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Research Article

Development of an Absolute Quantitative Real-Time PCR (qPCR) for the Diagnosis of *Aeromonas hydrophila* Infections in Fish

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Abstract

Aeromonas hydrophila is a Gram-negative motile bacillus responsible for substantial losses in global aquaculture, which also poses a risk to human health. Aeromonosis outbreaks are associated with excess organic matter in water supplies, failure in management, primary parasitic infestations, and the presence of powerful bacterial virulence factors. This study validated an adhesin gene from *A. hydrophila* (*ahaI*) using a quantitative real-time PCR assay based on SYBR Green I. The analytical specificity and sensitivity of the test was analyzed. The ability to identify and quantify *A. hydrophila* samples isolated using classical microbiology techniques and total DNA extracted directly from Nile tilapia, *Oreochromis niloticus*, kidney tissue experimentally infected with bacteria was also determined. The assay had 100% specificity and the sensitivity was 1.9 log gene copies of detection for total DNA from tissue. This study constitutes a valuable tool for the rapid and accurate diagnosis of *A. hydrophila* in intensive fish farming scenarios.

Keywords: Adhesin Gene; Molecular Diagnosis; Nile Tilapia; Septicemia

Abbreviations

PCR: Polymerase Chain Reaction; qPCR: Absolute Quantitative Real-Time PCR

Introduction

Aeromonas hydrophila is a Gram-negative straight chemo-organotrophic rods of approximately 0.8 - 1.0 × 1.0 - 3.5 µm in size, which are motile by single polar flagella. Although this microorganism is among the most common bacteria in freshwater habitats throughout the world, it is noted for causing haemorrhagic septicaemia in economically important hosts worldwide, including cichlids [1], catfish [2] and cyprinids [3]. Disease outbreaks are primarily associated with excess organic matter in water supplies, stress and primary infections [4].

It is suggested that *A. hydrophila* is enteropathogenic, causing disease by adhesive and enterotoxic mechanisms [5]. It is known that bacterial adherence to host epithelial cells plays an essential role in causing infections [6]. The adherence of a pathogen on the host surface is usually mediated by macromolecules known as adhesins, which refers to a surface molecule of a bacterium capable of specifically binding to a receptor on a substratum. Most bacterial species express more than one type of adhesin [7]. Although information on *A. hydrophila* adhesins is still very limited, previous work by Fang., *et al.* [7], showed that a major adhesin isolated from a fish pathogenic *A. hydrophila*, may be a conserved protein in the genus Aeromonas based on its ability to cross-inhibit serologically different *A. hydrophila.* Targeting this adhesin gene (*aha*I) constitutes an interesting and valuable study, not only to identify the specie, but also, enables future projects regarding recombinant adhesin as potential vaccine against aeromoniose.

Absolute quantitative real-time PCR (qPCR), a variation of polymerase chain reaction (PCR), has been used to identify *Aeromonas* spp. in wastewater effluent and catfish [8,9]. Real-time qPCR allows DNA quantification, has high sensitivity and specificity and is accurate in detecting small numbers of bacteria. Use of qPCR also eliminates the need for gel electrophoresis. The fluorescent dye SYBR Green I used for this technique intercalates into double-stranded DNA, remains stable at high temperatures, does not interfere with enzyme activity and binds to DNA molecules in a linear manner; SYBR Green I is also lower in cost compared with other intercalating agents on the market and is easy to use [10].

In this study, an absolute Real-Time qPCR assay based on SYBR Green I, targeting the major adhesin gene (*aha*I) for *A. hydrophila* was developed. The analytical specificity and sensitivity of this assay for detection of *A. hydrophila* was examined. Moreover, in an effort to reduce the time required for diagnosis, total DNA samples extracted directly from experimentally infected tilapia were assessed to demonstrate the effectiveness of the developed methodology.

