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Development of a recombinase polymerase amplification (RPA) fluorescence assay for the detection of enterocytozoon hepatopenaei (EHP)

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ABSTRACT

The emerging microsporidian parasite Enterocytozoon hepatopenaei (EHP) causes retardation of shrimp growth, leading to significant financial losses in shrimp aquaculture. Therefore, the development of an efficient and sensitive detection method will be conducive to the prevention and control of the shrimp parasite. In this study, we developed and evaluated a rapid real-time recombinase polymerase amplification (RPA) method that can detect EHP within 15 min at a constant temperature of 38.5 °C. The detection limit of this EHP RPA was 10 copies/μL of DNA molecules per reaction. The specificity of EHP RPA was tested, and the assay did not cross-react with white spot syndrome virus (WSSV), shrimp hemocyte iridescent virus (SHIV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), or *Vibrio parahaemolyticus*. Field and clinical applicability of this assay was evaluated using 61 field samples. The coincidence rate of the detection results for the clinical samples between RPA and qPCR was 95.1 %. In summary, the real-time RPA analysis provides an efficient and sensitive detection method for EHP.

1. Introduction

In recent years, the emerging microsporidian parasite Enterocytozoon hepatopenaei (EHP) in Penaeus vannamei has spread throughout Asia (Tang et al., 2016). EHP was first reported in Thailand in 2009 (Tourtip et al., 2009). In China, EHP has been identified in cultured shrimp since 2013 (Ma et al., 2019a). EHP was considered to be a new species belonging to the genus Enterocytozoon. The microsporidian is replicated in the cytoplasmic area of the tubule epithelial cells of the hepatopancreas (Tourtip et al., 2009). In general, EHP can slow the growth of shrimp without causing death (Santhoshkumar et al., 2017), and thus can cause a reduction in breeding output, thereby imposing a continuous threat to shrimp farming industries (Thamizhvanan et al., 2019). However, no pathognomonic signs can be detected, and it is difficult to identify the EHP infection by visual examination (Ma et al., 2019a).

Polymerase chain reaction (PCR), quantitative PCR (qPCR) methods and loop-mediated isothermal amplification (LAMP) are all highly sensitive and specific as they target and amplify DNA from field and clinical samples. All methods are widely used for the detection of microorganisms (Cai et al., 2018; Liu et al., 2018). However, the methods require complex instrumentation, costly cold-chain-dependent reagents, and extended time in endemic field settings.

Recombinase polymerase amplification (RPA) is a rapid and highly sensitive isothermal amplification method that utilizes a recombinase to facilitate the insertion of oligonucleotide primers into their complement

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in a double-stranded DNA molecule (Piepenburg et al., 2006; Lobato and O'Sullivan, 2018). This reaction can be performed at a relatively low and constant temperature in the range of 35 °C – 42 °C. Thus, RPA has great potential for a variety of applications in parasite detection. In this work, a simple, rapid, and specific detection method for EHP has been established.

2. Materials and methods

2.1. Clinical samples and viruses

A total of 61 field samples were collected from dead shrimp with suspected cases of EHP. The body length range was 5.6–8.9 cm, and the weight range was 2.2–3.7 g. The hepatopancreas tissues of shrimp were obtained from each shrimp and stored at −20 °C.

White spot syndrome virus (WSSV), shrimp hemocyte iridescent virus (SHIV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and *Vibrio parahaemolyticus* were obtained from Guangdong Pearl River Fisheries Institute and were stored at the Guangdong Laboratory Animal Monitoring Institute.

2.2. DNA extraction

DNA of WSSV, IHHNV, SHIV, EHP and *Vibrio parahaemolyticus* were extracted from 400 μL tissue fluid. According to the instruction of TGuide Virus DNA/RNA Kit (Tiangen Biotech, Beijing, China), 400 μL tissue solution, 40 μL protease K and 12 μL Carrier RNA solution were added to the sample tube, and then the sample tube was placed at hole 4 of the TGuide M48-TIANGEN (Tiangen Biotech, Beijing, China). Program 202 was selected to run. Positive plasmids of EHP was extracted by Plasmid Mini Kit I (50) (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. Viral genomic DNA and plasmids were stored at −80 °C.

