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Comparison between a TaqMan Polymerase Chain Reaction Assay and a Culture Method for *ctx*-Positive *Vibrio cholerae* Detection

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The main objective of the present work was to evaluate a real-time polymerase chain reaction (PCR) method to detect toxigenic *Vibrio cholerae* in *Pangasius hypophthalmus*, a freshwater fish cultured mainly in South East Asia. A FDA traditional culture method and a real-time PCR method of the *ctx* gene were used for detection of *V. cholerae* in spiked samples of pangasius fish. After an overnight enrichment of samples at 37 °C in alkaline peptone water, 2 cfu/25 g of fish was detected with both methods. Although both methods were very sensitive, obtaining results with culture methods may take several days, while real-time PCR takes only a few hours. Furthermore, with traditional methods, complementary techniques such as serotyping, although not available for all serogroups, are needed to identify toxigenic *V. cholerae*. However, with real-time PCR, toxigenic serogroups are detected in only one step after overnight enrichment.

KEYWORDS: *ctx* gene; culture fish; real-time PCR; *Vibrio cholerae*

INTRODUCTION

Monitoring, characterization, and enumeration of foodborne pathogens is a key aspect in food microbiology and food safety, and rapid methods for pathogen testing have been gaining interest for the food industry. These methods include antibody-based assays, genetic amplification methods, and sensor development (*1*−*7*).

The genus *Vibrio* belongs to the family Vibrionaceae, which also includes the genera *Aeromonas*, *Plesiomonas*, and *Photobacterium*. There are 30 species in the genus *Vibrio*; 13 of these are pathogenic to humans, including *Vibrio cholerae*, *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio cincinnatiensis*, *Vibrio hollisae*, *Vibrio vulnificus*, *Vibrio furnissii*, *Vibrio damsela*, *Vibrio metshnikovii*, and *Vibrio carchariae*. All of the pathogenic vibrios have been reported to cause foodborne diseases, although *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are considered the most significant pathogens. These agents are of great concern to the food industry, public health institutions, and consumers (*8*).

Nowadays, there are many culture-based and molecular methods for the characterization of different species of *Vibrio* (*9*−*12*). Methods for the differentiation of pathogenic serogroups of *V. cholerae* are scarce; the most common are those based on antisera agglutination. Within molecular methods, polymerase chain reaction (PCR) has become the most extensively used molecular technique (*13*).

V. cholerae is naturally present in tropical and temperate climates and still remains the leading cause of morbidity and mortality in many developing countries. Strains associated with cholera disease are those containing the *ctx*AB genes that encode for cholera toxin (CT). These include *V. cholerae* O1, the most common of them, *V. cholerae* O139, and *V. cholerae* O141 (*14*, *15*). Recently, *V. cholerae* serogroup O75 has also been identified as the causative agent of severe diarrhea in some patients in the Southeastern part of the United States (*16*).

Although European legislation concerning microbial criteria for foodstuffs (*17*, *18*) does not specifically consider the study of *V. cholerae*, we should take into consideration the possible presence of these pathogens in cultured fish products or in seafood. In this sense, it is important for health authorities to have a fast method to analyze fish products that come from areas where *V. cholerae* could be present or in cases of a food alert.

The FDA Bacteriological Analytical Manual (BAM) culture method uses an enrichment step in alkaline peptone water (APW) followed by isolation on the selective and differential medium, thiosulfate citrate bile salts sucrose (TCBS) agar. Sucrose-positive colonies are selected for biochemical tests (*19*). However, a further study based on antisera agglutination, expensive and not available for all serogroups, is needed to identify toxigenic *V. cholerae*.

