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Using a real-time quantitative polymerase chain reaction (PCR) method for reliable enumeration of *Aeromonas hydrophila* in water samples

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Aeromonas hydrophila is **an opportunistic pathogen of human and animals with a multifactorial pathogenesis. Control of *A. hydrophila* numbers in water is recommended for water quality estimation. Consequently, we developed a SYBR Green method for rapid quantification of *A. hydrophila,* targeting three genes: 16SrRNA gene, the cytolytic enterotoxin gene (*aerA*) and the heat-stable cytotonic enterotoxin gene (*ast*). The sensitivity and the specificity of the method were tested using 34 positive and negative controls. The improved level of detection was established with serial dilution of genomic DNA of type strain CIP 7614T. Linear relation between Ct-value and bacterial cell concentration were obtained. The limit of detection found in this study corresponds to 10 cells of *A. hydrophila*. Finally, the method was tested on seven artificially contaminated waters and on 30 unknown water samples. A significant similarity was observed when comparing results of SYBR Green method (concerning specially the 16SrRNA gene) with microbiological enumeration. Only 13% of natural samples were contaminated with *A. hydrophila*. The qPCR protocol developed in this study allows quantifying of *A. hydrophila* in water samples with a good level of detectability.**

Key words: *Aeromonas hydrophila*, water, real time PCR, SYBR Green.

INTRODUCTION

The genus *Aeromonas*, which is comprised of 22 species (Janda and Abbott, 2010), was recently classified as an independent family: *Aeromonadaceae* (Suarez et al., 2008). *Aeromonas* spp. are facultative anaerobic, Gram negative, oxidase positive and rod shaped bacteria (Popoff, 1984). They are widely isolated from fresh, chlorinated, marine and river water. Furthermore, they can be detected in food and clinical samples in which they can survive even at low temperature (Isonhood and Drake, 2002; Janda and Abbott, 2010).

Among the different species of *Aeromonas*, *Aeromonas hydrophila* is commonly associated with a large spectrum of human infections including gastroenteritis, septicaemia, skin and wound infections which are often fatal for young, elderly or immunocompromised individuals (Fraisse et al., 2008). It can affect several species of fish causing haemorrhagic septicaemia, a major freshwater disease affecting aquaculture worldwide (Paniagua et al., 1990; Rodriguez et al., 2008).

A. hydrophila is listed in the first and second contaminant candidate list (CCL 1 and CCL 2) of potential waterborne pathogens (US EPA, 2006). The European Community established a norm, fixing the maximal concentration of *A. hydrophila* as 20 Colony-Forming Unit (CFU)/100 ml of water leaving the proces-sing plant and 200 CFU/100 ml in distribution World Health Organization re-gisters *Aeromonas* in the Fourth Edition of Directives for Quality of Drinking Water System (Figueras and

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Table 1. Primers used for PCR detection and quantification of putative virulence genes in *Aeromonas* isolates. Melting temperatures (Tm) were obtained after analysis of control DNA extracted from *A. hydrophila* CIP 7614T.

Targeted gene	DNA sequence primer (5'-3')	Length of sequence (bp)	Tm (°C)	Reference
16SrRNA	F: 5'GGCCTTGCGCGATTGTATAT 3' R: 5' GTGGCGGATCATCTTCTCAGA 3'	103	81.1	Trakhna et al., 2009
Heat stable enterotoxin gene *ast*	F: 5'CGGCCCGGTCTACTACCA 3' R: 5'TGACCCCCTGATCCTTGATG 3'	65	79.7	This study
Aerolysin gene *aerA*	F: 5'CAAGGCTGATATCTCCTATCCCTATG 3' R: 5' GCCACTCAGGGTCAGGTCAT 3'	67	76.8	Trakhna et al., 2009

Borrego, 2010; Moyer, 1999; Van der Kooij, 1993; Villari et al., 2003). These values are based on the prevention and not on the impact of their presence in the drinking water for public health. The Standard methods for quantification of these pathogens require enrichment culture and subcultures on selective agar followed by phenotypic identification for up to 48 h (WHO, 2011). The Canadian procedure "MFLP-58B" for enume-ration of *A. hydrophila* on ice and water using a filtering membrane, require up to 48 h and three media to confirm the identification (Warburton, 1999). The introduction of nucleic acid-based methods such as the polymerase chain reaction (PCR) may allow to spare time and to detect an infinitesimal quantity of bacteria. PCR is a rapid, sensitive and specific technique and it has been used for the detection of pathogens like *Salmonella* (Nam et al., 2005), *Vibrio vulnificus* (Panicker and Bej, 2005), *Campylobacter jejuni* (Sails et al., 2003). PCR was also used for detection of *Aeromonas* spp. (Khan et al., 2009; Kingombe et al., 1999) and for the detection or charac-terization of virulent genes of *A. hydrophila* (Trakhna et al., 2009; Wang et al., 2003).

