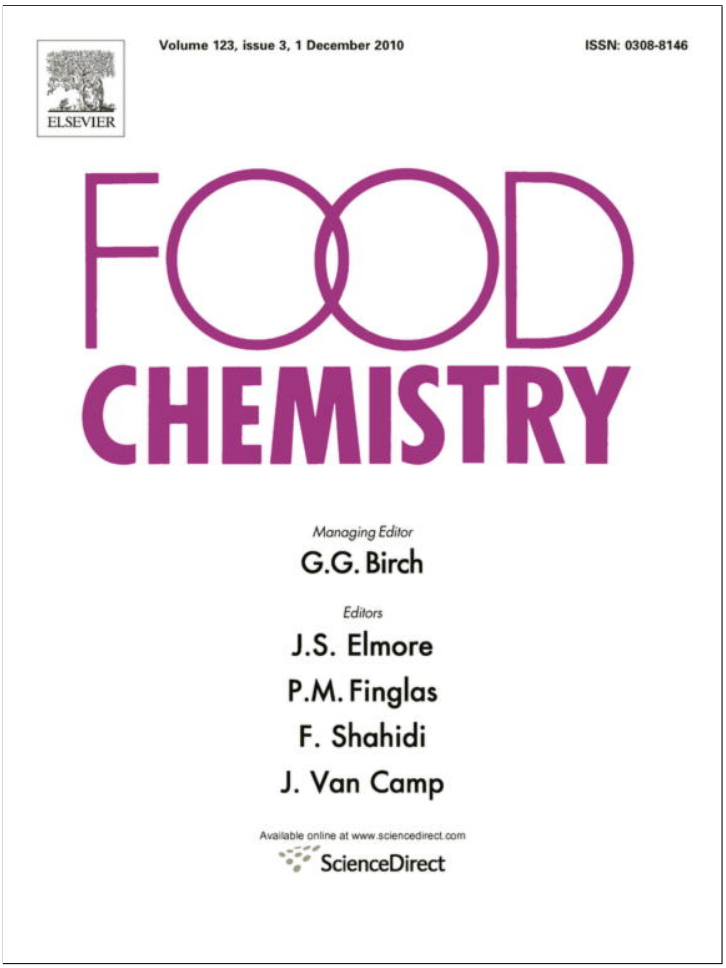

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Analytical Methods

Rapid and sensitive detection of *Vibrio parahaemolyticus* in sea foods by electrochemiluminescence polymerase chain reaction method

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ABSTRACT

Vibrio parahaemolyticus has been considered as one of the most important food-borne bacterial pathogens. Because of the safety concerns, detection and characterization of *V. parahaemolyticus* have attracted much attention. In this study, electrochemiluminescence polymerase chain reaction (ECL-PCR) method combined with universal probes hybridization technique was applied to rapid detection of *V. parahaemolyticus*, infected and uninfected sea foods for the first time. Whether the sea food samples were infected was discriminated by detecting the gyrase B (*gyrB*) gene. We detect *V. parahaemolyticus* both in artificially contaminated sea foods and natural samples. The experiment results show that the infected and uninfected sea food samples can be clearly identified and the detection limit for *V. parahaemolyticus* is 1.6 pg purified genomic DNA in the presence of 1 μg non-specific background DNA. The technique may provide a new means in *V. parahaemolyticus* detection due to its simplicity and high efficiency.

1. Introduction

The detection of pathogenic bacteria is important for our health and safety. The development of rapid and specific methods of detecting pathogenic bacteria in fields such as the food industry, clinical diagnosis and environmental control is required (Rahman & Sun, 2009). *Vibrio parahaemolyticus* is a gram-negative halophilic bacterium that naturally inhabits marine and estuarine environments. The organism can cause potentially serious infections in humans when raw or partially cooked seafood is consumed (Blake, Weaver, & Hollis, 1980; Rippey, 1994). Furthermore, seafood with only a small number of *V. parahaemolyticus* organisms can reach an infectious dose in only a few hours. *V. parahaemolyticus* food poisoning outbreaks all over the world include Japan, India, America, Russia and Southeast Asia (Antonio, Julio, Carlos, & Jaime, 2003; Blake et al., 1980; DePaola, Kaysner, Bowers, & Cook, 2000; Okuda et al., 1997). The increased frequency with which food-borne bacterial pathogens have been causing recurring outbreaks, sometimes with fatal infections. Therefore, methods for monitoring and routine screening for the presence of *V. parahaemolyticus* are necessary. Numerous techniques for *V. parahaemolyticus* detection have been developed (Gooding & Choudary, 1999). The most common method to detect *V. parahaemolyticus* is a culture procedure using enrichment media and subsequent isolation on selective plating media (Ray, Hawkins, & Hackney, 1978). Although it is necessary to obtain analytical results in the shortest time, traditional and standard bacterial detection methods may take up to 7 or 8 days to yield an answer. This is clearly insufficient, and many researchers have recently geared their efforts towards the development of rapid method. Improved identification methods based on new enrichment media (Pendru, Iddya, & Indrani, 2009; Ray et al., 1978) and selective fluorogenic (Miyamoto, Miwa, & Hatano, 1990) were also developed. Although the detection time has been shortened, the operation processes are still very complex. Enzyme-linked immunosorbent assays (ELISA) is also applied to *V. parahaemolyticus* detection (Honda, Yoh, Kongmuang, & Miwatani, 1985). Although it is a very sensitive method, the reaction of the ELISA method can be suppressed by some extractive in samples, thus effecting the detection results (Menzel, Jelkmann, & Maiss, 2002).