Another approach is to use molecular biology methods; in fact, there are several works concerning the application of PCR on the APW enrichment for detecting *V. cholerae*, but most are based on traditional PCR. However, methods based on real-time PCR, which is simpler and faster than conventional PCR assays, are scarce. **Table 1** shows published genes employed in real-time PCR for identification of *V. cholerae*. Only *ctx* operon can discriminate toxigenic from nontoxigenic *V. cholerae* (*20*, *21*). This gene is responsible for the production of the cholera toxin that induces

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Table 1. Previously Published Genes Used To Identify *V. cholerae* with Real-Time PCR Method

gene	description	size (bp)	dye	ref
*hly*A	hemolysin protein	70	TaqMan	*42*
ctx	cholera toxin	308	SYBR green	*20*
rtx	repeat in toxin	265		
rtx	repeat in toxin	120	SYBR green	*43*
*eps*M	extracellular secretion protein	145		
*tcp*A	toxin coregulated pilus	147		
*msh*A	mannose sensitive hemaglutinin	113		
rtx	repeat in toxin	120	Molecular Beacon	*44*
*eps*M	extracellular secretion protein	145		
*tcp*A	toxin coregulated pilus	147		
*omp*W	outer membrane protein	89		
ctx	cholera toxin	84	TaqMan	*21*

cholera disease. In the present work, *ctx* operon was selected for real-time PCR because it is specific for toxigenic *V. cholerae*.

The objective of the present work was to evaluate a real-time PCR technique to detect and quantify *V. cholerae* in pangasius fish (*Pangasius hypophthalmus*) spiked with toxigenic *V. cholerae* and to compare this technique with traditional culture methods. A real-time PCR method based on the method of Blackstone (*21*) was used in cultured fish products for the first time. Also, a commercial DNA extraction kit and three different pre-PCR conditions were evaluated as follows: (a) enrichment of samples in APW for 7 h before PCR, (b) enrichment of samples in APW for 19 h before PCR, and (c) no enrichment before PCR. Spiked samples a and b were also analyzed following the FDA culture method. A comparison was established between the FDA BAM culture method (*19*) and real-time PCR for toxigenic *V. cholerae* detection in fish products. Furthermore, a study of the presence of toxigenic *V. cholerae* was carried out by applying the real-time PCR technology.

MATERIALS AND METHODS

***Vibrio* Species Bacterial Strains.** *V. cholerae* O1 (CECT 514) and *V. parahaemolyticus* (CECT 511) were obtained from the Spanish Type Culture Collection (CECT); *V. cholerae* O139 CCUG 47460 was obtained from CCUG (Culture Collection, University of Göteborg). All were obtained in lyophilized format. They were grown in APW (10 g/L tryptic digest of casein and 10 g/L NaCl, pH 8.5), and stock cultures were stored at −80 °C.

For viable counts, inocula were prepared diluting the overnight culture in APW, and serial dilutions were streaked onto TSA plates (15 g/L bovine casein peptone, 5 g/L soya peptone, 15 g/L agar, and 5 g/L NaCl, pH 7.2). The plates were incubated at 37 ± 1 °C for 24 h, and colonies were counted.

***V. cholerae* Detection by Plating on Selective Agar.** The method used was based on FDA BAM (*19*). Two hundred twenty-five milliliters of APW was added to 25 g of pangasius, previously spiked with toxigenic *V. cholerae*, and homogenized for 30 s. Incubation was carried out at 37 ± 1 °C, and inocula were streaked onto TCBS agar-selective media (pH 8.6) at 7 ± 1 and 19 ± 1 h of incubation. Plates were incubated at 37 ± 1 °C for 24 and 48 h. Typical *V. cholerae* colonies in TCBS were yellow and round (2−3 mm of diameter), since most strains ferment sucrose. Biochemical confirmation was carried out using API20E from Biomérieux.

Toxigenic *V. cholerae* Detection by Real-Time PCR. DNA extraction was done using NucleoSpin Tissue kit (Macherey-Nagel) (*22*) following the manufacturer's instructions for Gram-negative bacteria. The real-time PCR cycling protocol for detection of *ctx* gene was done in a final volume of 25 μL that contained 1x qPCR MasterMix No ROX (Eurogentec), 250 nM sense and antisense primers (Integrated DNA Technologies), 100 nM nuclease probe 5′-labeled with FAM and Black Hole Quencher-1 on the 3′ end (Integrated DNA Technologies), and 5 μL of template. The fluorogenic probe and primer set were previously described by Blackstone et al. (*21*). Real-time PCR was run using Mx3005P QPCR System (Stratagene) with an initial denaturation/polymerase activation step of 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing and extension at 63 °C for 30 s, and 72 °C for 1 min.