Materials and Methods

Bacterial isolation and DNA preparation

In total, 33 strains of *A. hydrophila* isolated from different Brazilian commercial fish farms (Itajú-SP, Santa Fé do Sul-SP, Palmital-SP, Rio de Janeiro-RJ, Arealva-SP, Guaíra-SP, Porto Ferreira-SP and Jaboticabal-SP) were used. These isolates are part of the Aquatic Organism Pathology Laboratory (Lapoa, Caunesp/Unesp), the Aquatic Animal Disease Laboratory (LENAQ, APTA/Votuporanga) and the Microbiology Laboratory (Private Lab, Jaboticabal-SP). The strains were isolated from animals with clinical signs characteristic of aeromonosis, such as darkening of the dermis, corrosion of the fins, focal haemorrhages, particularly in the gills and opercula, ulcers, and abdominal distension, as characterized by Austin and Austin [11]. The isolates used were previously identified as *A. hydrophila* by Sebastião., *et al.* [12] using 16S rRNA gene sequencing. The strains used in this study are presented in table 1.

Strain	Reference	Organ	Fish	Origin
A111, A114, A120 e A125	Lapoa	Kidney	*Oreochromis niloticus*	SP
127FG	Lenaq	Kidney	*O. niloticus*	SP/PR
231413, 35142A, 201406B, 281442B, 37142A, 42142C, 42141D, 43142C, 49143A, DP09146, DP09148 e MI15141G	Private Lab	Kidney	*O. niloticus*	SP
37142B		Brain	*O. niloticus*	SP
1SIL	KJ561014	Kidney	*O. niloticus*	SP
5SIL	KJ561015	Kidney	*O. niloticus*	SP
A129	KJ561018	Kidney	*O. niloticus*	SP
ATCC7966	KJ561019	Water	-	RJ
A130	KJ561020	Skin	*O. niloticus*	SP
A122	KJ561021	Skin	*Pseudoplatystoma corruscans*	SP
A133	KJ561022	Skin	*O. niloticus*	SP
41FG	KJ561023	Kidney	*O. niloticus*	SP/PR
A128	KJ561024	Skin	*O. niloticus*	SP
A135	KJ561025	Skin	*Cyprinus carpio*	RJ
117FG	KJ561026	Kidney	*O. niloticus*	SP/PR
120FG	KJ561027	Kidney	*O. niloticus*	SP/PR
125FG	KJ561029	Kidney	*O. niloticus*	SP/PR
126FG	KJ561030	Kidney	*O. niloticus*	SP/PR
128FG	KJ561031	Kidney	*O. niloticus*	SP/PR

Table 1: Aeromonas hydrophila strains used in this study. PR: Paraná State; Brazil SP: São Paulo State; Brazil; RJ: Rio de Janeiro State; Brazil.

One colony from each bacterial strain was inoculated in a 2 mL tube containing TSB (Tryptic Soy Broth – Biolife, USA) medium. Cultures were incubated for 18 h (OD_{600} of 1.5) at 28°C. After incubation, 1 mL of bacterial culture was centrifuged at 10.000 x g for 1 minute. The supernatant was discarded, and the pellet was frozen at -20°C for subsequent DNA extraction. An Axyprep® miniprep kit was used to extract bacterial genomic DNA according to the manufacturer's instructions (Axygen Biosciences, Union City, CA, USA). DNA quantification was performed using fluorometry on a QuBit 2.0 system (Life Technologies, NY, USA).

Primer design

The primers used here were previously described by Fukushima., *et al.* [10], with modifications: the reverse primer was redesigned based on the sequence of the *aha*I gene from the strain *A. hydrophila* subsp. *hydrophila* ATCC 7966. Sequences are shown in table 2.

Primer	Sequence	T_m (°C)
*aha*I Forward	GAGAAGGTGACCACCAAGAACA	57.8
*aha*I Reverse	GAGATGTCAGCCTTGTAGAGCT	54.2

Table 2: Primer sequences (5' to 3') used to amplify the gene ahaI in *A. hydrophila*, yielding a 200 bp amplicon.

After manually designing the primers, the sequences were entered into the software Primer Express 3.0 to verify possible formation of simple dimers, primer-dimers and hairpins and to determine melting temperatures.