In our previous study (Trakhna et al., 2009), we esta-blished a technique just for the detection of *A. hydrophila* by using probes. Wang et al. (2008) has also elaborated a PCR triplex for the detection of this bacterium. In both works, the Taq-man-based real-time PCR assay was used and the detection was based only on the DNA concentration. In addition, The PCR assay developed by Wang et al. (2008) was specific only for pathogenic strains of *A. hydrophila*.

The aim of this study was to develop a method for *A. hydrophila* quantification (based on bacterial number) in water using a real time PCR, while minimizing the cost of analyses by using the chemistry SYBR Green instead of probes. This SYBR Green PCR is targeting segments of 16SrRNA specific for this species as well as *aerA* and *ast* genes to determine toxin-production genes.

After a validation of this method on pure DNA and pure culture, we analysed water sample using PCR and compared the results with those obtained by classical filtering membrane method.

MATERIALS AND METHODS

Bacterial strains

The bacterial isolates and the type strains used in this study are listed in Table 2. They are divided in three groups: 6 strains were obtained from Pasteur Institute collection, 21 were isolated from water samples and 6 clinical strains isolated from patients in the Strasbourg University Hospital by Dr Harf-Monteil (Trakhna et al., 2009). All bacteria were cultivated separately in 9 ml of Brain Heart Infusion medium (BHI, Difco) overnight at 30°C.

Natural and artificially contaminated water samples

A total of 30 samples were collected from distinct geographic sites in France, Sweden and Tunisia. For each sample, 500 ml of water were collected in sterile bottle and were stored at 4°C until analysis. Three of those samples were natural mineral water representative of water brands sold all over France. These samples were purchased at retail outlets in Beauvais (France). Seven samples of sterile water (500 ml) were contaminated with different concen-tration of *A. hydrophila*. Therefore, an overnight culture of the CIP 7614T strain was prepared, then, different volumes were added randomly to 500 ml of sterile water. One hundred milliliter of each sample were used for DNA extraction and PCR quantification, and 300 ml for microbiological enumeration (analysis were realised in triplicate).

Microbiological enumeration of *A. hydrophila*

One hundred milliliter of natural water samples and contaminated water were analysed in triplicate by the standard membrane filtration technique using 0.45 µm pore size membrane filter in a selective media: Ryan medium (Holmes and Sartory, 1993). All plates were incubated at 37°C and colonies counted after 24 h incubation. Presumptive *A. hydrophila* were defined as green colonies with a black center on Ryan medium. *A. hydrophila* iso-lates (5 colonies by sample) were confirmed using tests for oxidase, catalase reactions and finally with API 20NE gallery (Biomérieux, France).

DNA extraction

To study the sensitivity of primers, we used the DNA of the CIP7614T strain. This Purified genomic DNA was extracted using the "Promega wizard DNA purification KIT" (Promega, France), according to the manufacturer's instructions using 1 ml of broth culture. Serial 10-fold dilutions from 100 ng to 10 fg per 25 µl of

Table 2. Ct values obtained for different species in the SYBR Green assay with the three target sequences 16S rRNA, *aerA* and *ast*.

