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Research Article

Establishment of SYBR Green I Real-Time PCR for Detection of *Streptococcus agalactiae* in Aquaculture Waters

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Streptococcus agalactiae has a serious negative impact on tilapia aquaculture, and rapid detection of trace *S. agalactiae* in aquaculture waters and timely and effective sterilization measures could significantly reduce the probability of its outbreak in tilapia farming. Here, we established a fluorescence quantitative detection method of *S. agalactiae* in tilapia aquaculture waters based on the Streptococcus agalactiae CAMP factor (*cfb*) (GU217532.1) sequence of its CAMP factor. The results showed that the Ct value and logarithm of plasmid copy number presented a good linear relationship ($y = -3.49x + 38.78$; $R^2 = 0.997$) in the plasmid concentration range of 1.57×10^2–1.57×10^9 copies/μL, and the lowest concentration for sensitive detection of the established method was 1.57×10^2 copies/μL. The method exhibited high specificity for the detection of *S. agalactiae* and generated negative results when using the DNA of *Aeromonas veronii, Staphylococcus epidermidis, Aeromonas hydrophila,* and *Edwardsiella tarda* as templates for qPCR. Intra- and inter-group repeated experiments produced variation coefficients lower than 2%, indicating high stability and specificity of the method. The method was then used to detect *S. agalactiae* in two tilapia aquaculture waters. The results showed that the concentration of *S. agalactiae* was 12 copies/mL and 2 copies/mL in the two water samples, respectively. The method can directly detect trace *S. agalactiae* in the aquaculture water and facilitate the diagnosis, prevention, and control of *S. agalactiae* in tilapia aquaculture, which will greatly reduce the probability of large-scale outbreak of *S. agalactiae* and economic loss.

1. Introduction

Tilapia is one of the main varieties promoted by the Food and Agriculture Organization of the United Nations (FAO) and also the second widely cultured fish in the world [1, 2]. It is characterized by strong adaptability, rapid growth, high fertility, and a wide range of feed, as well as delicious meat with few intermuscular bones [3]. The increase in farming density of tilapia will significantly increase the risk of disease outbreak, and *Streptococcus agalactiae* is the biggest threat to tilapia [4]. The annual economic loss in tilapia aquaculture caused by *S. agalactiae* can exceed 250 million USD [5].

S. agalactiae is a Gram-positive pathogen also known as group B streptococcus (GBS). It has a wide range of hosts and global genetic lineage of transmission, with the capability of invading many species including mammals, fish, reptiles, amphibians, and birds [6, 7]. Currently, *S. agalactiae* is divided into 10 serotypes (Ia, Ib, and II–IX) based on the capsular polysaccharide, among which six serotypes (Ia, Ib, II, III, V, and VI) are widely distributed [8]. When infected by *S. agalactiae*, tilapia will show lower appetite and unstable swimming posture to swim round on the water surface, and its eyes will become white, muddy, swollen, and prominent [9]. Outbreak of *S. agalactiae* disease in tilapia farming is more likely to occur in relatively warm seasons mainly due to stress responses, such as high farming density, excessive temperature difference, and hypoxia, which will lead to deterioration of the aquaculture environment. Moreover, the disease can be horizontally spread through food and infected fish.

S. agalactiae disease of tilapia is highly infectious, causing high mortality and wide prevalence [10]. However, culture-based detection of *S. agalactiae* is time-consuming and of limited sensitivity. Therefore, a more efficient and sensitive method is needed for the detection of *S. agalactiae* in aquaculture waters.

Numerous methods have been developed to detect pathogenic bacteria. Compared with traditional PCR, real-time fluorescence quantitative PCR (RT-qPCR) has the advantages of high specificity, sensitivity, repeatability, and time efficiency [11–13]. RT-LAMP assay was used to detect the tilapia lake virus [14]. qPCR can quantify the DNA number and detect low levels of *S. agalactiae* based on the *groEL* gene [1]. A droplet digital PCR (ddPCR) was developed for rapid detection of *S. agalactiae* with high sensitivity and specificity using the *CpsE* gene [15]. A real-time recombinase polymerase amplification (real-time RPA) assay was used to detect *Aeromonas hydrophila* using the haemolysin gene [16]. Early detection of diseases and timely adoption of prevention and control measures are of great significance for aquaculture.