Real-time qPCR amplification

Real-time qPCR was performed using a thermocycler for real-time PCR (Applied Biosystems - Model Real time - 7500) and the marker used was SYBR Green I dye (Applied Biosystems, Life Technologies Ltd of Brazil). Amplification reactions were performed in 20 µL volumes consisting of 1 µL of each *aha*I primer (F and R), 10 µL of PCR SYBR Green I master mix, 2 µL of DNA and 6 µL of DEPC (Diethyl Pyrocarbonate) water. The PCR program consisted of an initial step at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15s and annealing at 60°C for 1 minute. At the end of each cycle, a DNA melting curve of the amplified products was performed between 65°C and 95°C to evaluate the melting temperature (Tm); readings were taken at 0.1°C increments. All samples were tested in triplicate.

A standard curve was generated from the synthetic gene Gene_aha_4490401, which was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pMA-T using SfiI and SfiI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. The *aha*I gene sequence is as follows: GAGAAGGTGACCACCAAGAACAAGTTCAAGTGGCCACTGGTAGGAGAAACCGAACTCTCCATCGAGATTGCGGCCAACCAGTCCTGGGCATCCCAGAACGGGGGCTCTACCACCACCTCCCTGTCGCAATCCGTGCGGCCAACTGTGCCGGCCCGCTCCAAGATCCCGGTGAAGATCGAGCTCTACAAGGCTGACATCTC (GeneArt® Gene Synthesis, Life Technologies, USA). The synthetic DNA was dissolved in RNase-free distilled water according to the

manufacturer's instructions and serially diluted (1:10) from 1 ng to 100 fg. The standard curve was generated using the same reaction conditions as the SYBR Green assay described above.

Analytical specificity test

The specificity of the assay for *A. hydrophila* was assessed by submitting DNA from 16 other bacterial species responsible for bacterial diseases commonly found in fish farms to the same qPCR reaction conditions described in Section 2.3. The following species were tested: *Acinetobacter johnsonii* (KJ561079), *Acinetobacter ursingii* (KJ561082), *Aeromonas jandaei* (KJ561032), *Aeromonas punctata* (KJ561034), *Aeromonas veronii* (KJ561046), *Citrobacter freundii* (KJ561090), *Citrobacter murliniae* (KJ561094), *Edwardsiella tarda* (KJ560940), *Enterococcus casseliflavus* (KJ560953), *Lactococcus garviae* (KJ560983), *Lactococcus lactis* (KJ561000), *Pseudomonas fulva* (KJ561097), *Pseudomonas nitroreducens* (KJ561103), *Streptococcus agalactiae* (KJ561059), *Streptococcus iniae* (KJ561076) and *Stenotrophomonas maltophilia* (KJ561111). *A. hydrophila* (KJ561025) was used as a positive control.

Analytical Sensitivity Test

DNA of *A. hydrophila* was serially diluted by a factor of 1:10 using nuclease-free water, with concentrations ranging from 1 ng to 100 fg. The regression curve was constructed by comparing the number of LOG gene copies and the Ct (cycle threshold) values. The slope values and R^2 were calculated based on the linear regression of the constructed standard curve. The conversion units (pg of DNA) for the amount of gene copies per µL was performed according to the below formula. The synthetic clone of *A. hydrophila* is known to be 2580 bp in size.

$$\text{Number of copies} = \frac{X}{\text{Clone size bp x } 649} x6{,}022\ x\ 10^{23}$$

Observations: Clone = plasmid + insert; 649 g/mol = MW of 1bp DNA; 6.022×10^{23} = Avogadro number; X = g/µL DNA.

Experimental infection assay

All procedures were approved by the Ethics and Animal Welfare Committee of the School of Agricultural Sciences and Veterinary Medicine of São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil under protocol number 011752/12.