Strain	Origin	Species	Ct value		
			16S rRNA	*ast*	*aerA*
***Aeromonas hydrophila* strains**					
CIP7614	Pasteur Institute Collection	*A. hydrophila*	+	+	+
CIP107274	Pasteur Institute Collection	*A. hydrophila*	+	+	+
CIP103561	Pasteur Institute Collection	*A. hydrophila*	+	+	+
VM	Intestinal organ of Mackerel	*A. hydrophila*	+	-	-
C1S	Salad (Lebanon)	*A. hydrophila*	+	-	+
PDC	Environmental sample (France)	*A. hydrophila*	+	-	-
JT	Environmental sample (France)	*A. hydrophila*	+	-	-
MBF	Environmental sample (France)	*A. hydrophila*	+	+	+
TUEI	Environmental sample (Tunisia)	*A. hydrophila*	+	+	+
PU6	Environmental sample (Tunisia)	*A. hydrophila*	+	-	+
TUA	Environmental sample (Tunisia)	*A. hydrophila*	+	+	+
603270765	stool	*A. hydrophila*	+	+	-
B13960	abscess	*A. hydrophila*	+	-	+
T64505	Blood culture	*A. hydrophila*	+	+	+
PAL	Aquatic plant(Lebanon)	*A. hydrophila*	+	+	+
***Aeromonas* non *hydrophila* strains**					
PREF	Fountain (France)	*A. caviae*	-	-	-
RI	Irrigation reservoir (France)	*A. sobria*	-	-	-
TUB	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUB2	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUP1	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUB3	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUB4	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUB5	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUL1	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUM1	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUM3	Environmental sample (Tunisia)	*A. sobria*	-	-	-
TUL2	Environmental sample (Tunisia)	*A. sobria*	-	-	-
606300204	Stool	*A. sobria*	-	-	-
B15650	Bile	*A. sobria*	-	-	-
608030635	Blood culture	*A. sobria*	-	-	-
Negative strains					
CIP 54:8	Pasteur Institute Collection	*Escherichia coli*	-	-	-
PsCEP	Onion	*Pseudomonas cepacia*	-	-	-
CIP75.2	Pasteur Institute Collection	*Vibrio parahaemolyticus*	-	-	-
CIPA232	Pasteur Institute Collection	*Proteus vulgaris*	-	-	-
Sterile water	-	-	-	-	-

Ct value < 35: +; Ct value >35 : -.

PCR reaction were amplified with SYBR Green assay, in triplicate. For water samples and standard curves construction DNA was extracted, by boiling method. Standard curves were established to quantify and determine the level of detectability of *A. hydrophila*. DNA was extracted using 1ml of a 10 serial dilutions of *A. hydrophila* culture. While, for the water samples (contaminated or natural), 100 ml, was used for DNA extraction. In both case, the cellsuspension was centrifuged for 10 min at 10 000 × *g*. The supernatant was discarded carefully. The pellet was resuspended in 100 µl of distilled water. The suspension was incubated for 10 min at 95°C and immediately ice-cold. The tube was centrifuged for 3 min at 3 000 ×*g* at 4°C. An aliquot of 5 µl of the supernatant was

used as the template DNA in the PCR. The concentration of DNA suspension was measured by spectrophotometry (biophotometer-plus Eppendorf). DNA was extracted from each strain and subjected to the real time PCR assay. PCR products sizes were confirmed by gel electrophoresis analysis (data not shown).

PCR primers

Primers used for quantification of A. hydrophila are listed in Table 1. Two primer sets were already designed: a species-specific region of the 16SrRNA *A. hydrophila* and a virulence (*aerA*) gene witch possess hemolytic, cytolytic and enterotoxic activities (Trakhna et al., 2009). A third sequence of the virulence gene encoding heat-stable cytotonic enterotoxin *ast* was added. Its detection is important for the industry, especially for the transformation of sea products.

To design specific primers for the SYBR Green assay, a multiple alignment analysis of sequences of the *ast* gene deposited in the NCBI GenBank database was performed using CLUSATLW software (Thompson et al., 1994). Then, PCR primers were designed from the output regions, using the primer express software version 2.0 (Applied biosystems, Foster City, CA). To ensure their specificity, primer sequences were searched with the BLAST program and synthetised by Eurogentec, France.

qPCR conditions

Real time PCR and data analyses were performed with the ABI PRISM SDS 7300 (Applied Biosystem). All reactions were performed in duplicate in 25 µl reaction volumes of universal master mix (Applied Biosystems) containing dUTP, uracil-N-glycolase and AmpliTaq Gold polymerase 12.5 µl; milliQ water 6 µl; forward primer 0.75 µL; reverse primer 0.75 µl and target DNA 5 µl. Standard amplification parameters were as follows: 50°C for 2 min for optimal AmpErase uracil-N-glycosylase activity and 95°C for 2 min to activate hot start AmpliTaq Gold polymerase, followed by 40 cycles, each of which included 95°C for 10 s and 60°C for 30 s. A final stage of dissociation is required for the PCR SYBR Green which includes 15 s at 95°C followed by 30 s at 60°C then 15 s at 95°C. All reactions were realised with a negative (water) and a positive control.