2.3. Primer and probe design

Primers and probes used in this study were designed based on the conservative SSU rRNA of the EHP genome. Twenty-six EHP sequences (from GenBank) based on SSU rRNA were aligned using MegAlign software (Tourtip et al., 2009; Liu et al., 2018). Four sets of primers and probes based on EHP (GenBank KF362130.1) were designed according to the recommendations from TwistDxCo. Ltd (https://www.twistdx.co.uk/en/support/rpa-assay-design-2). The primer sets determined were synthesized by Sangon Biotech (Shanghai) Co. Ltd (Table 1).

2.4. The construction of a standard recombinant plasmid

PCR was conducted in a total volume of 20 μL containing 10 μL of 2× Premix rTaq, 7 μL of ddH_2O, 1 μL of forward primer EHP-F4 (c = 10 μM), 1 μL reverse primer EHP-R3 (c = 10 μM), and 1 μL (10 ng) of DNA. The PCR products were purified using the Axygen Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and cloned into pMD18-T (TaKaRa, Tokyo, Japan). Three to five independent clones of each amplicon were sequenced. The plasmids were sequenced using an ABI 3730XL Sanger-based Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Positive plasmids were extracted using a Plasmid Mini Kit I (200) (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol and were stored at −20 °C.

Table 1
Sequences of primers and probe for the real-time RT-RPA assay.

Name	Sequence
EHP-F1:210−243	5′-CAATTAAAAGGTGGTGTTAAAAGCCATTGAG-3′
EHP-R1:325−353	5′-ATACGTCTAAGAGCATCGCTTTCGCCTCC-3′
EHP-F2:147−175	5′-CGGTAATTCCAACTCCAAGAGTGTCTATG-3′
EHP-R2:380−409	5′-AACTACAGCGGTGTCTAATCACTTTCGATA-3′
EHP-F3:127−155	5′-AGTTTTGGTGCCAGCAGCCGCGGTAATTC-3′
EHP-R3:421−449	5′-TCTCGCAACACCCAGCATTGTCGGCATAG-3′
EHP-F4:107−136	5′-CGTGAAGCAATTGGAGGGCAAGTTTTGGT-3′
EHP-R4:318−347	5′-CTAAGAGCATCGCTTTCGCCTCCGTTGGTC-3′
Probe:248−294	5′-TTGAGAGTAGCGGAACGGATAGGGAGCA/i6FAMdT /G/idSp//iBHQ1dT/ATAGGTGGGCAAAGA/C3Spacer-3′

2.5. Development of RPA assays

RPA was conducted in a total of volume of 50 μL containing 2.5 μL of Mg^{2+} buffer (c =280 mM), 2 μL of forward primer (c = 10 μM), 2 μL of reverse primer (c = 10 μM), 0.6 μL probe (c = 10 μM), 2 μL DNA template, 1 tube of basic reaction unit, and 38.9 μL of sterile water. The cycling parameter for the Rotor-Gene Q fluorescent quantitative PCR (Qiagen, Hilden, Germany) included 38.5 °C for 15 min (30 cycle; 1 cycle = 30 s). Optimal reaction conditions were defined after testing different primers and probe matches (a set of 16 different matches was made from four forward primers and four reverse primers), different primer ratios (c = 10 μM), seven primer concentration ratios (1.0 μL and 2.0 μL; 1.0 μL and 3.0 μL; 2.0 μL and 1.0 μL; 2.0 μL and 3.0 μL; 3.0 μL and 1.0 μL; 3.0 μL and 2.0 μL; 2.0 μL and 2.0 μL), different primer concentrations (c = 10 μM), (0.5 μL and 1.5 μL; 1 μL and 3 μL; 1.5 μL and 4.5 μL; 2.0 μL and 6.0 μL; 2.5 μL and 7.5 μL; 3 μL and 9 μL), different Mg^{2+} concentrations (c =280 mM) (1.5 μL, 2.0 μL, 2.5 μL, 3.0 μL, and 3.5 μL), and different probe concentrations (c = 10 μM) (0.3 μL, 0.6 μL, 0.9 μL, 1.2 μL, and 1.5 μL).