Modern molecular biology methods have more and more been used in pathogenic bacteria detecting due to their good specificity and high sensitivity. Polymerase chain reaction (PCR) procedures are clearly rapid and highly specific for detection of *V. parahaemolyticus* (Luan et al., 2008; Nordstrom, Vickery, Blackstone, Murray, & DePaola, 2007). In contrast to the long culturing process, bacterial genome DNA can be amplified by PCR in a short time. Detection using PCR takes much less time than traditional detection methods. Thus, PCR technology has the potential to enable the rapid and specific detection of pathogenic bacteria via specific amplification and detection. Therefore, if the PCR method allows detection of the nucleotide sequence specific to *V. parahaemolyticus*, it can facilitate identification of the organism. The gyrase B gene (*gyrB*) encodes the B subunit protein of DNA gyrase (topoisomerase type II). It is a single-copy gene and is essential for DNA replication. It

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has been used as a molecular taxonomic marker for bacterial species since 1995 (Yamamoto & Harayama, 1995). In 1998, Kasthuri et al. (Venkateswaran, Dohmoto, & Harayama, 1998) applied *gyrB* gene to *V. parahaemolyticus* PCR detection. So in this paper, *gyrB* gene is employed as the target of *V. parahaemolyticus.*

In PCR-based bacterial detection, PCR-amplified DNA must also be quickly and conveniently detected. Generally, the presence of amplified products can be confirmed by gel electrophoresis after PCR amplification. Several detection systems for pathogenic bacteria such as *Salmonella* based on the combination of PCR and gel electrophoresis have already been developed and commercialized. Gel electrophoresis is an easy method of detecting PCR products, but it cannot distinguish between specific amplified products and non-specific ones. Thus, gel electrophoresis is not sufficiently accurate to specifically detect PCR-amplified products. Fluorescence detection method, such as real-time PCR is also widely used (Tyagi, Saravanan, Iddya, & Indrani, 2009; Ward & Bej, 2006), although it has high sensitivity, it cannot be widely used since the expensive equipment and strict operating procedure.

Since Kenten et al. (1991) and Blackburn, Shah, & Kenten (1991) first applied DNA probe to ECL, this method has been widely used in DNA analysis (Lee, Yun, & Lim, 2007; Richter, 2004; Zhu, Tang, Xing, & Chen, 2008) and various areas ranging from chemical analysis to the molecular-level understanding of biological processes (Zhou, Xing, Zhu, Tang, & Jia, 2008). ECL combines chemiluminescence and electrochemistry, which is a chemiluminescent reaction of species generated electrochemically at an electrode surface. Initially, TBR and TPA are oxidized at the surface of an anode. TPA^{+*} immediately loses a proton and becomes a powerful reducer. When TPA^{*} and $Ru(bpy)_3^{3+}$ react, the latter enters an excited state by a high energy electron transfer from the electron carrier, TPA^{*}. Relaxation of $Ru(bpy)_3^{2+*}$ to the ground state results in a light emission, at 620 nm (Liu, Xing, Shen, & Zhu, 2004). Noticeable, $Ru(bpy)_3^{2+}$ is not consumed during the reaction and may be oxidized and excited repeatedly.

The key idea of the paper is to develop a novel method for rapid and sensitive detection of *V. parahaemolyticus* in sea foods through an ECL-PCR technique by introducing a pair of universal probes. An assay like this provides the ability to more rapidly detect *V. parahaemolyticus* in sea foods, which should benefit not only the seafood industry, but also the consumer by ensuring the safety of the food provided for consumption.

2. Materials and methods

2.1. Materials

β-Mercaptoethanol and cetyltrimethyl ammonium bromide (CTAB) were purchased from AMRESCO, Netherlands. Taq DNA polymerase and dNTPs were bought from Takara Biotechnology (Dalian) Co. Ltd., China. Six hundred base pairs of DNA Ladder were purchased from Guangzhou Dongsheng Biotech Co. Ltd., China. The streptavidin micro-beads (2.8 μm in diameter) were obtained from Dynal Biotech (Lake Success, NY, USA). TPA was acquired from Aldrich Chemical Company. The $Ru(bpy)_3^{2+}$ *N*-hydroxysuccinimide ester (TBR-NHS ester) was prepared according to previously published paper (Terpetschnig, Szmacinski, Malak, & Lakowicz, 1995).

Primers of *V. parahaemolyticus* has been reported before (Venkateswaran et al., 1998). The universal sequences and probes were designed by our lab. All primers and probes were synthesized by Shanghai Sangong Biological Engineering & Technology Services Co. Ltd. (SSBE), China, and the probes were labeled with TBR-NHS ester by our lab. The forward primer was 5′-TAACTGAATAGACTAAGACCGGCGTGGGTGTTTCGGTAGT-3′. The reverse primer was 5′-CTAATCAACGACCTTGTATCTCCGCTTCGCGCTCATCAATA-3′. The TBR-probe was 5′-TBR-TAACTGAATAGACTAAGAC-3′ and the biotin-probe was 5′-biotin-GATACAAGGTCGTTGATTAG-3′. Universal sequences in primers have been underlined.