Statistical analysis

The qPCR and plating data were analyzed with Microsoft Excel® Data Analysis Tool-Pak to test for differences in means with one-way ANOVA at a significance level of 0.05.

RESULTS

Specificity of real time PCR assay

Fifteen strains of *A. hydrophila* were included in the test of specificity of the primers against 14 *Aeromonas* non *hydrophila* species and 5 bacteria belonging to other families. For the 5 bacteria belonging to the other families (negative control), we had no positive results (no signal in 40 cycles) for the three genes. While, all *A. hydrophila* strains showed a positive result for 16SrRNA sequence. Results obtained with *aerA* and *ast* genes sequences were variable. In fact, we had positive results only for 9 of 15 (60%) for *ast*and and 11 of 15 (73%) for *aerA* (Table 2).

For *Aeromonas* non *hydrophila* strains, we obtained negative reactions for 16SrRNA sequence (no signal in 40 cycles). For the other two genes, we had obtained late duplication with CT values > 35; consequently, we had conceded that amplification is positive when a Ct value < 35. Above Ct value =35, results were unsure and it will be recommended to test a higher sample concentration (10 or 100-fold concentred) to confirm the result. However, it seems that some other *Aeromonas* than *hydrophila* own a part of *ast* and aerolysin genes.

Standard curves and amplification efficiencies for pure DNA

To determine the sensitivity of a SYBR Green assay, a pure extract of *A. hydrophila* DNA (CIP7614T) was used as a template. The amplification curves were linear in the range between 100 ng to 1 pg (Figure 1). The lower limit of quantification for the assay was set to be 1 pg per reaction. The slopes obtained were -3.29, -3.21 and -3.43 for 16SrRNA, *aerA* and *ast* respectively and consequently, very high efficiencies (> 95%) were obtained after calculation according to (Ibekwe et al., 2002). Based on the standard curves and the limit of detection of this assay, negative results were defined as those exhibiting CT values higher than 35.

After amplification, a melting curve analysis of the amplified fragment was carried out. The melting curves of *A. hydrophila* strains (Figure 2) were clearly different from those observed for the non-*hydrophila* strains (with only a weak signal). Thus, real-time PCR combined with melting curve analysis enabled the discrimination of *A. hydrophila* and non-*hydrophila* strains. For conformational reasons, the amplified products were analysed on agarose gel (3%) and we did not find any primer dimer (results not shown)

Real time PCR results of quantified genomic DNA of *Aeromonas hydrophila*

The standard curves were generated using mean Ct values for various concentrations of *A. hydrophila* CIP7614T. The slopes of standard curves were -3·33, -3·47 and -3·34 for 16SrRNA gene, *aerA* and *ast* respectively (Figure 3). The standard curves showed a linear correlation between Ct values and cell numbers ranging from 10^8 to 10^1 cells; the amplification efficiency was 99.7% for 16SrRNA, 99.3% for *aerA* and 94.2% for *ast* genes (calculated according to (Ibekwe et al., 2002)). The results show that the quantification limit of the SYBR Green assay was 10 cells.

Quantification of *A. hydrophila* number in water

Previous to environmental analysis, artificially contaminated samples (n=7) were enumerated by filtration and