Experimental Design

Sixty juvenile Nile tilapia, *O. niloticus* (19.7 ± 0.5 g, n= 60) were acquired from a commercial fish farm located in Porto Ferreira (SP). Ten percent of the batch was previously tested for *Aeromonas hydrophila* absence with the described Real-time qPCR methodology. Then, fish were equally distributed (3 fish per tank) into 20 43-L aquariums. Each aquarium was supplied with an external filter and aeration. Siphoning (in which one-quarter of the volume of water was exchanged) was performed every two days. A commercial diet containing 35% crude protein (Fri-Aqua, Trown Nutrition) was provided twice a day. Feed equivalent to 1% of the biomass was supplied. To acclimatize the fish, the temperature in the test room was maintained at 28°C for 7 days; the water temperature was 25°C ± 1. After the fish were infected with *A. hydrophila* strains, the temperature in the test room was maintained at 28°C during the day and at 21°C at night.

A. hydrophila experimental infection

Twenty *A. hydrophila* strains (231413, 126FG, A129, ATCC7966, 41FG, 117FG, 120FG, 125FG, A122, A111, A135, A120, A128, 35142A, A125, A133, 1SIL, 128FG, A130 and 127FG) were selected for this study. Bacteria were cultured in TSB medium (Tryptic Soy Broth, HIMedia, India) and centrifuged at 7000 x g for 30 min. The pellet was resuspended in 0.85% saline and centrifuged again at 7000 x g for 30 minutes. Bacterial suspensions were adjusted to a concentration of 1.5×10^7 CFU/mL (as determined from previous LC50 studies). For each strain, 100 µL of bacterial suspensions were injected intraperitoneally (fish were anesthetized with 1 mL/L clove oil per tank prior to injection).

Moribund fish showing hemorrhagic areas on their epidermis and fin corrosion were euthanized by thermal shock (20 - 30 minutes on ice). Ten milligrams of caudal kidney tissue were collected aseptically for DNA extraction. Kidney tissue was plated on TSA (Tryptic Soy Agar, HIMedia, India) to re-isolate bacteria. The same procedure was performed on fish that survived after seven days.

DNA extractions directly from the kidney tissue of tilapia experimentally infected with *A. hydrophila* were performed using the DNeasy Blood and Tissue kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. DNA integrity was verified on a 0.8% agarose gel and quantified by fluorometry (QuBit 2.0, Life Technologies, NY, USA). Genomic DNA was also extracted from re-isolated strains as described in Section 2.1. The same gene amplification reaction conditions and real-time thermocycler programs as described in Section 2.4 were used. The initial DNA concentration of tissue samples was 3 ng/µL.

Results

The optimization phase was performed *in vitro* cultured bacteria, the primer concentration that showed the lowest Ct and greater ΔRn was 800/800 nM. This concentration was selected, although a small NTC (no template control) resulted in dimer formation, as shown in figure 1. The presence of dimer formation in tests with SYBR Green is very common and difficult to avoid because SYBR Green can intercalate double-stranded DNA and detect nonspecific products [13]. A distinction could be easily made using the dissociation temperature, which on average was 86°C for A. hydrophila but 72°C for the dimer.

The Standard curve was built to test the efficiency of the primers and was accomplished by serially diluting (1:10) the plasmid DNA from *A. hydrophila* from 1 ng to 100 fg. The slope value was -3.507, the R^2 value was 0.999, and the efficiency was 92.824% (Figure 2).

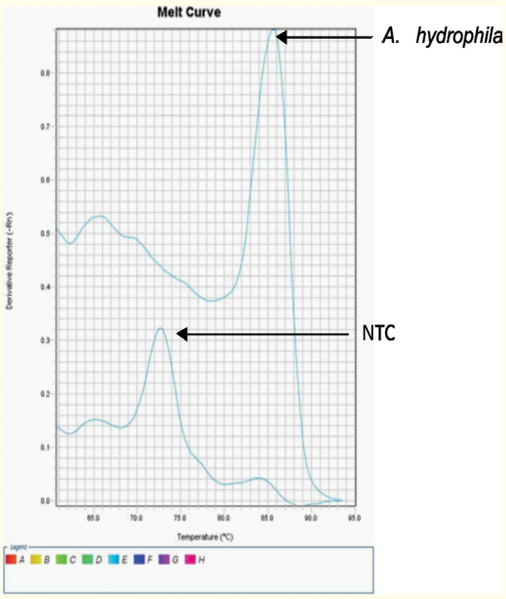

Figure 1: Dissociation curve showing the melting temperature of *Aeromonas hydrophila* and dimer formation in the NTC (No template control).