Due to the special characteristics of aquaculture, the outbreak of tilapia disease is a result of some complex interactions among tilapia, pathogen, and environment. Disease outbreak is generally a process from quantitative change to qualitative change. First, there is a small amount of *S. agalactiae* in the water, which will infect tilapia in a small range with slow reproduction, but no obvious disease phenomenon will be observed in this process. The feces of infected tilapia contain certain amounts of *S. agalactiae*, which can survive in pond water and reinfect other healthy fish [10]. Then, *S. agalactiae* will quickly increase to a threshold value in the water and directly infect healthy tilapia through the water. Because the physical barrier of tilapia is composed of mucus and scales, infected fish can hardly infect healthy fish directly. At this time, only a few early infected fish will show symptoms. With increasing number of *S. agalactiae* in pond water, the infection ability of tilapia will be gradually enhanced. If the feces and carcasses of infected tilapia are not cleaned up in time, *S. agalactiae* will be quickly released into the pond water again, which will eventually result in outbreak of *S. agalactiae* disease. *S. agalactiae* can survive for 48 h in the brain of dead fish [17], and high temperature will promote the spread of *S. agalactiae* in tilapia. Based on the outbreak process of *S. agalactiae* disease, early warning of *S. agalactiae* in aquaculture waters is of the utmost importance. Once *S. agalactiae* is detected, appropriate measures, such as sprinkling of chlorine disinfectant, can be taken to completely eliminate the pathogenic bacteria at the early stage of outbreak, which is of great significance for the sustainable development of tilapia aquaculture. The *cfb* gene, a specific gene in *S. agalactiae* that encodes the CAMP factor, is often used as a marker gene to detect the presence of *S. agalactiae* [18]. This study aims to establish an efficient method for the detection of *S. agalactiae* in aquaculture pond water, which will provide scientific and technical support for initial diagnosis of *S. agalactiae* disease and reduce the economic loss in aquaculture.

2. Materials and Methods

2.1. Bacterial Culture. *S. agalactiae*, *Aeromonas veronii*, *Staphylococcus epidermidis*, *Aeromonas hydrophila,* and *Edwardsiella tarda* were kept by our laboratory and grown at 37°C in brain heart infusion medium (BHI). Tilapia pond water samples were collected from rural areas near Liuzhou City.

2.2. Design and Synthesis of Primers and Optimization of Reaction Conditions. According to the sequence of *cfb* (Gene ID: GU217532.1) in GenBank, the full and partial regions of *cfb* primers were designed with primer 5.0 software and synthesized by GenScript Biotech Corp. SYBR Green I fluorescent PCR was carried out with the recombinant plasmid as the template. The concentration ratios of the F/R primers (0.4 mM: 0.6 mM, 0.5 mM: 0.5 mM, 0.6 mM: 0.4 mM) and the annealing/extension temperature (58°C, 60°C, 62°C) were optimized. The recombinant plasmid was used as the template for PCR amplification. The optimal reaction conditions were determined when the minimum Ct and maximum fluorescence value were obtained, and the melting curve showed no nonspecific product.

2.3. Extraction of Total DNA from Bacteria and Pond Water. All the liquid cultured strains of *S. agalactiae*, *A. veronii*, *S. epidermidis*, *A. hydrophila,* and *E. tarda* were collected, and their total DNA was extracted following the instructions of total DNA Extraction Kit (TIANamp Bacteria DNA Kit). 5 L water sample was obtained from tilapia aquaculture farm. Firstly, the water was filtered by 4 layers of gauze to remove coarse impurities. Then, filter the water several times by ordinary neutral filter paper (pore size: 30–50 microns), collect the filtered filter paper together, and rinse with sterile water again. Finally, the water sample was centrifuged at 4000 r/min for 10 minutes for concentration [19]. Total DNA of different water samples was extracted following the instructions of total DNA Extraction Kit. Finally, the total DNA was eluted with 50 μL buffer TE and stored at −20°C for future use.

2.4. Real-Time PCR. All the DNA isolates were analyzed in duplicate by real-time PCR according to the protocol of PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). A 160 bp fragment of the *S. agalactiae*-specific *cfb* gene was used as the target sequence. The SYBR Green Mix was purchased from Thermo Fisher Scientific. The 10 μL volume contained 5 μL SYBR Green Mix, 1 μL template DNA, 0.5 μL *cfb*-qF, 0.5 μL *cfb*-qR, and 3 μL ddH_2O. Each test included a positive control (*S. agalactiae* DNA as template) and a negative control (ddH_2O in place of DNA template). Real-time PCR was performed in LineGene 4800 (Bioer, Hangzhou). The optimum reaction program was 95°C for 2 min, 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s, for 40 cycles. The melting curve program was from 72°C to 95°C (increasing at 1.6°C per second and kept for 15 s), 95°C

to 60°C (decreasing at 1.6°C per second and kept for 1 min), and then 60°C to 95°C (increasing at 0.15°C per second and kept for 15 s). All data were analyzed on the LineGene 4800 software.