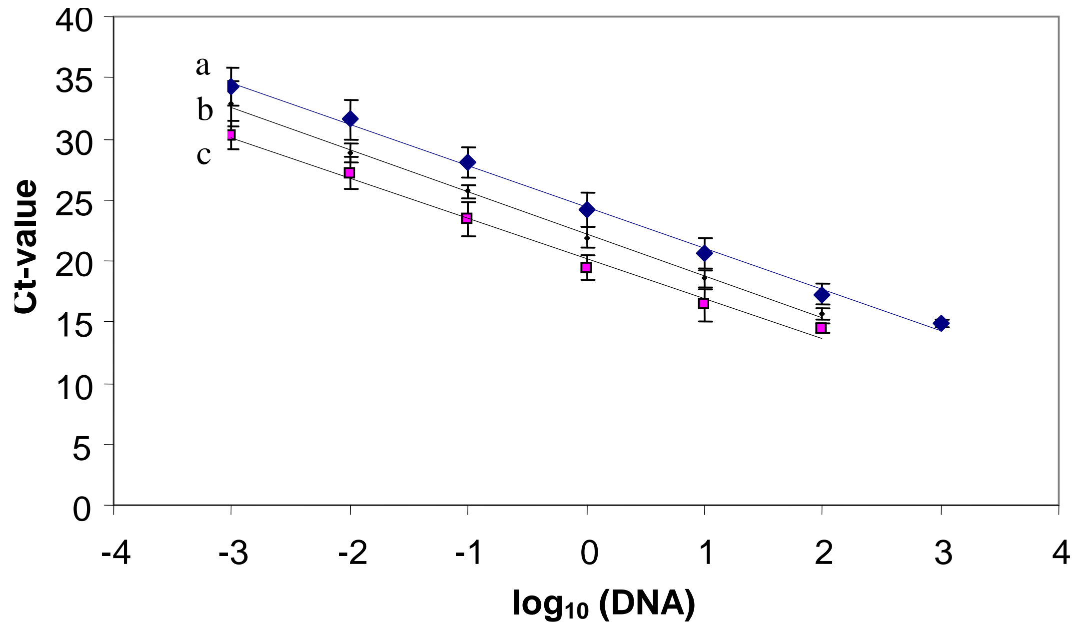

Figure 1. Amplifications of 10-fold serial dilutions of *Aeromonas hydrophila* genomic DNA in SYBR Green real time PCR. Standard curves plotted for log_{10} DNA concentrations versus the number of cycles required to reach the Ct, based on the mean of triplicate samples.
a: *aerA*, **b:** *ast,* **c:** 16SrRNA gene.

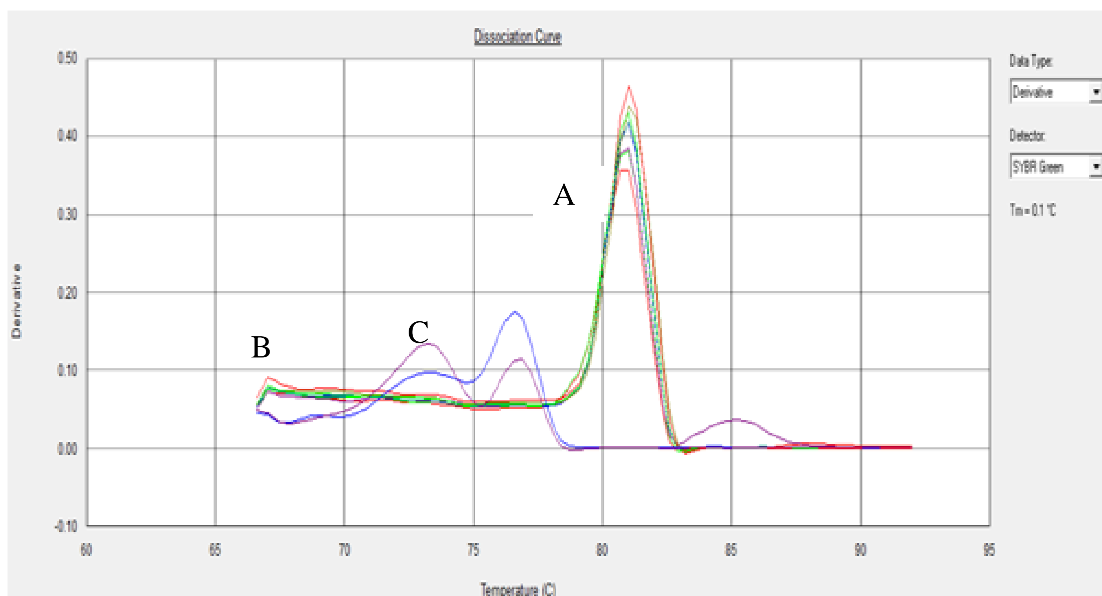

Figure 2. Exemple of melting curve analysis of *aerA* amplicons of different dilutions of the CIP 7614T strain (A) and non Aeromonas hydrophila strains (B and C)

PCR methods. All PCR results were very close to classical method and statistical ANOVA analyses did not show any significant difference (P > 0.05). However, when the bacterial concentration was low, standard error was higher. In order to evaluate the SYBR Green assay developed in the present study, the number of *A. hydrophila* in 30 unknown water samples were measured using the PCR assay and the membrane filtration enumeration method followed by identification with API gallery. The results are presented in Table 3.