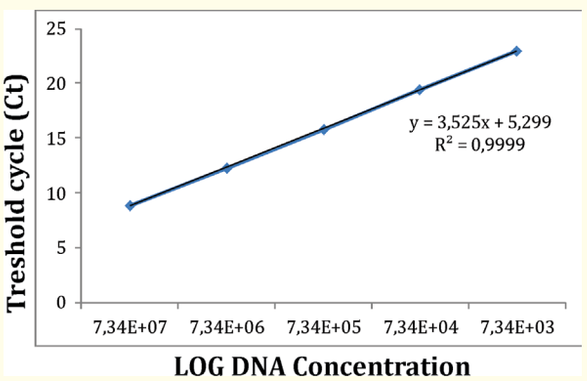

Figure 2: Standard curve generated from serial dilutions of plasmid DNA from *Aeromonas hydrophila* in the real-time qPCR assay. The data indicates the ratio between the copy number of the ahaI gene and the cycle threshold (Ct). Plasmid DNA from *A. hydrophila* was serially diluted from 1 ng to 100 fg and amplified with SYBR Green I.

The *A. hydrophila* isolates analyzed had varying levels of *aha*I transcript present (Figure 3). Of these measurements, 42.4% (n = 14) have a mean C_t value of 17, mean quantification of 5.9 gene copies (in log scale) and an expected melting curve temperature of 86°C. In contrast, amplification of *aha*I in 57.6% (n = 19) strains was delayed, with a mean C_t value of 31 and concentrations of 1.9 gene copies, a mean T_m of 86°C was maintained for these reactions, which implies that those samples are positive reactions but with moderate amounts of target nucleic acid.

The expected size of the PCR amplicons was confirmed by agarose gel electrophoresis. Figure 4 shows the 200 bp bands from *A. hydrophila* strains.

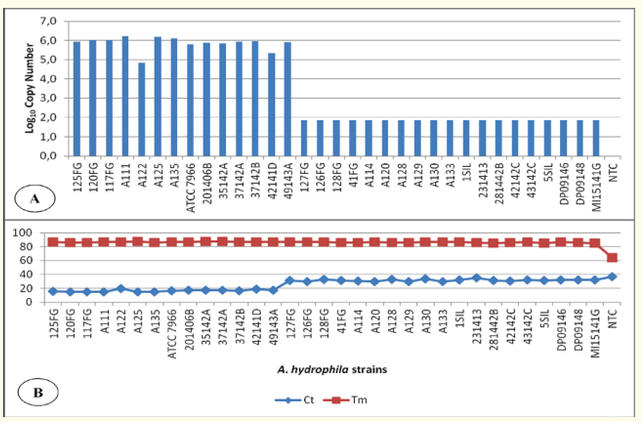

Figure 3: Quantification of *ahaI* gene of *Aeromonas hydrophila* strains in log scale (A). Cycle threshold (Ct) and Melting Temperature (Tm) of the same strains (B). NTC: no template control.

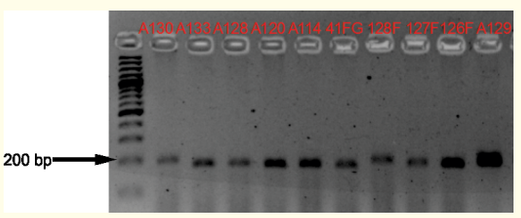

Figure 4: Agarose gel (3%) stained with ethidium bromide showing the amplification of the ahaI gene from *Aeromonas hydrophila* (A130 to A129). Molecular size marker: 1 kb plus (Invitrogen®).

The analytical specificity test was successful, as bacterial DNA other than *A. hydrophila* was not amplified. The Ct values of these reactions were above 33 and had variable T_m values, also multiple peaks were observed, as shown in table 3.