2.6. Analytical sensitivity and analytical specificity

Ten-fold serial dilutions of the EHP DNA standard from 10^4 copies/μL to 10^0 copies/μL were prepared and tested in three replicates for two independent runs to determine the sensitivity of the RPA assay. The threshold time was plotted against molecules detected, and a semi-logarithmic regression was calculated using Prism 7.0 software (GraphPad Software Inc., San Diego, California, USA). Moreover, a probit regression was performed to determine the limit of detection (LOD) of the RPA assay at 94 % probability.

The specificity of the EHP RPA assay was evaluated by testing a panel of pathogens including the WSSV, SHIV, IHHNV, and *Vibrio parahaemolyticus*. DNA extraction from one EHP-positive verified sample was tested concurrently as a positive control. No template served as a negative control.

2.7. Detection of field samples

The samples were tested to evaluate the sensitivity and specificity of the EHP RPA assay and were compared with qPCR to check for any nonspecific amplification (Liu et al., 2018). Briefly, a pair of specific primers, F157 :5′-AGT AAA CTA TGC CGA CAA-3′and R157: 5′-AAT TAA GCA GCA CAA TCC-3′, and a TaqMan probe: 5′-FAM-TCC TGG TAG TGT CCT TCC GT-TAMRA-3′were designed based on the SSU rRNA sequence of EHP. The TaqMan probe real-time PCR amplification system in 25 μL contained 2× Premix Ex Taq™ 12.5 μL (TaKaRa, Dalian, China), 10 μM primers F157 and R157 1 μL, 10 μM TaqMan probe 0.625 μL, 50 ng of DNA template, and nuclease-free water was added to make a volume of 25 μL. The amplification reaction was performed in a qPCR instrument Rotor-Gene Q fluorescent quantitative PCR (Qiagen, Hilden, Germany) and the reaction procedure was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The association between RPA threshold time (TT) and qPCR cycle threshold (Ct) values was analyzed by Prism 7.0 software (GraphPad Software Inc., San Diego, California, USA).

3. Results

3.1. Optimization of the EHP RPA assay

First, the relative performances of the candidate primer sets were evaluated and compared. A set of 16 different matches was assessed by the basic RT-RPA assay. As shown in Fig. 1, the combination of EHP-F1 and EHP-R4 was optimal based on the least Ct and the highest fluorescence value. Furthermore, according to the references, the fluorescence value could be affected by the primer ratios. In Fig. 2a and b, the experiment is to find the best ratio of primers. Radio of forward primer and reverse primer 1:2, 1:3, 2:1, 2:3, 3:1, 3:2 and 1:1 were tested in Fig. 2a. Radio of forward primer and reverse primer 2:3, 1:2, 1:3, 1:4, 1:5 and 1:6 were tested in Fig. 2b. The results showed that the optimal primer ratio was 1:3. Based on the primer ratio, the primer concentration needed to be confirmed in the system. As shown in Fig. 2c, the resulting primer concentration was forward primer (c = 10 μM) 1.5 μL and reverse primer (c = 10 μM) 4.5 μL in a 50 μL reaction system. In addition to the different primer concentrations being tested, the optimal Mg^{2+} concentration was achieved with 2.5 μL (c =280 mM), and the resulting probe concentration was 1.2 μL (c = 10 μM).

Finally, the optimal reaction system was 1.5 μL of forward primer (c = 10 μM), 4.5 μL of reverse primer (c = 10 μM), 2.5 μL Mg^{2+} (c =280 mM), 1.2 μL probe (c = 10 μM), 1 tube of basic reaction unit, 2.0 μL DNA template, and 38.3 μL sterile water.