TCBS agar culture media and sodium chloride violet purple enrichment broth were obtained from Guangdong Huankai Microbial science & Technology Co. Ltd. *V. parahaemolyticus*, *V. alginolyticus*, *V. fluvialis* and infected seafood samples were obtained from College of Life Science, South China Normal University (Guangzhou, China). Uninfected samples were bought from the local super market.

2.2. Apparatus

A custom-built ECL detection system has been described in our previous studies (Liu et al., 2004; Zhou, Xing, & Zhu, 2009). It is composed of an electrochemical reaction cell, a potentiostat (Sanming Fujian HDV-7C), an ultra high sensitivity single photon counting module (Channel Photomultiplier, Perkin–Elmer MP-962), a multi-function acquisition card (Advantech PCL-836), a computer and labview software. The electrochemical reaction cell contains a working electrode (platinum), a counter electrode (platinum), and a reference electrode (Ag/AgCl).

2.3. General

The basic principle of the method is outlined in Fig. 1. Briefly, the genome of *V. parahaemolyticus* was extracted and a 324-bases DNA fragment in *gyrB* was amplified by PCR. Two universal sequences were introduced to the 5′ terminal of primers, respectively. The universal serial of sense primer is complementary to the TBR-probe sequences and that of anti-sense primers is complementary to biotin-probe sequences. After PCR amplification, all the PCR products contain universal serials. So the amplified PCR products can hybridize with the TBR-probe and biotin-probe. Non-specific amplified products cannot hybridize with the TBR-probe and biotin-probe. After hybridization, biotinylated DNA were bound to streptavidin coated paramagnetic beads (Blackburn et al., 1991). This kept the biotinylated DNA in the electrochemical reaction cell of the instrument, while all other components were washed away. The TBR-probe would emit light on the anode surface (Yan, Xing, & Tan, 2004), the light was captured by an ECL detection system and then we can read it out through Labview software. Finally, we could confirm whether *V. parahaemolyticus* existed.

2.4. Preparation of samples and culture enrichment

V. parahaemolyticus were cultured in sodium chloride violet purple enrichment broth at 37 °C with shaking (Shiping, SPH-210A, China) and subcultured on TCBS agar for colony counting. Sea foods samples were shucked and homogenized with tissue homogenizer. A certain amount of *V. parahaemolyticus* was added to 50 mL sodium chloride violet purple enrichment broth containing 1 g sea foods tissue homogenate for seeded sea foods experiments. Unseeded tissue homogenizer in enrichment broth was used as a negative control.

2.5. DNA extraction

All the samples were grinded with tissue homogenizer, and subsequently the cetyltrimethyl ammonium bromide (CTAB) method was used for DNA sample extraction and purification. We extracted DNA from *V. parahaemolyticus, V. alginolyticus*, *V. fluvialis*, infected seafood samples and uninfected ones. Each DNA pellet was washed with cold 70% ethanol, dried and resuspended in 20 μL Tris–EDTA (pH 8.0) buffer. The DNA concentration and purity were determined using a biophotometer (Eppendorf, Hambury, Germany).

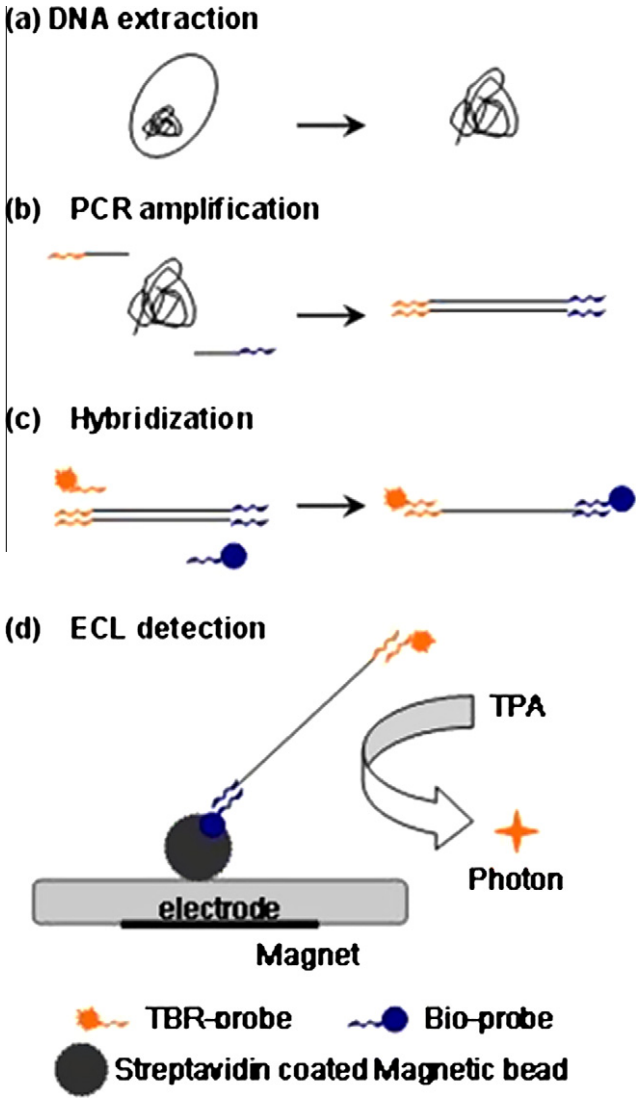

Fig. 1. The basic principle of this ECL-PCR method for detection of *V. parahaemolyticus*.