2.5. Establishment of the Standard Curve. With the total *S. agalactiae* DNA genome as the template, the *cfb* gene was amplified by conventional PCR with specific primer *cfb*-F/R (Table 1). After purification and sequencing, the PCR product of *cfb* was cloned into pMD18-T vector and transformed into *E. coli* DH5*α*. The recombinant plasmid was designated as pMD18-T-*cfb*. The plasmid was extracted with the plasmid extraction kit, and the plasmid concentration was measured with a micro-spectrophotometer with the following formula: plasmid concentration (copies/μL) = [6.02×10^{23} (copies/mol) × plasmid concentration (ng/μL) × 10^{-9})]/[plasmid length (2692 + 160 bp) × 650 (g/mol ∗ bp)] [20]. The reconstructed plasmid pMD18-T-*cfb* was diluted by tenfold serial gradient and used as the template, respectively. Under the optimized conditions, the specific product was amplified by fluorescent quantitative PCR. With logarithm of the relative copy number of each gradient of the recombinant plasmid as the abscissa and the Ct value as the ordinate, the standard curve was generated automatically by the qPCR system. All experiments were repeated three times.

2.6. Detection of Sensitivity and Specificity. To further evaluate the specificity of the established method, the total DNA was isolated from *S. agalactiae*, *A. veronii*, *S. epidermidis*, *A. hydrophila*, and *E. tarda* and used as templates for fluorescent quantitative PCR amplification, respectively. The sensitivity of the established method was verified with different dilutions of the reconstructed plasmid pMD18-T-*cfb* (1.57×10^1–1.57×10^9 copies/μL). Each test included a positive control (*S. agalactiae* DNA as template) and a negative control (ddH_2O in place of DNA template). The qPCR was performed with the conditions and concentrations described above. All experiments were repeated three times.

2.7. Repeatability and Stability Test. The same batch or different batches of reconstructed plasmid pMD18-T-*cfb* at different concentrations were used as positive templates, and ddH_2O was used as the negative control template. The fluorescent quantitative PCR was carried out as described above. All experiments were repeated three times. The variation coefficients within and between groups were calculated, respectively.

2.8. Determination of the Copy Number in Pond Water. The performance of the established method was evaluated using samples from two tilapia culture ponds in Liuzhou and Shatang, Guangxi Province, where outbreak of *S. agalactiae* disease just occurred. The water samples were concentrated at different folds and processed as described in Materials and Methods. The copy number was calculated according to the formula, $C = V * 10^{(38.78-y)/3.49} * V_{\text{total}}$, where C indicated the concentration of aquaculture waters per sample (copies/ml); V indicated the volume of total DNA per sample (μL); y indicated the cycle threshold (CT) value; and V_{total} indicated the volume of aquaculture waters per sample (ml). All water samples were determined three times.

3. Results

3.1. Design of cfb-Specific Primers. According to the *cfb* gene (GU217532.1) sequence of the CAMP factor in *Streptococcus agalactiae* GDzl in NCBI, we designed the primers targeting the full and partial regions of the *cfb* gene (Table 1).

3.2. Gene Amplification and Recombinant Plasmid Construction. The CAMP-factor encoding gene (*cfb*) was amplified, and result showed that the product was consistent with expectation and had no dimer (Figure 1(a)). The *cfb* gene fragment was recovered, sequenced, and connected to the pMD18-T vector to obtain the reconstructed plasmid pMD18-T-*cfb*, which was then verified by fluorescence quantitative primer *cfb*-qF/R (Table 1). The length was 160 bp (Figure 1(b)).

3.3. Establishment of the Standard Curve. Amplification curves of plasmids at different concentration gradients are presented in Figure 2(a). The results showed that there was a strong linear relationship between the logarithm of plasmid copy number from 1.57×10^9 to 1.57×10^2 copies/μL and the Ct value. The equation of the standard curve was $y = -3.49x + 38.78$ ($R^2 = 0.997$) (Figure 2(b)). Moreover, the melting curve was narrow with a single specific melting peak, indicating that there were no primer dimers and nonspecific product (data not shown).

3.4. Specificity Analysis. The specificity analysis demonstrated that only *S. agalactiae* was detected as positive, while other bacteria and the control were determined as negative (Figure 3), indicating that the established method has high specificity (Figure 3).

3.5. Sensitivity Analysis. The sensitivity analysis showed that the reconstructed plasmids at concentrations of 1.57×10^2–1.57×10^9 copies/μL could be effectively amplified, but the amplification of reconstructed plasmids at 1.57×10^1 copies/μL and blank template control was invalid, indicating that the minimum concentration of template copies for the established method was 1.57×10^2 copies/μL (Figure 4).

