted from triplicate samples by using C_t values of tenfold dilutions of template extracted from 8 × 10⁶ c.f.u. *A. salmonicida* NCIMB 1102 ml⁻¹. To confirm the results of the real-time PCR, 10 µl aliquots of PCR products were analysed by electrophoresis on ethidium bromide-stained 2 % agarose gels (Bio-Rad).

Sensitivity and specificity of the real-time PCR assay. The specificity of the real-time PCR was determined by comparing PCR products derived from *A. salmonicida* (*n*=16) and non-*A. salmonicida* strain (*n*=26) DNA templates. For sensitivity assays, DNA was extracted from pure cultures of *A. salmonicida* NCIMB 1102 (8 × 10⁶ c.f.u. ml⁻¹) to generate a standard curve used for enumeration of unknown samples.

Detection of seed *A. salmonicida* in fish tissue homogenate. Tissue samples (250 mg kidney, liver, spleen or intestine) from rainbow trout were homogenized in a 1 ml suspension of early stationary phase *A. salmonicida* NCIMB 1102 cells diluted in deionized water to a final concentration of 32, 320 and 3200 c.f.u. (g tissue)⁻¹. Homogenates were centrifuged at 12 000 g for 1 min and DNA was extracted using InstaGene matrix following the manufacturer's instructions. Enumeration of *A. salmonicida* by real-time PCR was compared with plate count values. Statistical analysis was based on Student's *t*-test using paired and equal variance of log c.f.u. g⁻¹. All statistics were performed using SPSS for Windows version 11.5 (SPSS).

Detection of *A. salmonicida* in naturally infected fish. Tissue samples (250 mg) of diseased fish associated with outbreaks on different fish farms were also tested. Samples were homogenized in 1 ml deionized water. Homogenates were centrifuged at 12 000 g for 1 min and DNA was extracted as described above.

RESULTS AND DISCUSSION

The fluorogenic primer method has several advantages over other methods, including ease of design and synthesis of the primers. The design of these primers is based on studies that demonstrate the effects of the primary and secondary structure of oligonucleotides on the emission properties of a conjugated fluorophore (Nazarenko *et al.*, 2002b). The primers are chemically synthesized oligonucleotides (22–29 nt) with a fluorophore attached to the C5 position of thymidine, which increases its fluorescence when incorporated into a double-stranded PCR product. This phenomenon results from (i) having the fluorophore close to the 3' end of an oligonucleotide with G or C at the 3' end, (ii) the existence of a G within a few bases of the label and (iii) the ability of the oligonucleotide to form a blunt-ended hairpin at temperatures close to the annealing temperature of the primer (Nazarenko *et al.*, 2002a).

Primer design

LUX fluorogenic primers were designed from a specific DNA probe for *A. salmonicida* (Hiney *et al.*, 1992), as 16S rRNA gene sequence analysis did not provide sufficient information for the delineation and identification of most species of the genera *Aeromonas*. It has been reported that *A. salmonicida* and *Aeromonas bestiarum* type strains differ by only two nucleotides, at positions 1011 and 1018 (Martínez-Murcia *et al.*, 2005).

A computer search using tBLASTX (Altschul *et al.*, 1997) for the matching sequences in GenBank including updates against the DNA probe for *A. salmonicida* (GenBank accession no. X64214) as a query sequence revealed more than 97 % similarity with plasmids from *A. salmonicida* subspecies listed in the NCBI genome database. The tBLASTX search also found that the DNA probe for *A. salmonicida* shared 47 % identity with the *YopP* gene from *Yersinia enterocolitica* (GenBank accession no. AF023202), which induces apoptosis in mouse macrophages (Mills *et al.*, 1997). Because of the similarity between the DNA probe for *A. salmonicida* and the *YopP* gene from *Y. enterocolitica*, the extreme 5'-end region of the sequence was targeted, as this is where the most sequence variation occurs between the two.