2.6. PCR amplification

The PCR of *V. parahaemolyticus* were carried out in 25 μL mixtures containing 1 μL of sample DNA, 2.5 μL 10× Taq polymerase buffer, 0.5 μL dNTP (10 mM), 15 pmol sense and anti-sense primers, 2.5 U Taq polymerase. The amplification reaction was performed on a commercial Mastercycler gradient PCR machine (Eppendorf, Hambury, Germany). Annealing temperature of *V. parahaemolyticus* PCR amplification is 55 °C, and the whole reaction contains 30 cycles.

2.7. Hybridization with probes

Hybridizations with biotin-probe and TBR-probe were performed by adding 20 μL of each to 10 μL of PCR products. The mixture was incubated for 5 min at 94 °C, then 1 h at 55 °C for *V. parahaemolyticus* in the PCR system.

2.8. ECL detection

Twenty microlitres of hybridization products was added to 170 μL Tris–EDTA buffers. Then, 10 μL of streptavidin coated magnetic beads was added. The mixture was then shaken at room temperature for 30 min. After washing and removing the supernatant, the samples were added to the ECL detection cell. Then, TPA was added to the reaction cell. A voltage of 1.25 V was applied across the electrodes and the signals of ECL were read using Labview software.

3. Results and discussion

3.1. Selection of oligonucleotide primers and probes

The primers of *V. parahaemolyticus* used in this paper have been reported before (Venkateswaran et al., 1998). Based on the genome sequences and primer sequences, two universal sequences, which were added at the 5′ terminals of the primers, were designed by our lab with the help of Primer Premier 5.0 software. Universal sequences in primers have been underlined. To verify whether the universal sequences would affect the specificity of PCR process, we amplified the *gyrB* gene of *V. parahaemolyticus* using the primers with or without universal serials. The amplified PCR products were analyzed by electrophoresis through a 2% agarose gel followed by staining with Goldview™ and observed under the gel image system (Quantity One™, Bio-RAD, CA, USA). As shown in Fig. 2, the specific band between 200 and 300 bp is examined when the PCR amplification is conducted with normal primers, compared with band between 300 and 400 bp when the PCR products amplified by primers with universal serials at the 5′ terminals. This result is coincident with the expected 285 bp PCR products of *gyrB* gene and 324 bp PCR products with two universal serials add to the 3′ and 5′ terminals, also indicating the validity and specificity of the primers.

3.2. Optimization of the method

Hybridization is an important step in this proposal. The hybridization efficiency of probes and PCR products has a great influence on the detection result. To obtain the highest hybridization efficiency, the concentration of probe and the pH value of hybridiza-

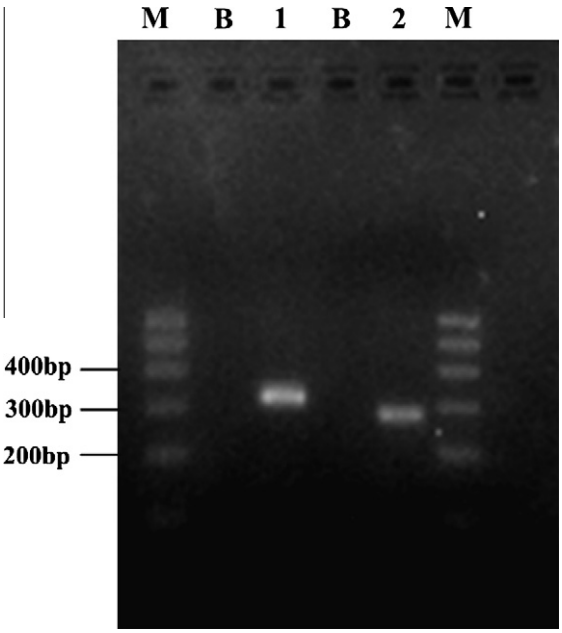

Fig. 2. Two percentage of agarose gel electrophoresis results. M, Marker; B, Blank; 1, PCR products amplified from primers without universal sequences; 2, PCR products amplified from primers with universal sequences.

tion buffer are optimized. Fig. 3(a) shows the effect of different probe concentrations to the hybridization process. When the concentration of probes is 0 – 20 times as much as that of primers, ECL intensity has a wide range increasing. While the rate exceeds 20, the increasing becomes flattened. The reason may be that the hybridization equilibrium was rapidly achieved with the increase of probe concentration, and the target sites saturated when high concentration of the probes were used. Taking both ECL signal intensity and detection cost into account, we set the concentration of probe as 20 times as primer's in the following experiments. pH value is also an important factor for nucleic acid hybridization. Suitable pH value can improve hybridization efficiency. In Fig. 3(b), the variety of ECL intensity on the pH range of 6.0 – 9.0 was studied. The ECL intensity increases with the increasing pH from 6.0 to 7.0, and decreases from 7.0 to 9.0. In the low pH value, a higher concentration of protonated species were produced for shielding phosphodiester backbones and permitting hybridization of DNA. However, if the pH is reduced below a critical level, the acidity will be too great to support hybridization between the nucleotide bases. In the high pH value, the repulsion is dominating between the negatively charged phosphate backbones. It therefore appears the maintenance of the pH near neutrality contribute to high hybridization efficiency. Therefore, we selected pH 7.0 as the optimal pH value.