3.2. Sensitivity and specificity of the EHP RPA assay

When the serial dilutions of EHP DNA were tested by the real-time recombinase polymerase amplification (RPA) assay, fluorescence signals were produced for a wide range of DNA molecule input levels. As illustrated in Fig. 3b, DNA copy number over a range of 0 log10 DNA copies to 4 log10 DNA copies per reaction produced positive fluorescence signals within 2.5–12.5 min. To evaluate the reproducibility of the real-time RPA assay, serial dilutions of DNA standard were tested for eight replicates. Three out of three (3/3) runs produced positive signals when DNA input was 10^4–10° copies per reaction, and 0/3 were positive when one copy of DNA per reaction was used. Semilogarithmic regression analysis was performed using the data from the three runs (Fig. 4a). The results showed that the real-time RT-RPA assay could detect EHP at concentrations as low as 10 copies/μL molecular DNA.

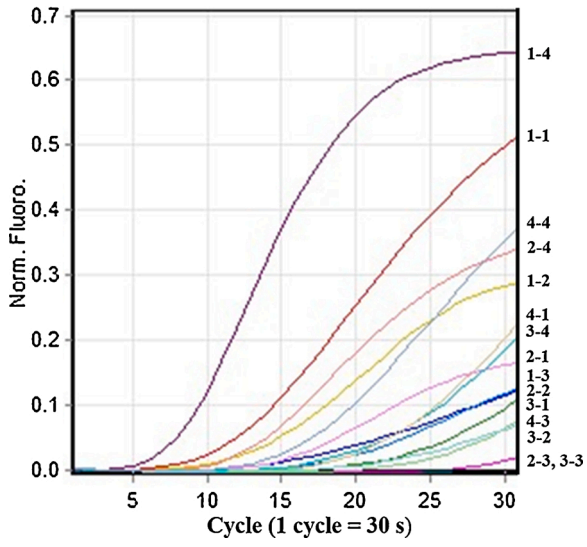

Fig. 1. Primer sets screening.
The initial sequence number represents the sequence number of the forward primer, and the latter sequence number represents the sequence number of the reverse primer. (The first number represents the forward primer and the second number represents the reverse primer, e.g., 1−1 : EHP-F1 and EHP-R1)

The specificity of the EHP RPA assay was determined using nucleic acids extracted from a panel of four shrimp pathogens. As shown in Fig. 3a, positive products were amplified only from EHP, and the curves of the WSSV, SHIV, IHHNV, and *Vibrio parahaemolyticus* showed no amplification. The specificity test of the EHP RPA revealed that the assay showed no cross-reactivity with other aquatic pathogens, indicating that the assay was highly specific.

3.3. Detection of field samples

A total of 61 field samples comprising 30 samples from the Guangdong Pearl River Fisheries Institute and 31 samples from a fish pond in Guangdong were tested for EHP by the RPA assay, and the results were compared with those from the gold standard qPCR. Out of the 30 samples, 6 tested positive in the RPA assay, and 5 were positive by qPCR. Among 31 samples, 8 were positive in the RPA assay, and 6 were positive by qPCR. The agreement between the EHP RPA assay and qPCR was 95.1 % (Table 2). Linear regression analysis of the 11 positive samples that were detected as EHP positive by both real-time RPA and real-time PCR showed that there was a good correlation between the TT and CT values (Fig. 4b). As revealed by the results, these two methods were consistent in clinical sample detection, suggesting the potential of RPA for clinical diagnosis.

4. Discussion

EHP is a newly emerged microsporidian parasite that poses a serious threat to shrimp aquaculture worldwide (Santhoshkumar et al., 2017; Chayaburakul et al., 2004). EHP infections cause slow growth of penaeid shrimp but do not result in the death of the shrimp. This can inflict significant economic losses in shrimp aquaculture (Jaroenlak et al., 2018). However, no effective approach has been reported to eliminate this disease. Thus, rapid and convenient diagnosis of EHP plays a key role in the intervention and implementation of preventive measures at the farm level (Salachan et al., 2017). In this study, we describe a simple, rapid, sensitive, and specific diagnostic tool for diagnosis of EHP using the RPA assay. Recombinase polymerase amplification (RPA) is a rapid, sensitive, and convenient amplification technique (Zeng et al., 2019). The reaction can be performed with minimal sample preparation, and the detection limit can be as low as 1–10 DNA target copies. The reaction can be performed in less than 15 min using simple isothermal amplification facilities and less-trained staff (Ma et al., 2019b).