The detection limit in the analytical sensitivity test of the selected primer pair was 20 fg (3.8 gene copies in log scale) for DNA from the bacterial isolates. At this concentration limit, no primer-dimer formation was observed.

Sample	Species	Quantification (pg)	Mean C_t	T_m (°C)
KJ561025	*Aeromonas hydrophila*	2.04	15.18 ± 0.04	86.08
KJ561079*	*Acinetobacter johnsonii*	0	34.93 ± 1.70	80/64
KJ561082	*Acinetobacter ursingii*	0	34.99 ± 1.50	73
KJ561032*	*Aeromonas jandaei*	0	32.91 ± 0.51	72/86/76
KJ561046*	*Aeromonas veronii*	0	31.82 ± 0.40	86/66
KJ561090	*Citrobacter freundii*	0	34 ± 1.20	73
KJ561094	*Citrobacter murliniae*	0	35 ± 1.74	72
KJ560940	*Edwardsiella tarda*	0	35.51 ± 1.35	73
KJ560953*	*Enterococcus casseli-flavus*	0	34.52 ± 1.97	86/66
KJ560983*	*Lactococcus garviae*	0	33.90 ± 0.62	73/86
KJ561000*	*Lactococcus lactis*	0	33.63 ± 0.92	66/73/75
KJ561097	*Pseudomonas fulva*	0	32.93 ± 0.77	73
KJ561103	*Pseudomonas nitrore-ducens*	0	34.64 ± 0.49	73
KJ561111	*Stenotrophomonas maltophilia*	0	34.74 ± 1.22	72
KJ561059*	*Streptococcus agalactiae*	0	30.89 ± 0.30	72/86
KJ561076*	*Streptococcus iniae*	0	28.60 ± 0.16	86/72
NTC	water	0	34.8	77.25

Table 3: Specificity assay of the A. hydrophila adhesin gene ahaI using a standard curve and DNA isolated from different piscine species. Mean threshold cycle (Ct) and melting temperature (Tm). A2 to A6: standard curve points; *Samples showing multiple peaks

Experimental infection

Forty-eight hours after the experimental infection, all the fish showed signs of corrosion of fins, red spots and skin darkening (Figure 5). During necropsy, the kidneys had a hemorrhagic-like appearance.

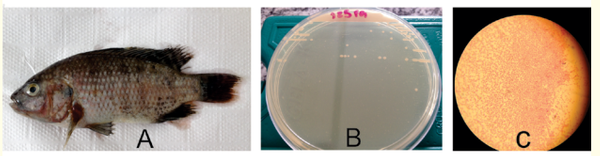

Figure 5: Nile tilapia with clinical signs of aeromonose at 48 hours after experimental infection with *Aeromonas hydrophila* strain 125FG. External clinical signs were as follows: corrosion of the fins and hemorrhagic spots on the epidermis (A). TSA plate containing *A. hydrophila* colonies re-isolated from kidney tissue (B). Gram-negative bacilli at 1000X magnification (C).

Strains of the bacteria could be re-isolated from kidney tissue on TSA after experimental infection and were identified by the described real-time qPCR of this study.

When quantification was performed using DNA extracted directly from the kidneys of infected fish (2 samples per aquarium), the concentration of *aha*I transcript was found to be 1.9 gene copies in log scale on average (Figure 6). Average C_t values were 31 and T_m was 85.5°C. Due to the low concentration of *A. hydrophila* present in the experimentally infected tilapia, it was not possible to detect its presence using conventional PCR, because the amount of bacterial DNA required for conventional PCR was not reached when DNA extracted directly from the kidneys. Notably, the detection of *aha*I in tilapia´s kidney experimentally infected with *A. hydrophila* was only possible using real-time qPCR.