The quantity of magnetic beads is critical to the ECL detection. The biotin-labeled DNA was linked to the surface of streptavidin-coupled beads though the highly selective biotin–streptavidin linkage (Monis & Giglio, 2006; Zhu et al., 2008). The unlinked DNA fragments can be washed away. The appropriate amount of magnetic beads can capture the entire special PCR products, so that improving the sensitivity and selectivity. But if the amount of magnetic beads is excess, the excessive beads will be absorbed on the surface of electrode, and hinder the reaction of TPA and TBR on the surface of electrode. We added 5 – 30 μL magnetic beads to the detection system, respectively. The ECL detection system gets the highest signal when the quantity of magnetic beads is 10 μL (Fig. 3(c)). So 10 μL beads are suitable to be added to each 20 μL hybridization products.

3.3. Detection of *V. parahaemolyticus* in a background of non-*V. parahaemolyticus*

3.3.1. Specificity of this method

To confirm this ECL-PCR method can specifically distinguish *V. parahaemolyticus*, we conducted a parallel experiment with *V. alginolyticus* and *V. fluvialis*. The detection results are displayed in Fig. 4. The signal of blank control is 96.14 ± 24.74 cps. The threshold value was calculated based on the mean of blank control plus

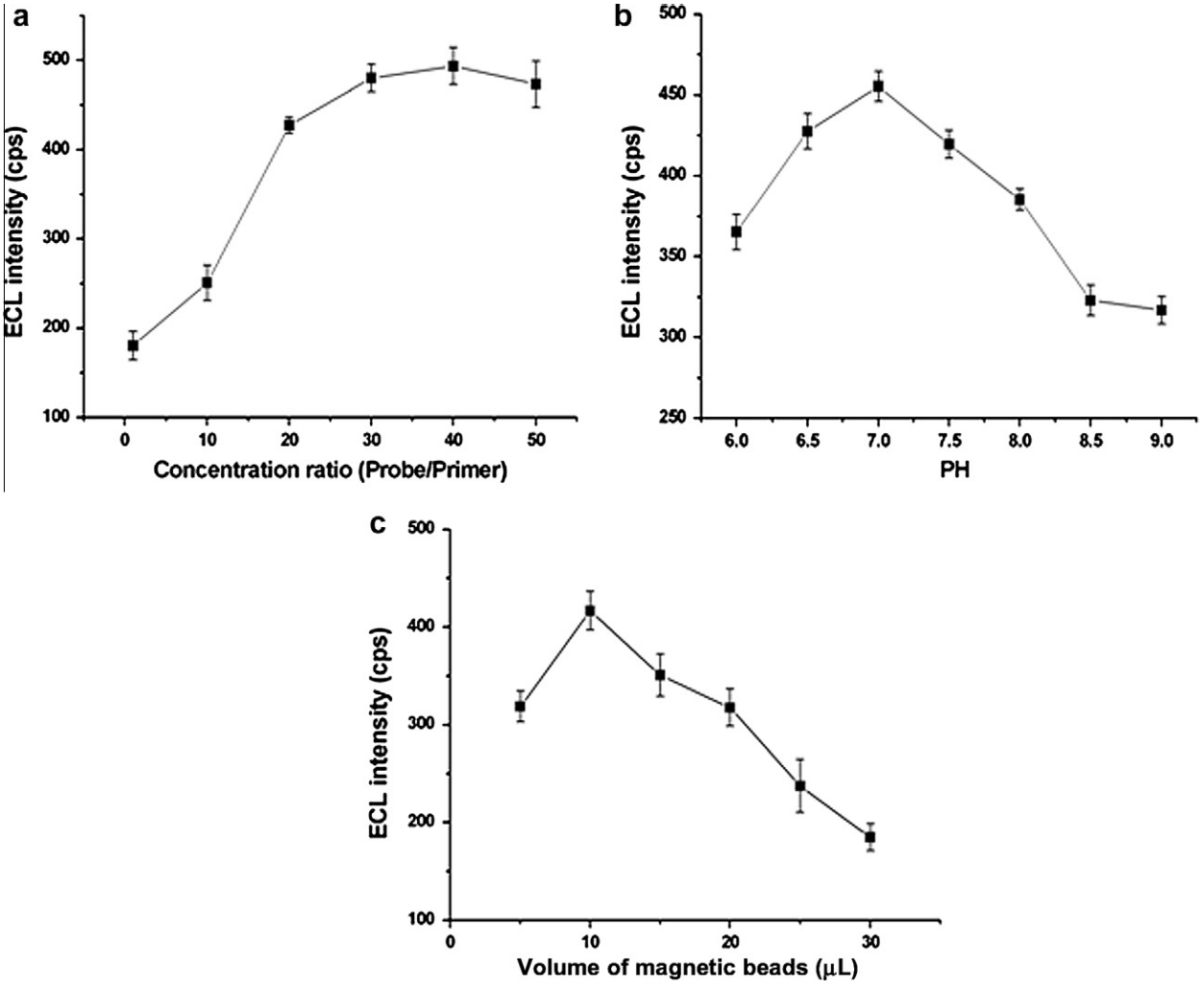

Fig. 3. Optimization of ECL detection. (a) Probe concentration optimization: the probes were added to hybridization system at concentrations of 1, 10, 20, 30, 40 and 50 times primer concentration (20 pmol). (b) Hybridization efficiency under different pH: hybridization running solution (pH 6.0 – 9.0, in 0.5 increment) was added to 10 μL PCR products. (c) The quantity of magnetic beads optimization: 5–30 μL magnetic beads in concentration of 10^5/μL were used to capture the hybridized products.