Specificity of detection

To improve the diagnosis capabilities for this bacterial pathogen, a real-time PCR assay was developed. Species specificity was confirmed by positive amplification of all 16 isolates of *A. salmonicida* and lack of a product from ten other strains of *Aeromonas* species (*Aeromonas encheleia*, *Aeromonas eucrenophila*, *Aeromonas sobria*, *A. bestiarum*, *Aeromonas veronii*, *Aeromonas allosaccharophila*, *Aeromonas culicicola*, *Aeromonas media* and *Aeromonas hydrophila*) and 16 strains of common bacterial fish pathogens (*Flavobacterium psychrophilum*, *Moritella viscosa*, *Photobacterium phosphoreum*, *Renibacterium salmoninarum*, *Vibrio tubiashii*, *Vibrio fluvialis*, *Vibrio splendidus*, *Vibrio furnissii*, *Vibrio pelagius*, *Vibrio aestuarianus*, *Vibrio anguillarum* and *Yersinia ruckeri*) (Table 1). The size of the amplified PCR product (131 bp) was verified by gel electrophoresis (not shown).

Byers *et al.* (2002) showed that primers PAAS1 and PAAS2, previously designed from the same sequence used in the present study (Hiney *et al.*, 1992), did not produce any false-positive reactions with 27 non-target bacterial DNA extracts and these primers were considered to be specific for *A. salmonicida*. However, the PAAS primer set correctly identified only 285/308 isolates as *A. salmonicida* and was therefore determined to have an *in vitro* sensitivity of 93 %. The failure of the PAAS primer set to identify 100 % of the *A. salmonicida* isolates appeared to be related to the primer target site, which had previously been shown to occur on a 6.4 kb cryptic plasmid (Sørum *et al.*, 1993) and to be present in approximately 90 % of *A. salmonicida* isolates (Mooney *et al.*, 1995). It is therefore reasonable to speculate that the

Fig. 1. Standard curve for quantification, plotted from triplicate samples using C_t values of tenfold dilutions of template extracted from 8×10^6 c.f.u. *A. salmonicida* NCIMB 1102 ml⁻¹.

specificity of the reaction could be related to the virulence of the strains, as all *A. salmonicida* strains used in the present study were isolated from disease outbreaks in *O. mykiss*, *Salmo salar*, *Salvelinus alpinus* and *Zoarces viviparus*. In addition, the probe sequence homology with the *YopP* gene from *Y. enterocolitica* could confirm this hypothesis.

Sensitivity of detection

The detection sensitivity of the real-time PCR assay was determined by testing triplicate sets of genomic DNA prepared from serial dilutions (8×10^1 to 8×10^6 c.f.u. ml⁻¹) of *A. salmonicida* NCIMB 1102. The results were reported as C_t values versus log starting quantities of DNA. Positive signals were found in all dilutions except those where the DNA concentration was below 5×10^{-4} ng μ l⁻¹ (80 c.f.u. ml⁻¹). The C_t values were also plotted against c.f.u. ml⁻¹ (8×10^1 to 8×10^6 c.f.u. ml⁻¹) in tenfold serial dilutions of the *A. salmonicida* NCIMB 1102 culture used

for extracting genomic DNA. Based on this approach, a correlation was observed between C_t and c.f.u. ml⁻¹ of the starting quantity of *A. salmonicida* NCIMB 1102 DNA (Fig. 1).

The standard curve demonstrated a C_t value of around 15 for 50 ng DNA (log 6.9 c.f.u. ml⁻¹) with a slope close to -3.53 , indicating good sensitivity. The high coefficient of determination ($R^2=0.99$) obtained in the assay showed a strong correlation between template DNA concentrations ranging from 0.5 pg to 50 ng and the ΔR_n signal and indicated that the assay is useful for quantitative measurements of *A. salmonicida*.

The real-time PCR detection limit for *A. salmonicida* DNA derived from pure culture was 0.5 pg PCR mixture μ l⁻¹, or the equivalent of 80 c.f.u. (ml culture)⁻¹, which would extrapolate to 16 c.f.u. per PCR assay, assuming 100% extraction efficiency.

Detection of *A. salmonicida* in fish tissues

The C_t values obtained from the analysis of tissue samples were extrapolated to the corresponding previously calculated standard curve. The presence of fish tissue did not affect real-time PCR detection of *A. salmonicida* NCIMB 1102, and the results of plate counts yielded a good correlation ($r=0.85$) between the two assays (Table 2). On the other hand, amplification products were not detected when tissues were inoculated independently with 32 c.f.u. (g culture)⁻¹.

