A culture independent method for the detection of *Aeromonas* sp. from water samples

Fadua Latif-Eugenín, Roxana Beaz-Hidalgo, María José Figueras

Unit of Microbiology, Medicine and Health Science School, Rovira i Virgili University, Reus, Spain

Abstract

The genus *Aeromonas* is present in a wide variety of water environments and is recognised as potentially pathogenic to humans and animals. Members of this genus are often confused with *Vibrio* when using automated, commercial identification systems that are culture-dependent. This study describes a polymerase chain reaction (PCR) detection method for *Aeromonas* that is culture-independent and that targets the glycerophospholopid-cholesterol acyltransferase (*gcat*) gene, which is specific for this genus. The GCAT-PCR was 100% specific in artificially inoculated water samples, with a detection limit that ranged from 2.5 to 25 cfu/mL. The success at detecting this pathogen in 86 water samples using the GCAT-PCR method was identical to the conventional culturing method when a pre-enrichment step was carried out, yielding 83.7% positive samples. On the other hand, without a pre-enrichment step, only 77.9% of the samples were positive by culturing and only 15.1% with the GCAT-PCR. However, 83.7% positive samples were obtained for the GCAT-PCR when the water volume for the DNA extraction was increased from 400 μL to 4 mL. The proposed molecular method is much faster (5 or 29 h) than the culturing method (24 or 48 h) whether performed directly or after a pre-enrichment step and it will enable the fast detection of *Aeromonas* in water samples helping to prevent a possible transmission to humans.

Introduction

The genus *Aeromonas* currently includes 30 species (Beaz-Hidalgo *et al.,* 2015) that are autochthonous aquatic microorganisms found widely in the environment (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013; Figueras and Beaz-Hidalgo, 2014, 2015). These bacteria have often been recovered from different types of water, such as raw and treated sewage, drinking water, seawater and fresh water (Emekdas *et al.,* 2006; Martone-Rocha *et al.,* 2010; Figueira *et al.,* 2011; Igbinosa and Okoh, 2013; Robertson *et al.,* 2014). *Aeromonas* is also common in healthy and in diseased fish (Beaz-Hidalgo *et al.,* 2010; Liu and Li, 2012; Chen *et al.,* 2012; Vega-Sánchez *et al.,* 2014), marine mammals (Pérez *et al.,* 2015), chironomid egg masses (Beaz-Hidalgo *et al.,* 2012) and food (Castro-Escarpulli *et al.,* 2003; Ottaviani *et al.,* 2006; Nagar *et al.,* 2013). In humans, *Aeromonas* is recognised as an opportunistic pathogen that can affect both immunocompromised and immunocompetent individuals, with gastroenteritis and wound infections being the most frequent clinical presentations, followed by bacteremia (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015).

The epidemiological relationships between *Aeromonas* strains recovered from drinking water and those from cases of diarrhoea have previously been established, thus reinforcing the waterborne and foodborne origin of *Aeromonas* infections (Khajanchi *et al.,* 2010; Pablos *et al.,* 2011). Therefore, having fast and reliable methods that can trace *Aeromonas* in water systems is essential because current biochemical methods are time-consuming and not always able to correctly identify the genus, being frequently confused with *Vibrio* sp. (Chacón *et al.,* 2002; Soler *et al.,* 2002; Lamy *et al.,* 2010). Presently, several genetic markers that allow molecular identification of *Aeromonas* to genus level have been proposed (Cascón *et al.,* 1997; Chacón *et al.,* 2002; Arora *et al.,* 2006; Balakrishna *et al.,* 2008; Robertson *et al.,* 2014). Among them, there is one that targets the *gcat* gene (237 bp) that encodes the glycerophospholipid-cholesterol acyl transferase. The latter was developed by Chacón *et al*. (2002) as an *Aeromonas* DNA genus probe that specifically hybridised with all 14 *Aeromonas* species recognised at that time. Later, the protocol was adapted by Soler *et al*. (2002) for a PCR reaction with an annealing temperature of 56°C. The *gcat* gene was characterised by Buckley *et al*. (1982) as a leading, lethal toxin of the species *Aeromonas salmonicida*. However, Chacón *et al*. (2002, 2003) demonstrated that this gene was present in all *Aeromonas* species and was used as a genus-specific genetic marker in the identification of the isolates in several studies (Chacón *et al.,* 2002, 2003; Soler *et al.,* 2002; Beaz-Hidalgo *et al.,* 2010; Puthucheary *et al.,* 2012). The aim of this study was to adapt the glycerophospholipid-cholesterol acyltransferase-polymerase chain reaction (GCAT-PCR) as a culture-independent method for the direct detection of *Aeromonas* from water samples.