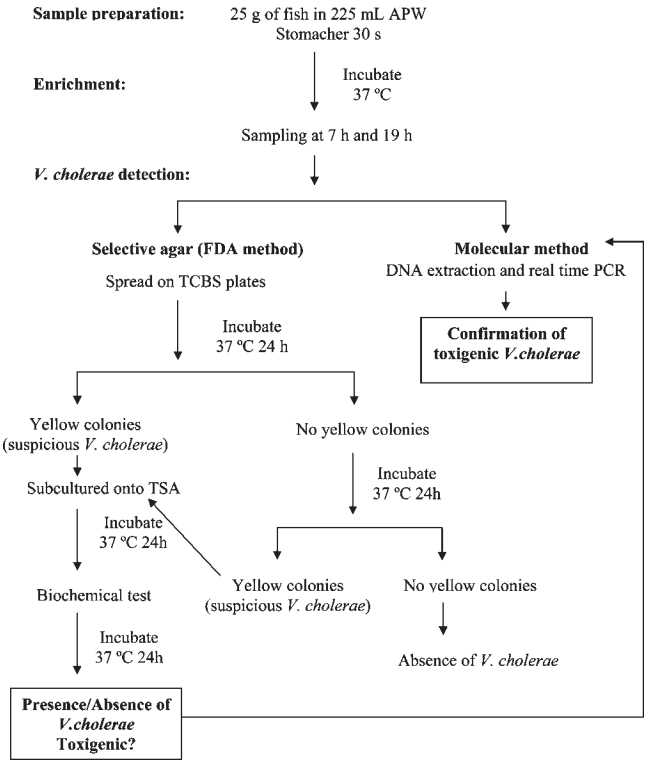

Figure 1. Diagram showing comparison of FDA-BAM culture method and real-time PCR.

The template for the generation of standard curves from pure cultures was prepared by growing *V. cholerae* O1 and O139 as described above. DNA was extracted from 1 mL of pure cultures, and 10-fold serial dilutions in water of DNA were used as a template for real-time PCR in triplicate and repeated three different times for each serotype. The standard curve was generated by plotting the log value of the calculated cfu per reaction versus the cycle threshold (Ct). The efficiency of the real-time PCR assay was calculated using the following formula: $E = (10^{[-1/\text{slope}]} - 1) \times 100$.

Comparison of Real-Time PCR and Plating on TCBS. The application of each one of these methods was carried out as it is detailed in **Figure 1**. Fish samples used for this study (*P. hypophthalmus*) were previously analyzed for *V. cholerae* by plating on TCBS, giving negative results.

First, nine samples of 25 g of pangasius were inoculated with 2.25 mL of 10-fold serial dilutions of a 20 h culture of *V. cholerae* O139 (7.5×10^7 cfu/mL), and then, 225 mL of APW was added to inoculated fish and homogenized for 30 s in a stomacher. One milliliter of the homogenized of each dilution was taken, and DNA was extracted using the NucleoSpin kit and analyzed for *V. cholerae* detection by means of real-time PCR as described above ($t = 0$). Homogenized samples described above were incubated at 37 ± 1 °C and analyzed after 7 ± 1 ($t = 7$) and 19 ± 1 h ($t = 19$) by real-time PCR and by traditional plating on TCBS, and results were compared.

Screening for *ctx*-Positive *V. cholerae* in Fish Samples. Twenty-nine pangasius fillets from different batches and culture areas were analyzed by means of the FDA BAM method and also by using a real-time PCR method. Fish samples were kept frozen until analyzed.

RESULTS

Sensitivity Studies of Real-Time PCR Method for Detection of *ctx*-Positive *V. cholerae*. Conditions described in the Materials and Methods were employed for the detection of *V. cholerae* O1 and O139. Both serogroups gave positive results, confirming the sensitivity of the real-time PCR method. Nontoxigenic *V. cholerae* and *V. parahaemolyticus* were also tested, but no amplification signal was obtained.