Recently, many methods have been developed to detect EHP infection, including transmission electron microscopy, histological observation (Cai et al., 2018), PCR assay (Jaroenlak et al., 2016), in situ hybridization (Tang et al., 2015), real-time PCR (Liu et al., 2018) and LAMP (Cai et al., 2018; Sathish Kumar et al., 2018). All of these techniques require complicated assay procedures, expensive reagents, and well-trained staff (Sathish Kumar et al., 2018). Furthermore, the methods require complex instrumentation, cold-chain-dependent reagents that are costly, and extended time in endemic field settings.

However, the new recombinase polymerase amplification (RPA) method does not require changing the temperature to unchain, anneal, and extend the DNA double strand. The results can be obtained at a constant temperature environment of 35 °C – 42 °C in about 15 min. RPA has been applied to viral pathogens of aquatic organisms, such as WSSV (Xia et al., 2014), IHHNV (Xia et al., 2015), and SHIV (Chen et al., 2019), and of Schistosoma such as haematobium (Rostron et al., 2019), Perkinsus beihaiensis (Wu et al., 2019), Burkholderia pseudomallei (Peng et al., 2019), Brucella (Gumaa et al., 2019), and Glaesserella parasuis (Zhang et al., 2019).

In this study, a real-time RPA assay was successfully developed for detecting the SSU of EHP, and the test was more sensitive than the present qPCR assay (Piamsomboon et al., 2019). In addition, we evaluated the reaction concentration relationship between the forward and

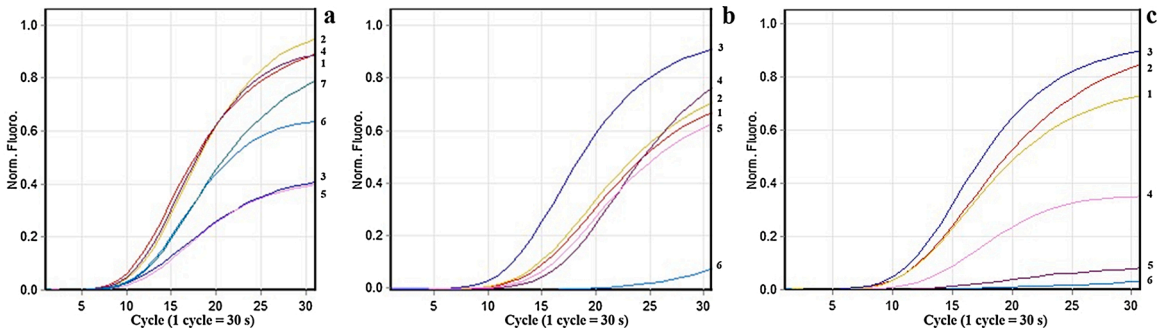

Fig. 2. Optimization and identification of RPA reaction conditions for EHP detection.
The first number represents the forward primer and the second number represents the reverse primer, e.g., 0.5 μL and 1.0 μL represents 0.5 μL EHP-F1 and 1.0 μL EHP-R4.
(a) Primer ratio optimization. The curves represent the different matches of primer ratios.
1: 0.5 μL and 1.0 μL; 2: 0.5 and 1.5 μL; 3: 1.0 μL and 0.5 μL; 4: 1.0 μL and 1.5 μL; 5: 1.5 μL and 0.5 μL; 6: 1.5 μL and 1.0 μL; 7: 1.0 μL and 1.0 μL.
(b) Primer ratio optimization II. The curves represent the different match of primer ratio
1: 1.0 μL and 1.5 μL; 2: 1.0 μL and 2.0 μL; 3: 1.0 μL and 3.0 μL; 4: 1.0 μL and 4.0 μL; 5: 1.0 μL and 5.0 μL; 6: 1.0 μL and 6.0 μL.
(c) Optimization of primer concentration. The curves represent the different matches of primer ratios.
1: 0.5 μL and 1.5 μL; 2: 1.0 μL and 3.0 μL; 3: 1.5 μL and 4.5 μL; 4: 2.0 μL and 6.0 μL; 5: 2.5 μL and 7.5 μL; 6: 3.0 μL and 9.0 μL.