In order to check the potential application of this PCR in natural infections, some samples from natural outbreaks were processed (Table 3). These preliminary results showed

Table 2. Quantification of *A. salmonicida* from inoculated tissues

Sample	Inoculum (log c.f.u. g ⁻¹)	Plate count* (log c.f.u. g ⁻¹ ± SD)	Real-time PCR† [log c.f.u. g ⁻¹ ± SD (C_t ± SD)]
Kidney	3.5	3.64 ± 0.14	3.53 ± 0.06 (29.1 ± 0.56)
Kidney	2.5	2.57 ± 0.12	2.66 ± 0.04 (32.6 ± 0.42)
Spleen	3.5	3.56 ± 0.06	3.30 ± 0.07 (30.1 ± 0.78)
Spleen	2.5	2.48 ± 0.12	2.32 ± 0.11 (34.1 ± 0.77)
Liver	3.5	3.55 ± 0.08	3.26 ± 0.08 (30.3 ± 0.08)
Liver	2.5	2.49 ± 0.12	2.44 ± 0.07 (33.4 ± 0.10)
Intestine	3.5	3.53 ± 0.14	3.30 ± 0.11 (30.1 ± 0.70)
Intestine	2.5	2.48 ± 0.08	2.23 ± 0.06 (34.5 ± 0.07)
All tissues‡	1.5	1.12 ± 0.25	ND

ND, No amplification detected.

**A. salmonicida* levels were determined by plate counts on tryptic soy agar spread plates.

†Real-time PCR determination of *A. salmonicida* concentrations was based on the mean of duplicate samples. Concentrations were derived from a standard curve using the mean of triplicate C_t values for serial tenfold dilutions of DNA extracted from known concentrations of bacteria.

‡Individual samples of kidney, spleen, liver and intestine.

Table 3. Quantification of *A. salmonicida* from naturally infected tissues

Sample	Real-time PCR* [$\log$ c.f.u. $g^{-1} \pm SD$ ($C_t \pm SD$)]
Kidney	3.60 ± 0.17 (29.8 ± 0.22)
Kidney	4.02 ± 0.16 (28.2 ± 0.42)
Liver	3.70 ± 0.14 (29.1 ± 0.10)
Liver	3.90 ± 0.16 (28.6 ± 0.08)
Spleen	ND
Spleen	2.71 ± 0.12 (33.5 ± 0.18)

ND, No amplification detected.

*Real-time PCR determination of *A. salmonicida* concentrations was based on the mean of duplicate samples. Concentrations were derived from a standard curve using the mean of triplicate C_t values for serial tenfold dilutions of DNA extracted from known concentrations of bacteria.

that bacterial concentrations were in the normal range of values.

The sensitivity obtained in this study was not greatly different from that previously reported in the literature, in which detection limits per PCR were 6 c.f.u. in pure culture (Del Cerro *et al.*, 2002) and 30 c.f.u. (Del Cerro *et al.*, 2002) and 200 cells g^{-1} (O'Brien *et al.*, 1994) in tissue samples. In addition, a limit of detection of 2.4 cells per PCR has been reported using a slot-blot hybridization technique (Hiney *et al.*, 1992) and a threshold limit for detection of 10^3 c.f.u. ml^{-1} using a normal ELISA test (Adams & Thompson, 1990).

The relative quantification of DNA using fluorogenic primers is comparable in sensitivity and dynamic range to other published methods of quantification, such as TaqMan probes (Best *et al.*, 2005), molecular beacons (Xi *et al.*, 2003) and DNA-binding dyes (SYBR Green) (De Medici *et al.*, 2003). However, direct comparisons among methods for quantification are difficult as these methods are functionally different and may be affected differently by various factors.

In conclusion, the fluorogenic primer method is rapid, uses fewer toxic chemicals and is a useful tool for the sensitive and accurate detection of *A. salmonicida* in fish tissue. However, further studies should be performed in order to establish diagnostic agreement with serological methods at different stages post-infection.

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