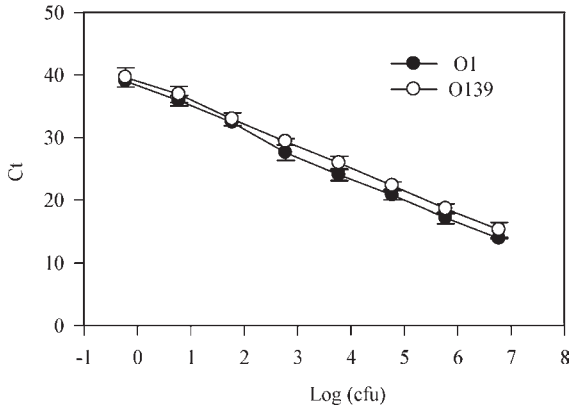

Figure 2. Standard curves showing correlation between log value of cfu of 10-fold serial dilutions in water of *V. cholerae* DNA and Ct values obtained. Values are the means ± SEMs of three experiments by triplicate.

Table 2. Detection of O139 *V. cholerae* in Spiked Fish Samples Enriched 7 and 19 h at 37 °C by Real-Time PCR and Culture in Selective Medium[a]

		7 h of enrichment			19 h of enrichment		
sample	cfu/25 g of fish	real-time PCR	TCBS (24 h)	TCBS (48 h)	real-time PCR	TCBS (24 h)	TCBS (48 h)
P0	1.7×10^8	+	+	+	+	+	+
P1	1.7×10^7	+	+	+	+	+	+
P2	1.7×10^6	+	+	+	+	+	+
P3	1.7×10^5	+	−	+	+	+	+
P4	1.7×10^4	+	−	+	+	+	+
P5	1.7×10^3	−	−	+	+	+	+
P6	1.7×10^2	−	−	+	+	+	+
P7	1.7×10^1	−	−	−	+	+	+
P8	1.7×10^0	−	−	−	+	+	+
	negative control	−	−	−	−	−	−

[a] TCBS plates were read at 24 and 48 h.

Studies of Real-Time PCR Method with Pure Cultures of *V. cholerae* O1 and O139. To determine the efficiency of the real-time PCR for *ctx*-positive *V. cholerae* detection in food samples, standard curves were created (see **Figure 2**). DNA from pure cultures of *V. cholerae* O1 and O139 was extracted with NucleoSpin kit as described in the Materials and Methods. Once extracted, DNA was diluted in water and subjected to real-time PCR to construct standard curves. The linearity range was from 10^7 to 10^0 cfu covering 8 orders of magnitude for both serotypes. The efficiency of the reaction was 92 ± 3% for the O139 serotype and 99 ± 3% for O1. The correlation coefficient was 0.99 in both cases. This experiment was repeated three times for each serotype.

Correlation between the Real-Time PCR Analysis and Plate Spread on TCBS. Twenty-five grams of pangasius fillets were spiked with serial dilutions of *V. cholerae* O139 following the protocol described in Materials and Methods and analyzed using two different procedures: the culture method based on BAM and the real-time PCR method used in this study. Results obtained with both methods were compared (see **Table 2**).

After 7 ± 1 h of enrichment, an aliquot of each sample was streaked on TCBS, and also, 1 mL was taken to be analyzed with real-time PCR. After 24 h of incubation at 37 ± 1 °C, growth of the yellow colonies in dilutions 0 (P0), −1 (P1) and −2 (P2) was observed. When incubation time at 37 ± 1 °C increased to 48 h, growth of the yellow colonies was observed in all of the dilutions except for −7 (P7) and −8 (P8). Real-time PCR after 7 ± 1 h of enrichment gave positive results in all of the dilutions except for −5 (P5), −6 (P6), −7 (P7) and −8 (P8).