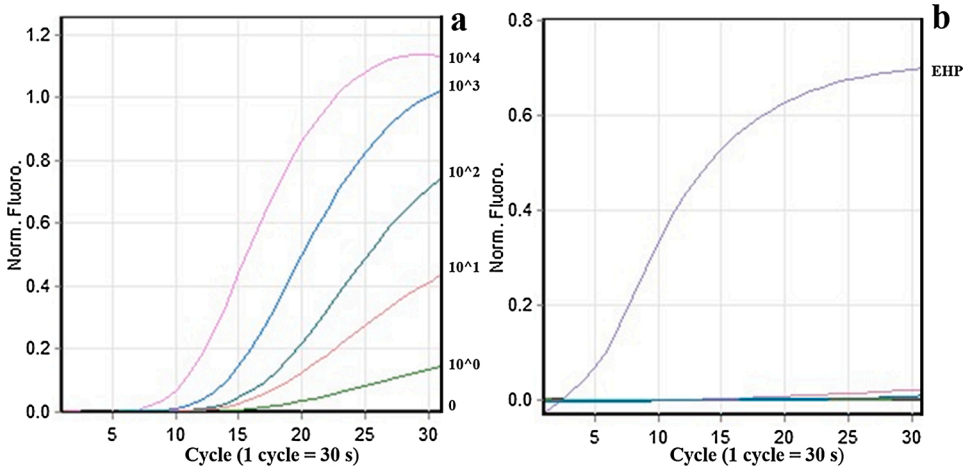

Fig. 3. Specificity and sensitivity.
(a) The sensitivity of the real-time RT-RPA assay. The number represents copies of DNA and N represents negative samples.
b) The specificity of the real-time RT-RPA assay. EHP, WSSV, SHIV, IHHNV, *Vibrio parahaemolyticus* and the negative samples.

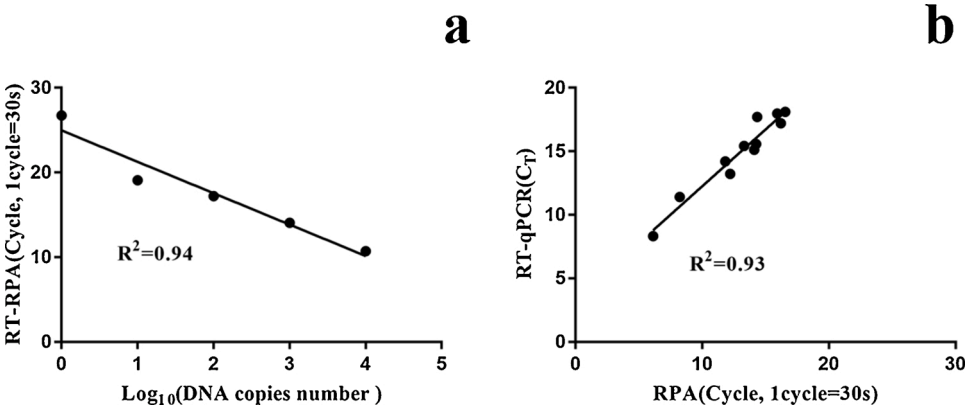

Fig. 4. Performance of the EHP RPA assay.
(a) Semi-logarithmic regression of the data collected from three runs using the DNA standard analyzed by GraphPad Prism 5.0.
(b) Linear regression analysis of RT-RPA threshold time (TT, y-axis) and RT-PCR cycle threshold (CT) values (x-axis) determined by Prism software.

Table 2
Coincidence rate of RPA and qPCR.

		qPCR			CR
		Positive	Negative	Total	
RPA	Positive	11	3	14	
	Negative	0	47	47	95.1 %
	Total	11	50	61	

reverse primers and probes. In general, most primer concentrations are employed in a ratio of 1:1 (Ma et al., 2019c), whereas according to the characteristics of RPA primer and probe design, the base and fluorescent groups in front of the IDSP group will be cleaved off by activity of 5′-3′endonuclease (Lobato and O'Sullivan, 2018; Kim and Lee, 2017). The quenched group after the IDSP group will then remain on the amplification product. However, based on asymmetric PCR (Poddar, 2000), the concentration ratio of the primers in the RPA was 1:3, which was conducive to the amplification of single-stranded DNA bound to the probe and improved the binding rate between the probe and the template, thus making the RPA reaction more efficient. This experiment established the most appropriate ratio between the forward primer and the reverse primer in the RPA reaction of EHP. The optimal ratio increased the detection sensitivity and improved the detection efficiency to some extent. Therefore, the fluorescent group and the quenched group can be more efficiently separated to emit fluorescence.