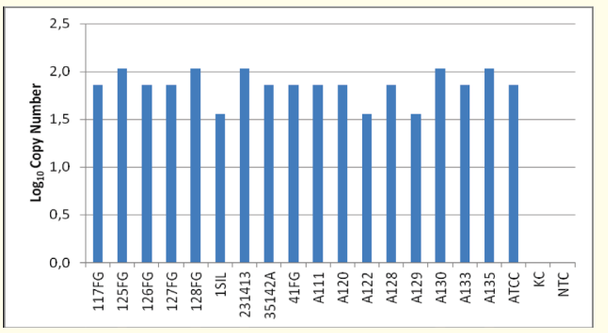

Figure 6: Quantification of the adhesin gene *ahaI* from DNA extracted directly from the kidneys of tilapia experimentally infected with various *A. hydrophila* strains in log scale gene copies. KC (kidney control) corresponds to DNA extracted from the kidneys of healthy tilapia. NTC (no template control).

Discussion

Currently, the genus *Aeromonas* comprises 25 species [14] although *A. hydrophila* is the most frequently isolated species in fish farms worldwide (61.5%), according to Rodrigues [15], it is necessary to make the correct distinction between species for a quick and accurate diagnosis that leads to appropriate treatment. Molecular techniques such as real-time qPCR appear as the alternative less labour intensive, more cost-effective and easily adapted for high-throughput processing when compared to methods such as culture, serology and conventional PCR [16]. In this study, an absolute real-time qPCR for *A. hydrophila* was developed based on the adhesin (*aha*I) gene. Moreover, an *in vivo* infectivity trial of various strains of *A. hydrophila* was performed in tilapia to validate the developed diagnosis methodology.

Following initial assay optimization, specificity and sensitivity of the assay was undertaken. Primer set was found to be 100% specific for the *aha*I gene present in the 33 *A. hydrophila* strains obtained from various Brazilian regions analyzed in this study, and the 16-other species evaluated, including *A. veronii, A. punctata* and *A. jandaei*, which did not amplify. The analytical sensitivity of the assay for purified DNA was 20 fg (3.8 log gene copies), comparable to the literature published [16,17].

The DNA intercalate used was SYBR Green which is not gene specific. Therefore, even the smallest amount of contamination in the water or the presence of dimers will produce fluorescence that can be misinterpreted as amplification [18]. Controls measuring the formation of primer-dimers were performed by analyzing the melting curve after the PCR reactions. As primer-dimer products are typically smaller than the target product, they dissociate at lower temperatures and are easily recognized in melting curve analyses [19]. It should be noted that the melting temperature of the positive sample of *A. hydrophila* in this study was 85 ± 1°C; the melting temperature of the negative control was below 72°C. This significant difference in melting temperatures increases the likelihood of distinguishing primer-dimers from the target product.

Koch´s postulate was also demonstrated: the clinical signs and gross pathological changes described from the affected tilapia from the experimental trial are in agreement with previous reports [20-22].

As the objective of the described methodology was to eliminate the need for bacterial isolation in order to decrease the time for diagnosis, DNA was extracted directly from the kidney tissues of the experimentally infected tilapias. Successfully, the aeromoniose diagnosis could be acquired within four hours (1.5 hours for DNA extraction and 2.5 h for real-time qPCR). In this assay, the detection limit was 1.9 log gene copies. It is known that one of the technical limitations of real-time qPCR is the detection of products at extremely low concentrations (1 genome copy, for example). These issues can be resolved by substituting non-specific intercalating agents by specific ones, although more expensive, such as the TaqMan system [16,23] or other molecular technique like digital PCR [24].

This method significantly reduced the time required compared to conventional PCR, which normally takes 3 days to complete, because of its dependency on bacterial isolation. Real-time qPCR also decreased the cost of analysis per sample by 26% (excluding equipment and skilled labor costs, in Brazil) compared to conventional PCR [25].

Conclusions

We have demonstrated that real-time qPCR system is specific and sensitive for the detection *A. hydrophila* in fish, which constitutes a valuable tool for the diagnosis of aeromoniose. This technique reduces time and costs compared to traditional microbiology methods and conventional PCR. It can be readily adopted for a variety of purposes including diagnostic testing, health certification, measure the efficacy of vaccines and antibiotic therapy. Such data can also assist in epidemiological studies aimed at identifying factors that predispose populations to disease outbreaks.

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