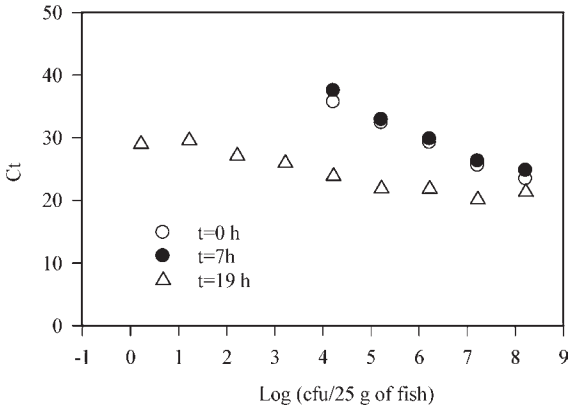

Figure 3. Standard curves showing correlation between log value of cfu of 10-fold serial dilutions in 25 g of pangasius of O139 *V. cholerae* and Ct values obtained. Three different incubation times at 37 °C were analyzed.

After 19 ± 1 h of enrichment, another aliquot was streaked on TCBS, and also, 1 mL was taken to be analyzed with real-time PCR. After 24 h of incubation at 37 ± 1 °C, growth was observed in all of the studied dilutions. Real-time PCR gave positive results for all of the dilutions analyzed. The negative control (non spiked pangasius fillets) was analyzed at 7 and 19 h, and no growth of specific colonies was observed with both methods. The limit of detection of both the culture method and the real-time PCR method was 2 cfu/25 g of fish after enrichment in APW at 37 ± 1 °C for 19 ± 1 h. The same results were obtained for pangasius fillets spiked with serial dilutions of *V. cholerae* O1.

Figure 3 shows real-time PCR results obtained for pangasius fish fillets spiked with serially diluted cells of *V. cholerae* O139. Without enrichment ($t = 0$), the limit of detection was established in 10^4 cfu/25 g of fish. Similar results were obtained after 7 h of enrichment. However, with 19 h of enrichment, 2 cfu/25 g of fish was detected. Three different tests with 10 replicates for each one were done to find the detection level of the method after 19 h of enrichment. Results were similar in all cases, obtaining a detection level of 2 ufc/25 g of fish.

Commercial Fish Samples. Twenty-nine samples of frozen pangasius were analyzed following the FDA culture method and the real-time PCR method used in this work. Fifteen were negative for *V. cholerae* with both methods, and 14 samples were positive with traditional culture methods, but no toxigenic *V. cholerae* was found using a real-time PCR method.

DISCUSSION

Molecular Methodology for Detection of Toxigenic *V. cholerae*. Nucleic acid-based methods have been developed for *V. cholerae* identification and offer a useful alternative to culture methods (*23*, *24*). There are some previous works about *V. cholerae* detection by traditional PCR; some of them involved a single gene target (*25*, *26*), while others employed two (*23*, *27*−*30*), four (*31*), or even seven genes (*32*). Different types of genes were employed, for instance, sequences encoding outer membrane proteins, virulence and regulatory genes, or genes involved in O-antigen biosynthesis. However, all of these studies rely on conventional PCR, which requires product characterization by gel electrophoresis, which is time-consuming and laborious.

However, real-time PCR enables the detection of reaction products through fluorescence, which provides a quicker and more sensitive method for the detection of a diverse range of bacteria and has revolutionized pathogen detection in microbiology. This technique allows visualization of the amount of PCR product formed during the amplification process, introducing fluorescent dyes or probes in the reaction.

As it is shown in **Table 1**, only Fukushima and Blackstone real-time PCR methods (*20*, *21*) detect toxigenic *V. cholerae* amplifying the *ctx* operon (serogroup identification). Other genes are used only for species identification.

Detection chemistries used were different in both studies; Fukushima used SYBR Green I dye, and Blackstone used a TaqMan fluorogenic probe. SYBR Green I dye binds to any double-stranded DNA, including nonspecific sequences, and it may generate false-positive signals. On the contrary, for TaqMan probes, specific hybridization between probe and target is required to generate a fluorescent signal, significantly reducing background and false positives.