3.6. Repeatability and Stability Test. Reconstructed plasmids at three dilution gradients (1.57×10^4–1.57×10^6 copies/μL) were selected to carry out fluorescent quantitative PCR amplification at the same time point and at different time points. As a result, the intra-assay CV value was 0.30%, 0.55%, and 0.99%, and the inter-assay CV value was 0.69%, 1.75%, and 0.90%, respectively, all of which were lower than 2% (Table 2). These results demonstrated that the established method has high repeatability and stability.

Table 1: Primers used in this study.

Primer	Sequence (5′-3′)	Length (bp)
cfb-F	CGGGATCCGAACTCTAGTGGCTGGTGCA	768
cfb-R	CCGCTCGAGCTGTTTGAAGTGCTGCTTGTAAT	768
cfb-qF	GTTATTCGCATTTTAGATCCATT	160
cfb-qR	TCCAGATTTCCTCTTATCAAGTTTT	160

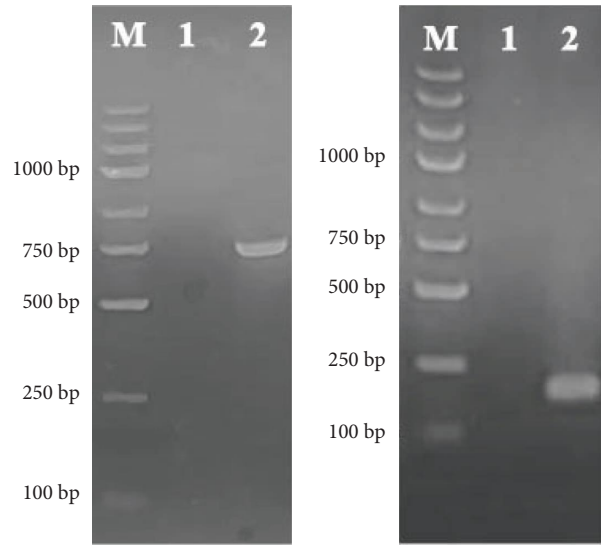
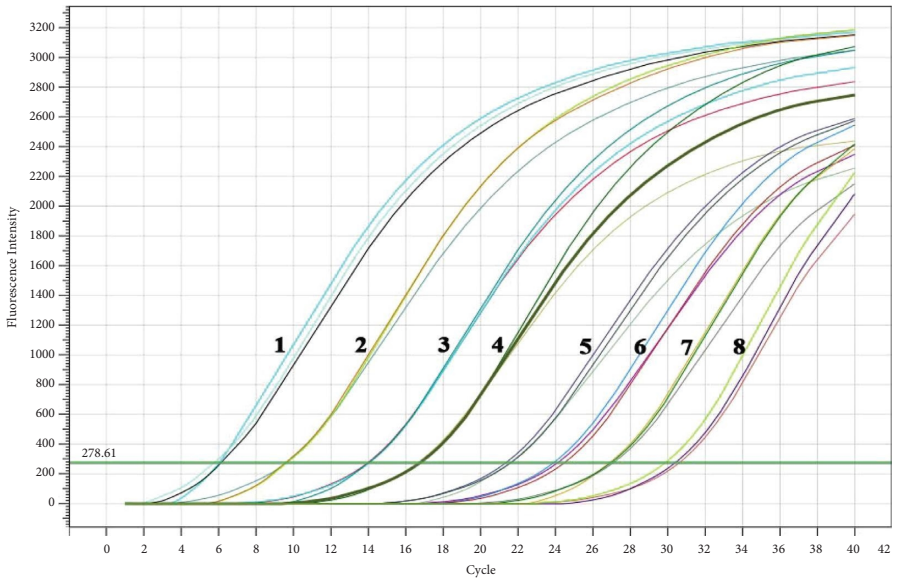
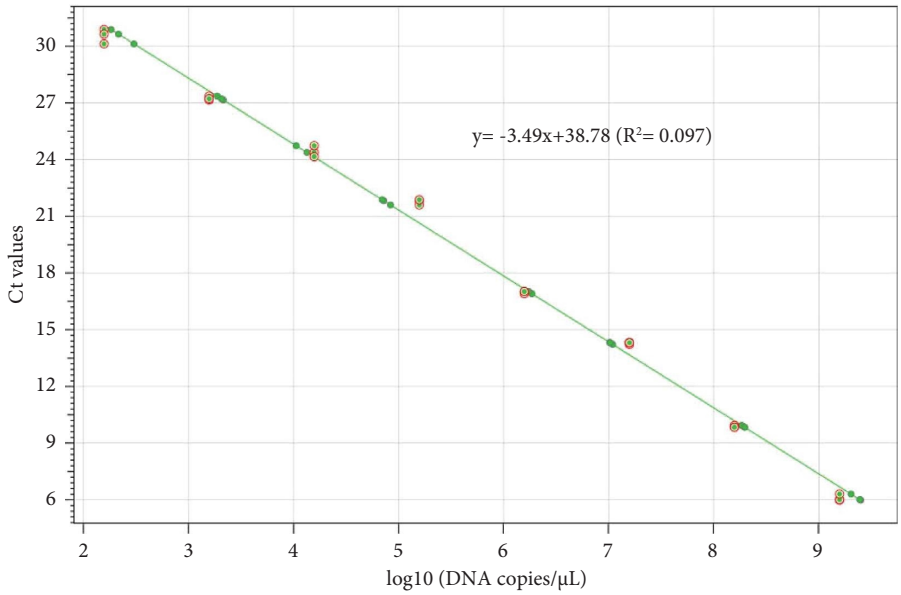