Correspondence: María José Figueras, Unit of Microbiology, Medicine and Health Science School, Institut d'Investigació Sanitària Pere Virgili (IISPV), Rovira i Virgili University, Sant Llorenç 21, 43201 Reus, Spain.
Tel: +34.9777.59321 - Fax: +34.9777.59322.
E-mail: mariajose.figueras@urv.cat

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Materials and Methods

Polymerase chain reaction specificity and sensitivity in artificially inoculated water samples

Five reference strains (*Aeromonas hydrophila* CECT 839^T, *Plesiomonas shigelloides* CECT 597, *Vibrio parahaemolyticus* CECT 588, *Escherichia coli* CECT 744, and *Pseudomonas aeruginosa* CECT 110^T) were used to evaluate the specificity of the PCR to detect *Aeromonas* in artificially inoculated, distilled water samples. All strains were inoculated into 10 mL of Trypticase Soy Broth (TSB; Difco, Leeuwarden, The Netherlands) and incubated at 30°C for 24 hours, then concentrations were adjusted to an optical density of 1 (measured with a spectrophometer at 450 nm), which corresponded to a concentration of approximately 10^8 cfu/mL. To verify this, serial 10-fold dilutions were performed in Buffered Peptone Water (BPW) from 10^8 to 10^0 cfu/mL for each strain. From each dilution (100 μL) were plated in triplicate onto Trypticase Soy Agar (TSA; Difco) medium and incubated at 30ºC for 24 hours. The concentration (cfu/mL) of each dilution was estimated by calculating the average of three plate counts. Three flasks (A, B and C) of 100 mL each of distilled water were artificially inoculated with 1 mL of a bacterial suspension (10^8 cfu/mL) that contained:

A) *A. hydrophila* CECT 839^T alone; B) *A. hydrophila* CECT 839^T, *P. shigelloides* CECT 597, *V. parahaemolyticus* CECT 588, *E. coli* CECT 744 and *P. aeruginosa* CECT 110; and C) the same microbes as B but without *A. hydrophila* CECT 839^T.

From each flask inoculated with the different bacterial mixtures, 10-fold serial dilutions were performed, and 400 µL of each dilution was used for DNA extraction using the InstaGene Matrix (Bio-Rad, Hercules, CA, USA) protocol. The GCAT-PCR amplification was made in a final volume of 50 µL containing 5 µL of DNA, 1 µL of each primer (GCAT-F (5'-CTCCTGGAATCCCAAGTATCAG-3'), and GCAT-R (5'-GGCAGGTTGAACAGCAGTATCT-3') described by Soler *et al*. (2002) at 15 µM, 5 µL of dNTP mix (Applied Biosystems, Carlsbad, CA, USA) at 10 mM, 0.5 µL Taq DNA Polymerase (5 U/µL; Invitrogen, Carlsbad, CA, USA), 5 µL of 10X PCR Buffer (Invitrogen), 1.8 µL of 50 mM MgCl (Invitrogen), and 30.7 µL of milliQ water. PCR conditions consisted of an initial denaturation step at 95°C for 3 min followed by 35 cycles of amplification in which denaturation, annealing and elongation temperatures were 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, respectively. A final elongation of 72°C for 5 min was included in the programme. Amplification products were analysed on 2% (w/v) agarose gels in 1X TRIS Borate EDTA (TBE) buffer after staining with Red Safe TM nucleic acid staining solution (INtRON Biotechnology, Seongnam, Korea). Bands at the expected size (237 bp) were considered a positive result.

The sensitivity of the GCAT-PCR was determined by performing dilutions (10^6-10^0) of water samples artificially inoculated with *Aeromonas* (flasks A and B), while the specificity of this method was also assessed by inoculating a bacterial mixture without *Aeromonas* into distilled water samples (flask C). Furthermore, specificity was corroborated from five randomly selected positive samples from which their bands (237 bp) were cut out and purified using GFX™ PCR DNA and Gel