Recently, a real-time PCR assay was employed to detect EHP; the method had a sensitivity of 40 copies per sample (Liu et al., 2018). We established that the present RPA assay showed a higher sensitivity of 10 copies per reaction compared to qPCR. As expected, the isothermal RPA was performed at 38.5 °C, whereas the two-step thermal cycling qPCR had to be conducted at 60 °C – 94 °C. The low and constant reaction temperature of RPA paves the way for simpler devices and for mobile pond-site and point-of-care detection of EHP. In addition, the detection limit of RPA is as low as 10 copies of EHP DNA per reaction, which is comparable to that of qPCR. The reaction time of RPA was less than 15 min; by contrast, approximately 35 cycles were required in the qPCR assay in order to achieve a similar detection sensitivity as in the RPA. Obviously, the RPA assay could save much more time in comparison with the qPCR. Compared with LAMP, RPA was more efficient and convenient. A real-time LAMP assay for EHP has been reported in 2018 (Sathish Kumar et al., 2018). Although both of them reaction time and sensitivity were similar, different reaction time and different plasmid concentration in RPA were more discriminative than in LAMP. This phenomenon can illustrate the method of RPA is more sensitive and dependable than LAMP. On the other hand, LAMP reaction temperature (60−65 °C) is higher than RPA (35−40 °C). In term of primer design, the method of LAMP needs more primer (3 pairs) than RPA (only 1 pair). Therefore, RPA assay was the preferred approach.

In our study, the nucleic acids extracted from three infected tissues samples by the magnetic bead-based kit were negative by RT-qPCR and positive by RT-RPA. The sequence of the three samples were tested and were positive samples indeed, which were all 138bp in length. However, no fluorescent signals in qPCR was observed for the extracts of the three infected tissues samples, which may suggest the samples had high levels of inhibitors (Ma et al., 2019c). One of the advantages of RPA over PCR was tolerance for amplification inhibitors, which has been proved by two previous studies (Moore and Jaykus, 2017; Krõlov et al., 2014).

There is a recent publication on EHP RPA (Zhou et al., 2020). This article was described an RPA assay for EHP detection, which could be performed at 30 °C for 40 min and sensitivity of that was 8×10^2 copies/μL was lower than that of qPCR and qRPA. In addition, that results were confirmed by the journal. For this manuscript, the fluorescent probe was utilized in the assay and firstly reported the concentration ratio of the forward and reverse primers, which could enhance the amplification efficiency. Then, the sensitivity of our EHP-RPA was consistent with qPCR. As the fluorescent probe was used in the reaction system, the resulting could be confirmed with visualizations as soon as 15 min.

To summarize, we developed a real-time RPA assay to amplify the SSU of EHP. The method can detect EHP within 15 min at 38.5 °C, and the detection limit of this EHP RPA was 10 copies/μL. Furthermore, EHP RPA did not cross-react with healthy shrimp DNA, WSSV, SHIV, IHHNV, or *Vibrio parahaemolyticus*. Compared with qPCR, the RPA assay is more convenient and less costly. It provides the possibility of rapid clinical detection of EHP infection.

CRediT authorship contribution statement

Gen Li: Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Feng Cong:** Resources, Supervision, Funding acquisition. **Weiyou Cai:** Resources. **Jinhui Li:** Methodology, Validation, Writing - review & editing. **Miaoli Wu:** Writing - review & editing, Data curation. **Li Xiao:** Resources. **Xiaoliang Hu:** Resources, Writing - original draft. **Weiwei Zeng:** Resources. **Dongsheng He:** Resources, Supervision, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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