Figure 1: Gene *cfb* amplification and construction of recombinant plasmid. (a) Amplification and detection of complete *cfb* gene. M, Marker DL5000; 1, negative control without template; 2, complete *cfb* gene was amplified by *cfb* F/R primer with the template of total *S. agalactiae* DNA genome. (b) Validation of *cfb* fluorescent quantitative primer *cfb*-qF/R. M, Marker DL5000; 1, negative control without template; 2, partial fragment *cfb* gene was amplified by *cfb*-qF/R with the template of the reconstructed plasmid pMD18-T-*cfb*.



(a)

Figure 2: Continued.



(b)

FIGURE 2: Amplification curve of real-time PCR using SYBR Green I assay and the standard curve. (a) Amplification curve of real-time PCR for detection of tenfold serial dilutions of recombinant plasmid pMD18-T-*cfb* after 40 cycles. Curves 1–8: 1.57×10^9, 1.57×10^8, 1.57×10^7, 1.57×10^6, 1.57×10^5, 1.57×10^4, 1.57×10^3, and 1.57×10^2 copies/μL pMD18-T-*cfb*, respectively. (b) Standard curve generated from the mean of Ct values against $\log_{10}$ of tenfold serial dilutions of recombinant plasmid pMD18-T-*cfb*.

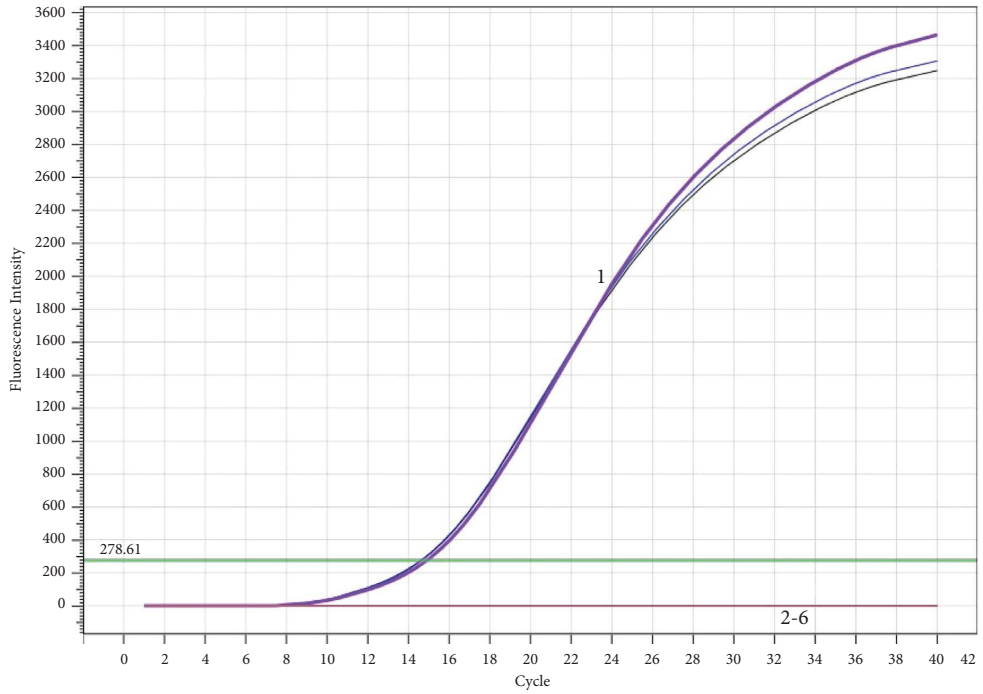


FIGURE 3: Specificity analysis of the real-time PCR using SYBR Green I. Curve 1: total DNA genome isolated from *S. agalactiae* DNA. Curves 2–5: total DNA genome isolated from *Aeromonas veronii, Staphylococcus epidermidis, Aeromonas hydrophila*, and *Edwardsiella tarda*, respectively. Curve 6: negative control without template. The primer *cfb*-qF/R was used for the specificity analysis.