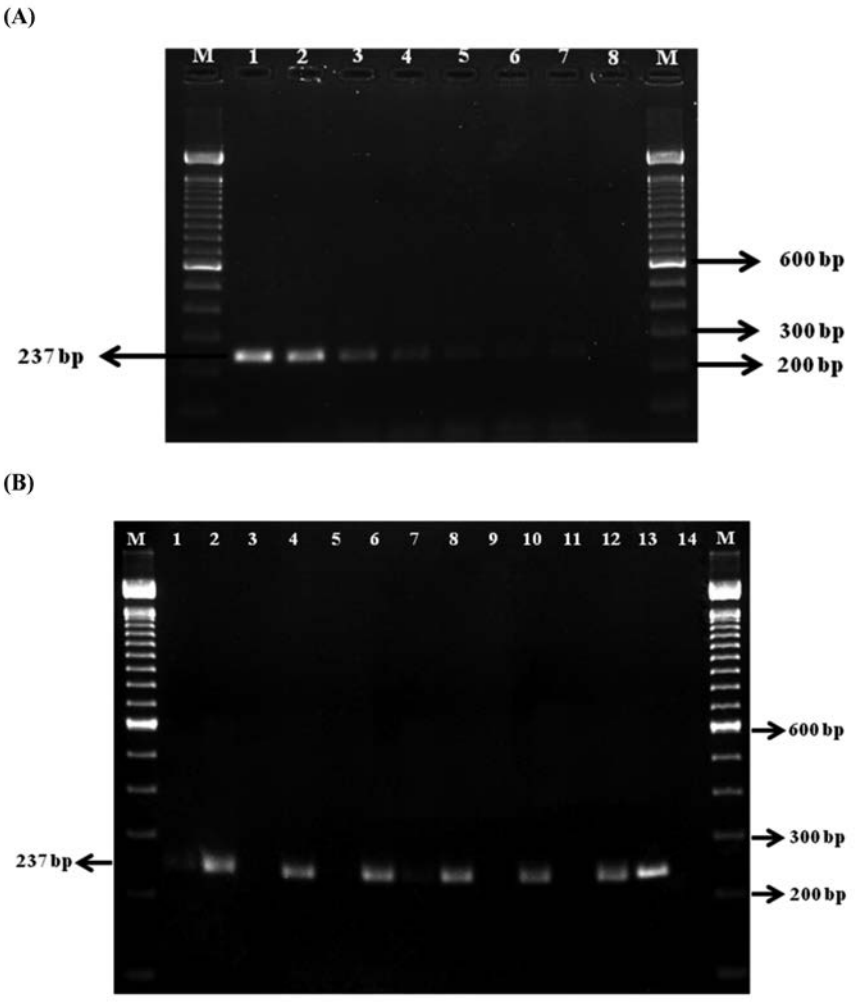

Figure 1. A) Sensitivity and specificity of the glycerophospholipid-cholesterol acyltransferase-polymerase chain reaction (GCAT-PCR) protocol using DNA from the artificially inoculated unenriched distilled water samples with different concentrations of mixed cultures of *Aeromonas hydrophila* CECT 839^T, *Plesiomonas shigelloides* CECT 597, *Vibrio parahaemolyticus* CECT 588, *Escherichia coli* CECT 744, and *Pseudomonas aeruginosa* CECT 110T. Lanes: 1-7, bacterial concentration ranged from 2.5x10^6 to 2.5x10^0 cfu/mL; 8, negative control; M, molecular weight ladder (100 to 2072 bp; Invitrogen, Carlsbad, CA, USA). B) Molecular detection of *Aeromonas* sp. by GCAT-PCR in water samples (lanes 1 to 12). Lanes: 1,3,5,7,9,11, unenriched samples; 2,4,6,8,10,12, enriched samples with alkaline peptone water at 30°C for 24 hours; 13, positive control; 14, negative control; M, molecular weight ladder (100 to 2072 bp; Invitrogen). Numbers on the left indicate the size of the GCAT amplified product (237 bp), and numbers on the right indicate the position of the molecular size marker.

Table 1. Detection of *Aeromonas* in eighty-six water samples by culturing and by the glycerophospholipid-cholesterol acyltransferase-polymerase chain reaction.

Water samples (n)	Number of positive samples (%)			
	Culturing		GCAT-PCR	
	Direct	Enrichment	Direct*	Enrichment
Sewage (68)	55 (80.9)	60 (88.2)	12 (17.6)	60 (88.2)
River (12)	12 (100)	12 (100)	1 (8.3)	12 (100)
Sea (6)	0 (0)	0 (0)	0 (0)	0 (0)

GCAT-PCR, glycerophospholipid-cholesterol acyltransferase-polymerase chain reaction. *These results were obtained when 400 µL of water were used for the DNA extraction, but when this water volume was increased 10-fold (4 mL) all samples (72, 83.7%) were positive for the presence of *Aeromonas*.

Band Purification Kit (GE Healthcare, Little Chalfont, UK) and sequenced. The identity of the obtained sequences was determined by BLAST with the sequences of the *gcat* gene deposited in the NCBI database.

Polymerase chain reaction sensitivity in natural water samples

A total of 86 water samples, including waste water (n=68), river water (n=12) and seawater (n=6), were collected from Catalonia, north-east Spain, between February 2012 and September 2013.