Taking these facts into consideration, Blackstone's method was selected for toxigenic *V. cholerae* identification in the present study. This method was employed previously in shellfish, sediments, ballast water, milk, potato salad, and bottled water, but it had never been employed before in cultured fish or fish products (*21*, *33*). Although *ctx* is an excellent indicator of the virulence potential of the bacteria, we must take into account the evidence of horizontal gene transfer between *V. cholerae* and some other *Vibrio* spp. such as *V. mimicus* (*21*). That is the reason why *ctx* has also been found in this species. The fact that the method proposed could detect any serogroup of *Vibrio* spp. that produces the CT (cholera toxin) could alert scientists and health authorities to the possibility of a new epidemic strain.

The most challenging aspect of real-time PCR-based detection is that the detection limit could be affected by inhibitory compounds of different matrixes, and then, inhibitors present in samples should be considered. Inhibitors may interfere in different ways, degrading DNA template or affecting DNA polymerase. Also, an adequate selection of the DNA extraction method and of the enrichment media is necessary (*34*, *35*). In this study, the NucleoSpin Tissue kit was selected to extract DNA from fish samples to avoid real-time PCR inhibition. This differs from previous results with other matrixes (*21*, *33*) and demonstrates that the adaptation of the PCR system to a food matrix, although it is a time-consuming process, is essential for the success of a PCR-based detection system. Diluted DNA from food samples or bacterial colonies previously isolated on selective agar media are also used to avoid real-time PCR inhibition (*36*).

BAM Culture Method versus Real-Time PCR Detection. Cultured fish harvested in waters contaminated with pathogenic *V. cholerae* can act as reservoirs and may spread the disease (*37*). However, as it was found in this work, fish often contain *V. cholerae* that lack the *ctx* gene or other vibrios that produce similar colony morphologies on TCBS, making isolation of the toxigenic strains difficult. Additional biochemical tests and molecular methods must be performed to confirm the presence of *ctx*-positive serogroups. Also, sometimes, cells can enter in a viable but noncultivable (VBNC) state (*38*); in these cases, cells will not be detected with culture methods (*39*, *40*). It has been suggested that the VBNC state accounts for the seasonal nature of cholera outbreaks because of survival for long periods, for example, in river sediments (*41*). Thus, assays allowing rapid differentiation of viable and nonviable cells may be of great importance in the future, particularly if routine end product testing is prioritized (*35*). Time needed to give positive results could be considered a disadvantage of culture methods, and it is not feasible to hold a food product for distribution for that length of time.

It must be noticed that in our study both real-time PCR and culture methods had the same limit of detection of 2 cfu/25 g of fish after the enrichment in APW at 37 ± 1 °C for 19 ± 1 h. However, the real-time PCR method used in this work for the detection of toxigenic *V. cholerae* in culture fish takes approximately 4 h, while the culture method takes at least 3 days for negative samples or up to 5 days for positive samples, and this may result in considerable loss of perishable foods. Also, it does not give any information about toxigenic serogroups, and additional techniques are needed to identify pathogenic strains. From our point of view, the characterization of these serogroups is very important since it would not be necessary to reject and destroy millions of tons of fish suitable for human consumption. Moreover, as we detected in this study, there is a very low probability of finding toxigenic *V. cholerae* in cultured fish; then, serotyping better than species identification is completely necessary.

It must be added that for routine laboratory analysis, the development of a standardized PCR protocol is necessary, and rapid methods like this real-time PCR method are adequate for monitoring these microorganisms in food. Also, inhibitors of samples should be considered when analyzing different matrixes, but the use of traditional methods remains an important component of sample analysis for testing laboratories.

While agencies such as AOAC International accept negative rapid screening results as definitive, positive samples must be confirmed by culture techniques, which remain an important component of sample analysis in testing laboratories. Nevertheless, our results on the detection of toxigenic *V. cholerae* by real-time PCR suggest that this technique could constitute a fast and reliable method for the confirmation of positive samples in routine monitoring of microorganisms in food and efforts must be dedicated to develop standardized protocols optimized for different types of matrixes.

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