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 erythrodematitis 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:** Ct, 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 Autonomic 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 Jose 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 (Biolog 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 (0.5 % tryptone, 0.05 % yeast 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′-GGTTACGACTT-3′) on a MegaBACE 500 sequencer following the manufacturer's protocols (Amershams 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/HC1 (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 A260/A280 ratios (Gene Quant pro RNA–DNA calculator; Amershams 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

<p>| Table 1. Bacterial strains detected by real-time PCR |</p>
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 501</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 502</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 503</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 504</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 505</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 793</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida MT-004</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida NCIMB 1102</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. salmonicida CLFP 4814</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. achromogenes 2320</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. achromogenes NCIMB 1110</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. achromogenes 265/87</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. achromogenes F-661-2/89</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. achromogenes 870626-1/1C</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. masoucida Fin 3</td>
<td>+</td>
</tr>
<tr>
<td>A. salmonicida subsp. masoucida NCIMB 2020</td>
<td>+</td>
</tr>
<tr>
<td>Aeromonas encheleia CECT 4342</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas europaea CECT 4224</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas sobria CECT 4245</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas bestiarum CECT 4227</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas veronii CECT 4257</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas allosacharophila CECT 4199</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas catticina CECT 5761</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas media CECT 4237</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas hydrophila S-123-91-1</td>
<td>−</td>
</tr>
<tr>
<td>Aeromonas hydrophila S-228/91</td>
<td>−</td>
</tr>
<tr>
<td>Vibrio tubishii LMG 10936</td>
<td>−</td>
</tr>
<tr>
<td>Vibrio fluvialis NCIMB 2249</td>
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</tr>
<tr>
<td>Vibrio splendidus NCIMB 2251</td>
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<tr>
<td>Vibrio furnissii Vib 293</td>
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<tr>
<td>Vibrio pelagius Vib 305</td>
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<td>Vibrio aestuarianus Vib 281</td>
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<td>Vibrio anguillarum ATCC 19264</td>
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<td>Moritella viscosa M 147/92</td>
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</tr>
<tr>
<td>Photobacterium phosphoreum NCIMB 844</td>
<td>−</td>
</tr>
<tr>
<td>Yersinia ruckeri ATCC 29473</td>
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</tr>
<tr>
<td>Y. ruckeri 146</td>
<td>−</td>
</tr>
<tr>
<td>Y. ruckeri 147</td>
<td>−</td>
</tr>
<tr>
<td>Flavobacterium psychrophilum NCIMB 1947</td>
<td>−</td>
</tr>
<tr>
<td>F. psychrophilum NCIMB 1826</td>
<td>−</td>
</tr>
<tr>
<td>Renibacterium salmoninarum Rs 146</td>
<td>−</td>
</tr>
<tr>
<td>R. salmoninarum Rs 147</td>
<td>−</td>
</tr>
</tbody>
</table>

+, 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′-CGGAACGTAAACCTTGGTCATTTT(FAM)G-3′) and (5′-ATGGTTATGGGAGGCAC-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/HC1 (pH 8.4), 100 mM KCl, 10 mM Tris/HC1 (pH 8.0), 1 mM EDTA].
Real-time PCR detection of Aeromonas salmonicida

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.

RESULTS AND DISCUSSION

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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.

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 x 10⁶ c.f.u. ml⁻¹) to generate a standard curve used for enumeration of unknown samples.

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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 cunicola, 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 (Sorum 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

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 (Ct) was calculated as the cycle number at which the reaction became exponential. The Ct 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 Ct values of tenfold dilutions of template extracted from 8 x 10⁶ c.f.u. ml⁻¹ A. salmonicida NCIMB 1102. 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).

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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 × 10^6 to 8 × 10^0 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 ml^(-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 μl^(-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

![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).](image)

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

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

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.
ACKNOWLEDGEMENTS

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