3.7. Determination of Copy Number in Aquaculture Waters. Water samples were collected from tilapia aquaculture waters in Liuzhou and Shatang, Guangxi Province, respectively. The total DNA was collected and the concentration was measured, and the concentrations of DNA samples in Figures 5(a) and 5(b) were 81.176 ng/μL and 75.487 ng/μL, respectively. The copy number of *S. agalactiae* was determined by fluorescence quantitative PCR. The

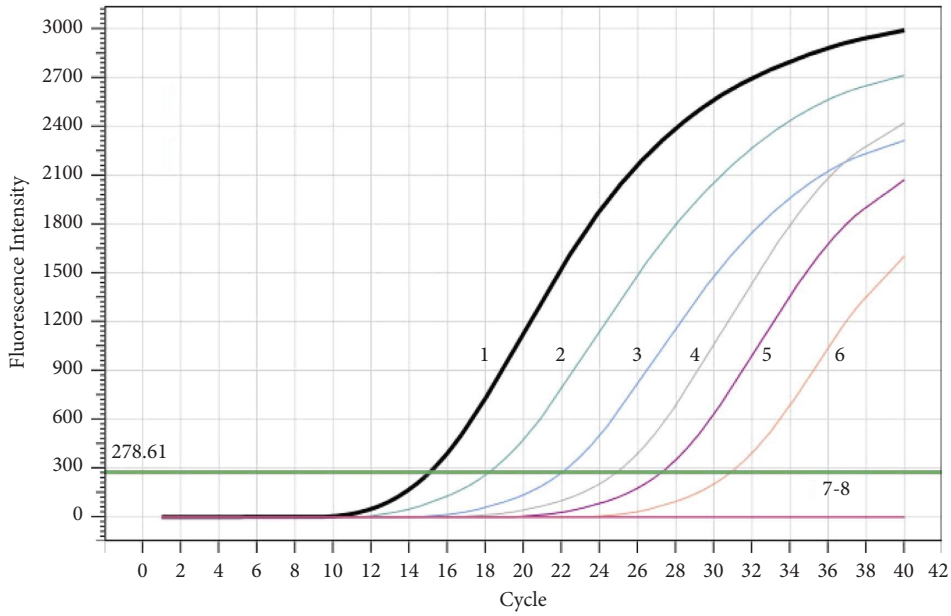


Figure 4: Sensitivity analysis of the real-time PCR using SYBR Green I. Amplification curve of real-time PCR for detection of tenfold serial dilutions of recombinant plasmid pMD18-T-*cfb* after 40 cycles. Curves 1–6: 1.57×10^7, 1.57×10^6, 1.57×10^5, 1.57×10^4, 1.57×10^3, and 1.57×10^2 copies/μL pMD18-T-*cfb*, respectively. Curve 7: 1.57×10^1 copies/μL pMD18-T-*cfb*. Curve 8: negative control without template. The primer *cfb*-qF/R was used for the specificity analysis.

Table 2: Repeatability and stability test of real-time PCR assay.

Template concentration of standard (copies/μL)	Intra-group repetition		Inter-group repetition	
	Mean ± SD[a]	CV (%)[b]	Mean ± SD[a]	CV (%)[b]
$1.57 * 10^6$	16.96 ± 0.05	0.30	16.81 ± 0.12	0.69
$1.57 * 10^5$	21.76 ± 0.12	0.55	21.24 ± 0.37	1.75
$1.57 * 10^4$	24.41 ± 0.24	0.99	24.31 ± 0.22	0.90

[a]Calculation of copy number was based on average Ct values. [b]CV is the coefficient of variation, which is a normalized measure of the dispersion of probability distribution. It is defined as the ratio of standard deviation to average, that is, CV = (standard deviation/average MEAN) × 100%.

results showed that the amplified Ct value of 3000-fold concentrated Liuzhou water sample was 28.80 (Figure 5(a)). According to calculation of the standard curve, the copy number concentration of *S. agalactiae* was 7.24×10^2 copies/μL. There were a total of 36200 copies of *S. agalactiae* in 50 μL total genome, and therefore the concentration of *S. agalactiae* was 12.07 copies/mL in Liuzhou water sample. Moreover, the amplified Ct value of 2000-fold concentrated Shatang water sample was 32.02 (Figure 5(b)). According to calculation of the standard curve, the copy number concentration of *S. agalactiae* was 87.09 copies/μL. There were 4354.5 copies of *S. agalactiae* in 50 μL of total genome, and thus the concentration of *S. agalactiae* in Liuzhou tilapia culture water was 2.177 copies/mL. These results demonstrated that the fluorescence quantitative PCR detection method could accurately detect *S. agalactiae* in aquaculture waters.