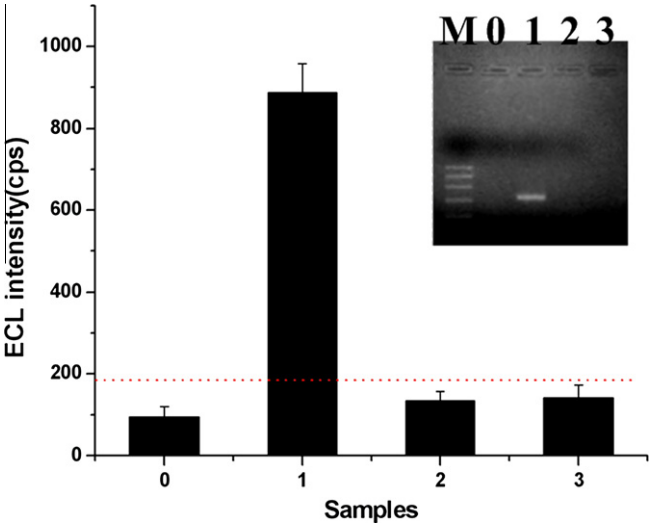

Fig. 4. Specificity of this ECL detection method. 0, Control; 1, *V. parahaemolyticus*; 2, *V. alginolyticus*; 3, *V. fluvialis*.

three times the standard deviation (SD) (DiCesare et al., 1993), which was 170.36 cps in the experiment. *V. parahaemolyticus* get a high signal of 877.98 ± 69.91 cps, while the signal of *V. alginolyticus* and *V. fluvialis* are 133.56 ± 22.54 and 140.78 ± 30.79 cps, respectively, which are below the threshold value. The agarose gel electrophoresis results are also shown in Fig. 4. A specific band between 300 and 400 bp is examined in lane 1, which is represent for *V. parahaemolyticus*, while no band appears in lanes 2 and 3, which is represent for *V. alginolyticus* and *V. fluvialis*, respectively. So *V. parahaemolyticus* can be well discriminated with this method, which is coincident with the agarose gel electrophoresis results in Fig. 4.

3.3.2. Effect of background non-specific bacteria

In the testing of natural samples, they may contain varieties of non-specific bacteria, which may greatly exceed the number of *V. parahaemolyticus*. To verify whether these non-specific bacteria will impact the testing process, the ability of detecting *V. parahaemolyticus* bacterial in the presence of non-specific bacteria (*V. alginolyticus* and *V. fluvialis*) was tested by adding non-specific bacteria to *V. parahaemolyticus*. Pure culture of *V. parahaemolyticus* was diluted to 10^5 CFU/mL. Approximately 1×10^5 to 1×10^8 CFU of an equal mixture of *V. alginolyticus* and *V. fluvialis* was added