In environment samples, the population of *A.*

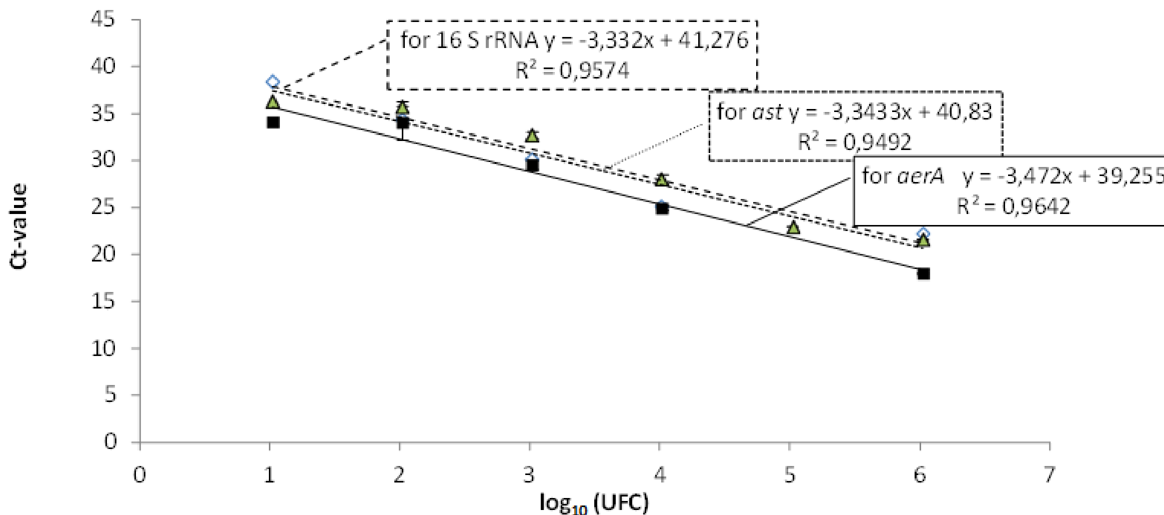

Figure 3. Standard curves for the culture of *Aeromonas hydrophila* ; Ct values were plotted against $\log_{10}$ cell numbers in a CIP7614T culture ◇16S rRNA, △: *ast*, b ■: *aerA*

hydrophila, estimated with the PCR assay using the primers of 16SrRNA sequence, was generally similar to that obtained with the membrane filtration method (identification was confirmed by Api gallery analysis). Results obtained with the other two genes (*ast* and *aerA*) were not specific and not quantitative for *A. hydrophila*. In these samples, classical microbial analyses have shown the presence of other *Aeromonas* species such as *Aeromonas sobria* (Table 3).

DISCUSSION

Real time PCR has been shown very useful in detection and quantification of pathogenic bacteria in different media: food products, clinical samples and water. In a recent work (Trakhna et al., 2009), we have established a method for rapid identification of *A. hydrophila* using a TaqMan assay with 16SrRNA gene and *aerA* genes. In this paper, we tried to establish quantification and less cost method using a SYBR Green assay.

The primer sets for 16SrRNA gene analysis used in this assay provided a good level of specificity for the *A. hydrophila* species. In fact, the sequence targeted seems specific to the species. This was demonstrated by using a variety of non *A. hydrophila* species. For the two others targeted genes (*aerA* and *ast*), the Ct-values obtained for others than *Aeromonas* genus are negative. However, late amplifications were obtained for some *A. sobria* and *Aeromonas caviae* and this observation seems to be frequent when bacterial strains were very close. Indeed, Singh and Sanyal (1992) and Kingombe et al. (1999) demonstrated that the *aerA* gene which codes the structural protein is present at all the strains of *Aeromonas* and that the conditions which end in the expression remain to clarify. Albert et al. (2000) detected the *ast* gene in different species of *Aeromonas*.

The identity of the PCR product from a sample can be confirmed by performing a melting curve analysis comparing its melting temperature with Tm of the product from the positive control. Nam et al. (2005) developed a qPCR for detection of *Salmonella* from environmental water samples and obtained a Ct-value mean of 37.9 ± 2.9 for the detection of non *Salmonella* strains.

The amplification of these two toxin genes (*aer* and *ast)* can be used besides for detection of potential virulent strains of *Aeromonas* spp. For the specific virulent *A. hydrophila* detection and quantification they must be used in combination with the 16SrRNA gene.