4. Discussion

To date, many methods have been developed and used for quantitative detection of viruses, parasites, and bacterial pathogens in tissues and the environment [21–24]. These methods generally have higher sensitivity and accuracy than traditional culture-based detection methods. *S. agalactiae* is one of the main pathogenic bacteria for tilapia [25]. At present, the prevention and control of *S. agalactiae* mainly depend on antibiotics and chemical drugs to control the early infection of pathogenic bacteria, whereas long-term use of chemical drugs may lead to drug resistance of pathogenic bacteria, drug residual, and some problems related to environmental and food safety. Rapid diagnosis will facilitate more accurate identification of *S. agalactiae*. Many detection methods have been reported for *S. agalactiae* in tilapia. However, these detection methods are mostly based on the tissues and organs of tilapia and are generally time-consuming and rather complex, which may lead to missing of the best treatment opportunity [1, 15, 26, 27]. Spread of *S. agalactiae* through culture water easily causes the outbreak of *S. agalactiae* disease in tilapia. Therefore, direct detection of *S. agalactiae* in the aquaculture water can provide important support for the prevention of *S. agalactiae* in tilapia.

The *cfb* gene in *S. agalactiae* encodes the specific CAMP factor. Hence, *cfb* is usually used as a marker gene for the detection of *S. agalactiae*. In this study, based on the maker gene *cfb*, a SYBR Green I fluorescence quantitative detection