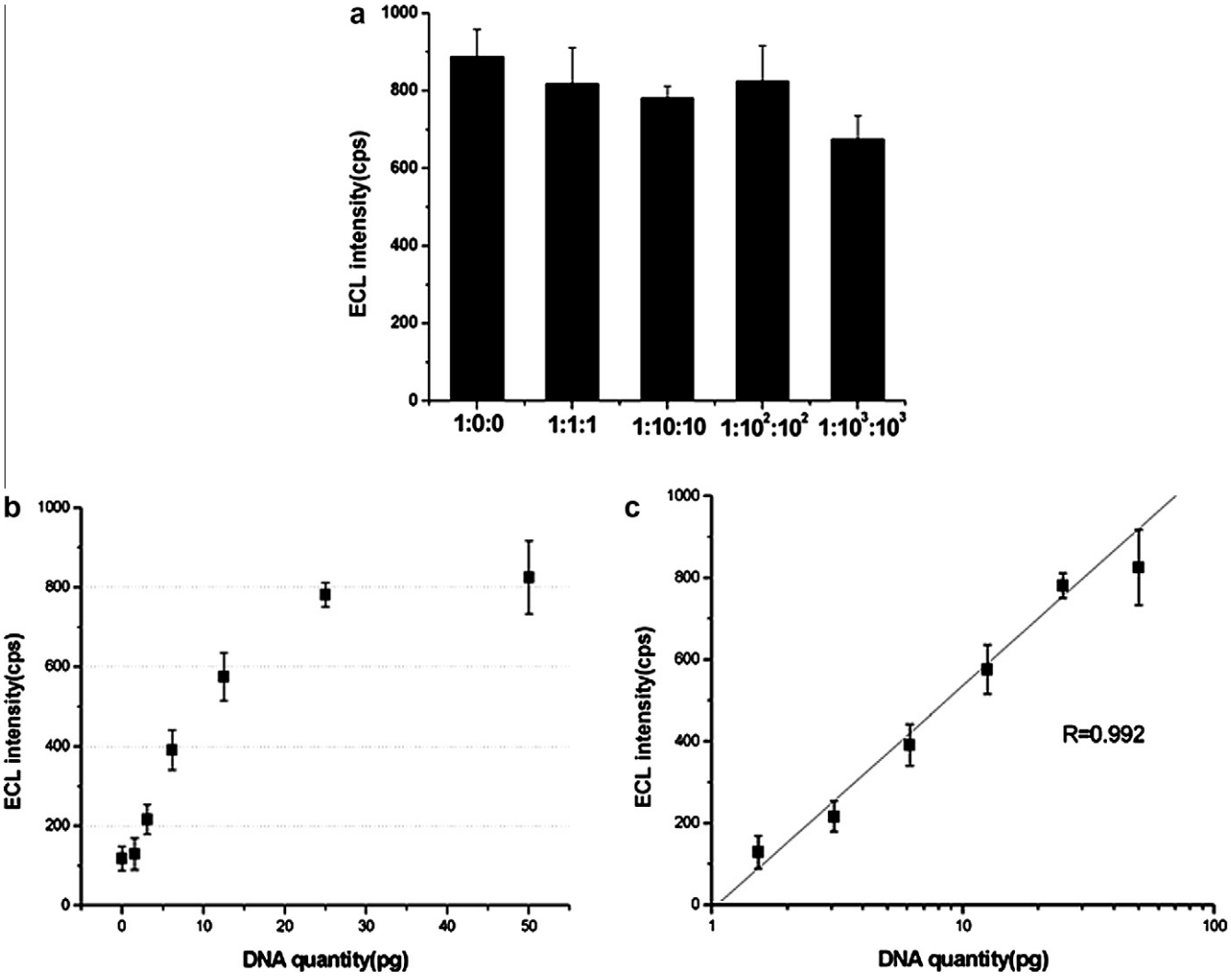

Fig. 5. Effect of non-*V. parahaemolyticus* background. (a) The effecting of non-specific DNA to the detection results. The ratio of *V. parahaemolyticus*:*V. alginolyticus*:*V. fluvialis* is 1:0:0, 1:1:1, 1:10:10, $1:10^2:10^2$, $1:10^3:10^3$, respectively. (b) Sensitivity of this ECL detection method with different quantities of *V. parahaemolyticus* DNA templates in the present of 1 μg other DNA (The ratio of *V. alginolyticus*:*V. fluvialis* is 1:1). (c) Calibration curve for the ECL system.

as background. The ratio of *V. parahaemolyticus*:*V. alginolyticus*:*V. fluvialis* is 1:0:0, 1:1:1, 1:10:10, $1:10^2:10^2$, $1:10^3:10^3$, respectively (Fig. 5(a)). The ECL signal of *V. parahaemolyticus* in the absence of non-specific bacteria is 869.66 ± 65.47 cps. Even though when the concentration of *V. alginolyticus* and *V. fluvialis* reaches to 10^8 CFU/mL, the ECL signal has a small change (675.21 ± 60.36 cps). So, the presence of background bacteria at a level of 10^8 CFU/mL has little influence on the *V. parahaemolyticus* detection.

3.3.3. Sensitivity of detection in the background non-specific DNA

The sensitivity of the assay was determined by using purified genomic DNA. Genomic DNA extracted from *V. parahaemolyticus* with 2-fold serially diluted in Tris–EDTA buffer (pH 8.0) to obtain amounts ranging from 50 to 1.6 pg. The external non-specific DNA was tested by adding different amounts of an equimolar mixture of DNA from *V. alginolyticus* and *V. fluvialis* to the system. The total amounts of external bacterial DNA added to the system were 1 μg. A PCR mixture containing no DNA was used as a negative control. As shown in Fig. 5(b), the minimum level of detection of purified *V. parahaemolyticus* genomic DNA in the background of 1 μg non-specific DNA is 1.6 pg. Standard curve (Fig. 5(c)) shows a good linear correlation between ECL signals and the amounts of genomic DNA.

3.4. Detection of V. parahaemolyticus in artificially contaminated sea foods and natural samples

In most of the detection methods, samples usually need to be enriched overnight to acquire enough amounts of bacteria (Ward & Bej, 2006). Given the excellent sensitivity of current assay, it may be allows us to reduce the enrichment time feasible. For testing the feasibility, pure cultures of *V. parahaemolyticus* was 10-fold serially diluted. One microlitre of diluted cultures were added in oyster homogenates. The mixture was enriched in 37 °C and detected with ECL-PCR method after 0, 1, 2, 4 and 8 h. The results are shown in Fig. 6(a). When 10 CFU/mL original *V. parahaemolyticus* culture was added, it can be detected in less than 4 h. When original *V. parahaemolyticus* culture was increased to 10^3 CFU/mL, it took only 1 h for enrichment to identify the bacterium. Some artificially contaminated sea foods were further detected to confirm the optimized enrichment time. Shrimp, oyster, shellfish and *Pseudosciaena* crocea are infected with 1 mL 10 CFU/mL original *V. parahaemolyticus* and enrichment for 4 h. As shown in Fig. 6(b), compared with the blank control's ECL signal