In agreement with previous studies (Albert et al., 2000; Ottaviani et al., 2011), we found a high heterogeneity in the distribution of toxin genes among the tested strains. The two genes seem to be conserved among the genus. Indeed, Chacon et al. (2003) confirmed that aerolysin gene is more frequent in clinical than environmental isolates of *Aeromonas* spp. This result is in concordance with our results and explains the fact that we did not detect the two virulence genes in all *A. hydrophila* species.

The population of *A. hydrophila* in water samples was both detected by the SYBR Green assay developed in this study and the membrane filtration enumeration method. Detection and quantification after the 16SrRNA sequence amplification is very close to classical and filtration method and is validated. The detection limit of the SYBR Green assay in bacteria culture and in artificially contaminated water samples was 10 cells/100 ml. This limit is higher than acceptable that for concentration of *A. hydrophila* in water distribution system (200 CFU/100 ml).

A. hydrophila was revealed in 13% of the samples tested (4/30). It appears that the presence of *A. hydrophila* is not very frequent in the environment, at least during the period of sampling (May-July). Pablos et

Table 3. Numeration of population of *A. hydrophila* in contaminated water and water samples by SYBR Green assay and membrane filtration method.

Sample	Origin	Results of PCR quantification (calculated log_{10} (CFU/100 ml)			log_{10} (CFU/100 ml) by filtration
		16S rRNA gene	*ast*	*aerA*	
Contaminated waters					
Sterile water		N	N	N	N
W1		4.12±0·02	3.87±0·09	4.08±0·65	3.62±0·00
W2		4.79±0·82	4.25±0·48	4.19±0·10	4.62±0·01
W3		5.71±0·48	5.16±0·36	5.35±0·05	4.92±0·01
W4		5.52±0·21	5.90±0·02	6.02±0·22	5.40±0·02
W5		1.60±0·21	1.51±0·06	1.54±0·21	1.50±0·05
W6		2.61±0·31	2.39±0·17	2.41±0·25	2.47±0·03
W7		0.89±0.40	0.99±0.45	1.09±0.30	0.77±0.18
Water samples					
Tunisian samples					
TUF2	Fountain	N	N	N	N
TUR2	River	N	N	N	N
TUF1	Fountain	1.94±0·00	0.64±0·05	N	0.95±0·04*
TUR3	River	N	N	N	N
TUR1	River	N	N	N	N
TUF3	Fountain	N	N	N	N
French samples					
FMF	Fountain	N	N	N	N
FMF1	Fountain	N	N	N	N
FMF2	Fountain	N	N	N	N
FM2	Pond	N	N	N	N
FMM	Fountain	1.28±0·09	N	N	1.43±0·02*
FL1	Fountain	N	N	N	N
FL2	Fountain	N	N	N	N
FFJ	Fountain	N	N	N	N
FO	Fountain	N	N	N	N
FPD	Lake	N	N	2.16±0·16	N†
FMI	Pond	N	N	N	N
FMF2	Pond	N	N	N	N
FE2	mineral w.	N	1.53±0·43	2.04±0·53	N†
FE1	mineral w.	1.42±0·11	1.50±0·05	2.05±0·17	1.34±0·05*
FE3	mineral w.	N	1.90±0·33	2.27±0·70	N†
FIR	Irrigation	N	N	N	N
FIRR	Irrigation	N	N	N	N
FVE	Fountain	N	N	2.25±0·25	N†
FJH	Fountain	N	1.67±0·11	2.22±0·60	N†
FCV	Fountain	N	1.67±0·79	2.15±0·67	N†
FFI	Fountain	N	1.47±0·12	2.15±0·16	N†
Sweden samples					
SB	Tap w.	0.78±0·71	N	3.03±0·41	0.69±0.12*
SF	Fountain	N	N	N	N
SMP	Pond	N	N	N	N

w: Water , * *A. hydrophila* identified by Api gallery, N† *Aeromonas* sobria confirmed and identified by Api gallery, N not *A. hydrophila*.

al. (2009) have recently evaluated the presence of *Aeromonas* in public drinking water in Spain and found that 26.5% of the samples were positives. Except two strains, all *Aeromonas* were recovered between October and early March. A similar study was accomplished by Emekdas et al. (2006) and the percentage of *Aeromonas* positive samples was only 4%.

Conclusion

This alternative method, based on 16srRNA, can be used to obtain rapid response for detection and quantification of *Aeromonas hydrophila*. However, due to the variety of water, we recommend regularly validating this qPCR against traditional culture-based methods.

Other applications may be considered, like detection and quantification hospital water samples to prevent nosocomial infections.

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