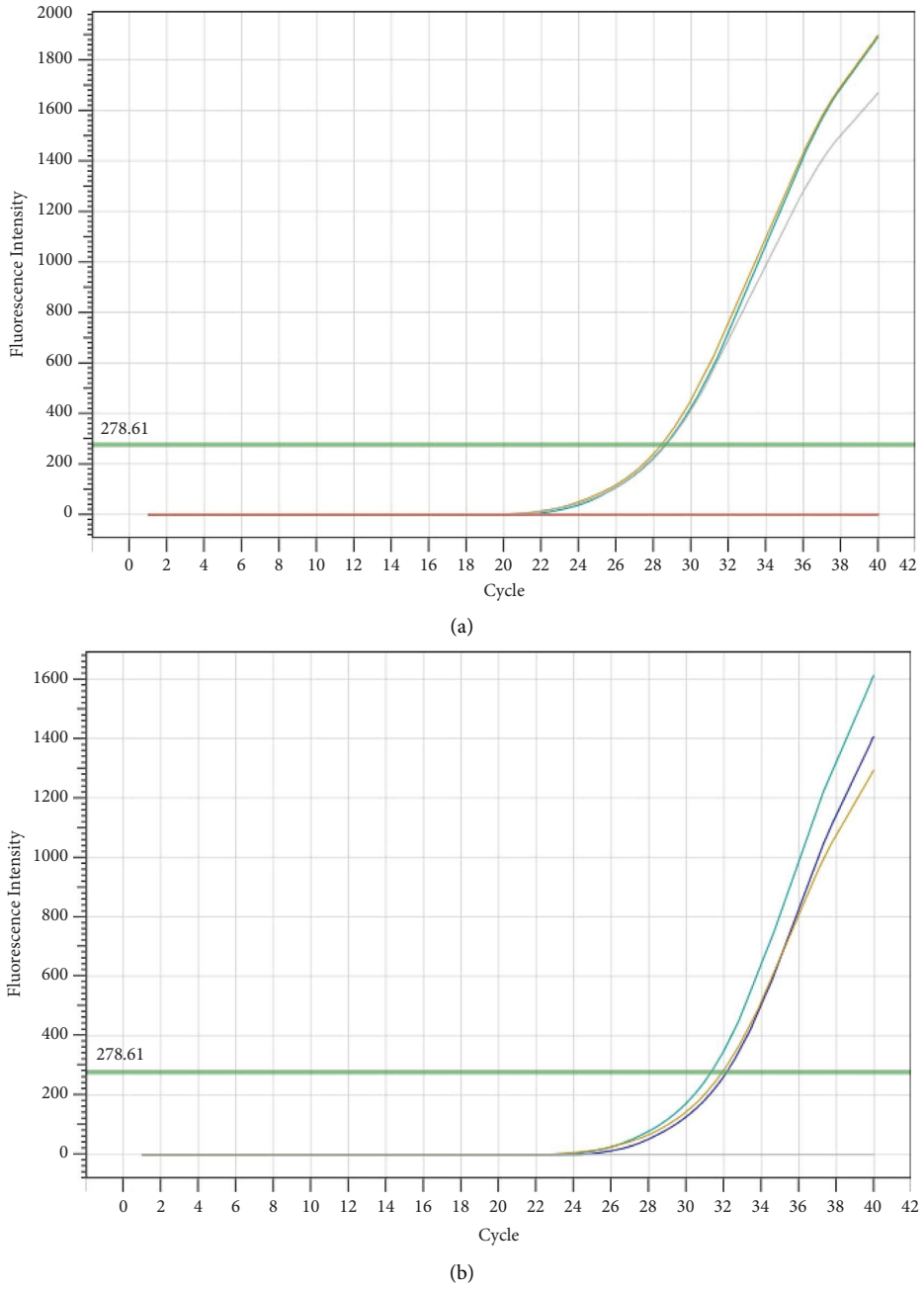


FIGURE 5: Determination of the gene *cfb* copy number of *S. agalactiae* in aquaculture waters. (a) Amplification curve of Liuzhou water sample using real-time PCR method. Liuzhou water sample was concentrated by 3000 folds. (b) Amplification curve of Shatang water sample using real-time PCR method. Shatang water sample was concentrated 2000 folds. The red baseline and the gray baseline are negative controls. The concentrations of DNA were 81.176 ng/μl and 75.487 ng/μl used for Liuzhou and Shatang, respectively. The primer *cfb*-qF/R was used for the specificity analysis. All experiments were repeated three times.

method for *S. agalactiae* in aquaculture waters was established by optimizing the reaction conditions and testing the specificity, sensitivity, and repeatability. The experimental results revealed that the established method has good specificity, with a stand curve of $y = -3.49x + 38.78$ ($R^2 = 0.997$) (Figure 2(b)), and the lowest limit of detection for the *cfb* gene of *S. agalactiae* was 1.57×10^2 copies/μL (Figure 4). The variation coefficient of repeated experiments within and between groups was lower than 2%, demonstrating a higher repeatability of the method. The method was then used to detect two aquaculture water bodies, which were concentrated by 60,000 folds and 40,000 folds, respectively (Figure 5(a)). The results showed that *S. agalactiae* was present in both water bodies. It happened that the two farms had just experienced an outbreak of *S. agalactiae* disease, indicating that the established method can be applied to actual aquaculture production.

There are some differences between fluorescent quantitative PCR and culture-based detection methods. The qPCR detection method has an obviously higher accuracy. Some researchers have reported that qPCR analysis can detect the presence of bacteria in samples that are detected as negative by standard culture-based methods. For example, the fluorescence quantitative method could detect the presence of *S. pneumonia* in 47 of 49 samples detected to be negative by culture-based methods (cell concentration $< 1 \times 10^4$ CFU/mL) [28]. The standard culture-based method can only detect living bacteria, while the qPCR method can detect both living and dead bacteria, which can account for the higher accuracy of the qPCR detection method [1]. Several factors may explain the difference between qPCR and standard culture-based methods, including aggregation, viability, and VBNC (viable but nonculturable) state [29]. Our current detection method needs to be combined with traditional culture methods in order to be better applied. In addition, qPCR method has the characteristics of strong sensitivity and high specificity, and special attention should be paid to avoiding the influence of environmental factors during the detection process. When the pond water was filtered and concentrated, it may cause the loss of some bacterial bodies in the water sample. When extracting the total DNA, the cell fragmentation of the bacterial bodies cannot be destroyed completely. Furthermore, the method requires professional equipment and trained technicians, making it difficult to be applied and popularized for ordinary farmers. Therefore, it is urgent to develop a more convenient, rapid, and sensitive detection method in the future.

In summary, the developed qPCR method can be used to detect the quantity of *S. agalactiae* in aquaculture water to prevent the disease of tilapia. The method can not only efficiently detect and monitor pathogenic bacteria but also help quantitative research on the dynamic changes in pathogenic bacterial infection, which can provide some guidance for farmers to take timely measures to control the pathogenic bacteria and reduce economic loss caused by the disease of tilapia.

Data Availability

The nucleotide sequence cfb reported in this article has been submitted to GenBank with accession number GU217532.1.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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