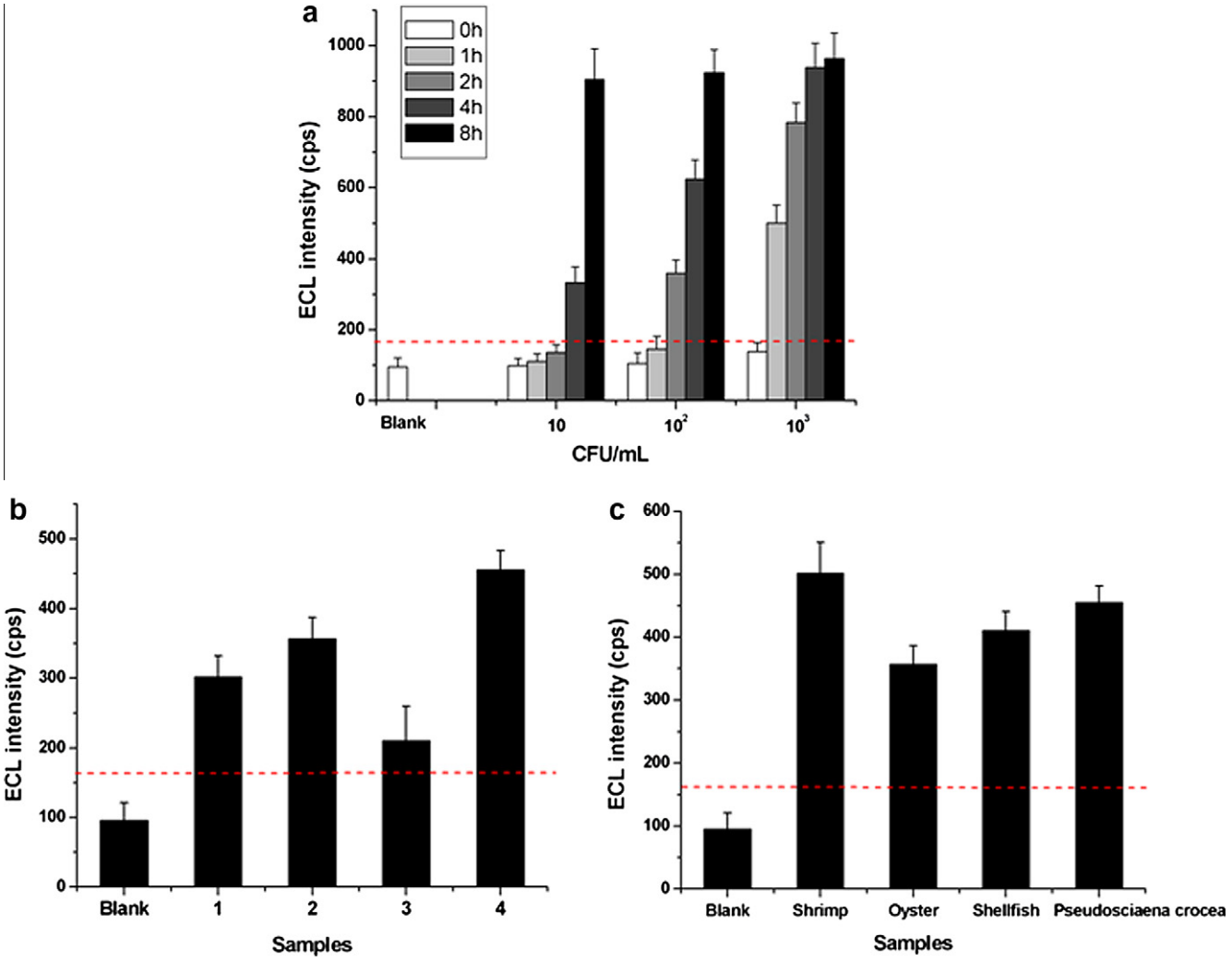

Fig. 6. The detection results of infected samples. (a) The detection results of artificial infected oyster samples. The copies of *V. parahaemolyticus* in tissue homogenate of shrimp are 10, 10^2 and 10^3, respectively. Samples in different culture time (0–8 h) are detected with PCR-ECL method. (b) The detection results of artificial infected sea foods samples. The four samples are shrimp, oyster, shellfish and *Pseudosciaena crocea*, respectively. (c) ECL detection results of infected samples. 1–2, infected oyster samples; 3–4, infected shrimp samples.

(95.45 ± 24.84 cps), the ECL intensity of four artificially contaminated samples were 501.69 ± 48.65, 356.55 ± 30.15, 410.62 ± 30.44 and 455.31 ± 25.67 cps, respectively. The samples can be well differentiated. So, enrichment duration of 4 h was sufficient to detect *V. parahaemolyticus* in sea foods homogenates. To determine whether *V. parahaemolyticus* could be detected in naturally contaminated samples with our ECL-PCR assay, we examined natural infected samples of shrimps and oysters (Fig. 6(c)). Samples 1 and 2 are shrimps; samples 3 and 4 are oysters. According to the blank control signals (98.63 ± 23.25 cps), the threshold value is set as 168.38 cps. ECL detection results of samples 1 – 4 are 301.69 ± 30.44, 367.76 ± 28.41, 210.62 ± 48.66 and 461.31 ± 28.71 cps, respectively, which are above the threshold value. So, we thought this method can clearly detect whether *V. parahaemolyticus* is existed or not.

4. Conclusion

ECL-PCR is a useful detection method because of its demonstrated combination of speed and sensitivity, both of which are critical to any assay for the detection of *V. parahaemolyticus*. Results indicate that our ECL-PCR system can successfully detect *V. parahaemolyticus* in natural sea foods samples. It can provide sensitive, specific and quantitative detection of *V. parahaemolyticus* in seafood without the elaborate process of characterization of bacteria by conventional microbiological methods. The test could be completed in about 4 h from the time of sample preparation. Its speed and facility will make it adaptable for identification of many bacterial pathogens and provide the potential for its adaptation for direct detection in many types of sea foods. Rapid detection like this should provide the seafood industry with early warning of potential health risks associated with potentially contaminated seafood and allow appropriate measures to prevent disease outbreaks to be swiftly undertaken. The use of an assay like this should be beneficial to both industry and consumer health.

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