Detection of *Aeromonas* using the GCAT-PCR (237 bp) in natural water samples was evaluated directly from the DNA extracted from the water, by direct culture incubated at 30°C for 24 h and by culture after an enrichment step at the same incubation conditions. In the case of the direct GCAT detection and direct culturing, 10 mL of water diluted in 90 mL of BPW were used to avoid a high bacterial load that could generate too much DNA template and inhibit the PCR reaction. For the enrichment, the same dilution (1:10 vol/vol) was performed in alkaline peptone water (APW-A) supplemented with ampicillin (10 mg/L) to which almost all *Aeromonas* are resistant. Finally 100 μL of each dilution was inoculated on three culture media Ampicillin Dextrin Agar (ADA), Starch Ampicillin Agar (SAA) and Bile Irgasan Brilliant Green modified (BIBG-m) from which colonies were verified with the GCAT-PCR as was described previously (Soler *et al*., 2002).

For the molecular detection of *Aeromonas* sp., 400 μL of the enriched and non-enriched samples were used for the DNA extraction to perform the GCAT-PCR as described above. To avoid the different molecules contained in the water samples interfering with the PCR reaction, the pellet obtained after the centrifugation of the 400 μL was washed three times using 1 mL of milliQ water (Millipore, Billerica, MA, USA) each time. For each wash, the pellet was resuspended in 1 mL of milliQ water and centrifuged at 13.000 RPM for three minutes. In order to study if the non-detection of *Aeromonas* in many of the non-enriched samples was associated with the water volume used to extract DNA, the latter was increased 10-fold (from 400 μL to 4 mL).

Results

Polymerase chain reaction specificity and sensitivity in artificially inoculated water samples

The DNA extracted from water samples inoculated with *A. hydrophila* CECT 839^T yielded the expected amplification product of 237 bp of the GCAT (Figure 1A) and when this PCR product was sequenced it showed the highest similarity (98-99%) with sequences of the *gcat* gene of *A. hydrophila* CAHH2 (KM201324), *A. hydrophila* CAHH13 (KP159601) and *A. veronii* B565 (CP002607) deposited in the NCBI database. No amplification was found for the DNA extracted from water inoculated with bacterial mixtures without *A. hydrophila*. The lowest detection limit of *Aeromonas* in artificially inoculated distilled water samples (flask A and B) ranged between 2.5 and 25 cfu/mL (Figure 1A).

Polymerase chain reaction sensitivity in natural water samples

Table 1 shows that 83.7% (72/86) of the water samples were positive for the presence of *Aeromonas* sp. both by culturing and by the GCAT-PCR method after a pre-enrichment step. Without a pre-enrichment step the number of positive samples was lower, 77.9% (67/86) by culturing and 15.1% (13/86) by the GCAT-PCR (Figure 1B). To improve these results we increased 10-fold (from 400 μL to 4 mL) the water volume used for the DNA extraction. This new sample volume was applied to all water samples that showed negative results with the original protocol (using 400 μL of the water sample for the DNA extractions) and then all 72 water samples (83.7%) were positive for the presence of *Aeromonas*.

Discussion

This adapted GCAT-PCR method for the direct detection of *Aeromonas* without culturing, was highly sensitive (2.5-25 cfu/mL) when distilled water samples were inoculated with *A. hydrophila* CECT 839^T alone or together with other bacterial species. These results are similar to those of other studies that have detected *Aeromonas* (10 cfu/mL or gr) by PCR using 16S rRNA and *gyrB* genes in inoculated food (raw chicken and raw milk) and drinking water samples (Arora *et al.,* 2006; Balakrishna *et al.,* 2008; Robertson *et al.,* 2014).

The 100% specificity obtained in the present study corroborates previous results, in which colonies or isolates were verified as belonging to the genus *Aeromonas* when screening for the presence of the *gcat* gene (Chacón *et al.,* 2002, 2003; Beaz-Hidalgo *et al.,* 2010; Puthucheary *et al.,* 2012).

The same 72 (83.7%) water samples were positive for *Aeromonas* after enrichment with APW-A using the GCAT-PCR detection and cultures. However, the sensitivity of the two methods (culturing and molecular) was lower when the samples were not pre-enriched (Table 1) with only 77.9% of the samples being positive by culturing and only 15.1% with the GCAT-PCR. The comparatively poor efficiency of the GCAT-PCR was probably due to the detection limit, because when we increased the water volume for the DNA extraction from 400 μL to 4 mL, the percentage of positive samples increased from 15.1 to 83.7%.

The main advantage of the proposed molecular method is that the time required to detect *Aeromonas* is reduced from 24 hours (culturing method) to only 5 hours (including DNA extraction, PCR and electrophoresis) for the unenriched water.

Conclusions

Considering that water is the transmission route for *Aeromonas* infections, this new method will enable fast detection of the bacteria in order to be able to implement strategies that will avoid further dissemination of the bacteria in drinking, irrigating or recreational water systems.

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