On-Site Detection of *Enterocytozoon hepatopenaei* in Shrimp: A Rapid and Reliable Colorimetric PSR Method

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Abstract
Enterocytozoon hepatopenaei (EHP) is a microsporidian parasite that significantly threatens global shrimp farming, particularly affecting species like Penaeus monodon and Penaeus vannamei. Early detection of EHP is crucial for effective disease control in aquaculture environments. This study aimed to develop a rapid, sensitive and specific diagnostic method for identifying EHP in shrimp populations through an isothermal and direct colorimetric polymerase spiral reaction (PSR) assay. The PSR assay was optimized for performance, yielding the best results at 65°C for 40 minutes. We validated the specificity of our PSR primer set by testing it against a range of aquatic pathogens, confirming no cross-reactivity.

Furthermore, the limit of detection with a 95% probability was remarkably low, at just 7.142 copies of the target gene per reaction. Clinical trials on shrimp samples yielded encouraging results, with the direct colorimetric PSR method achieving 100% sensitivity and specificity compared to traditional qPCR assays. Notably, the PSR assay eliminates the need for DNA extraction, facilitating on-site detection at shrimp farms. In summary, the newly developed colorimetric PSR assay offers a quick and reliable option for detecting EHP, enhancing disease management in shrimp aquaculture through its simplicity, accuracy and cost-effectiveness.

Keywords: EHP, colorimetric PSR, on-site detection, aquatic pathogens.

Introduction

Enterocytozoon hepatopenaei (EHP) is a microsporidian parasite that poses significant challenges to shrimp aquaculture worldwide[5,31,37,40]. Discovered in 2009, EHP primarily infects the hepatopancreas of shrimp, an essential organ responsible for digestion and nutrient absorption[4,5,12]. This parasite predominantly affects species within the Penaeidae family, such as the giant tiger prawn (*Penaeus monodon*) and the whiteleg shrimp (*Penaeus vannamei*) which are commonly cultivated in shrimp farming[3,41]. The impact of EHP on infected shrimp includes stunted growth, reduced feed intake and a pale or yellowish hepatopancreas[1,39]. Severe infections can lead to diminished growth rates, heightened vulnerability to secondary infections and significant economic losses[1,11,24,28].

Managing EHP effectively requires a multifaceted approach involving stringent biosecurity measures, consistent monitoring for early detection and the implementation of targeted treatment protocols[5,24].Traditional diagnostic methods for EHP include histological inspection, *in situ* hybridization and various forms of polymerase chain reaction (PCR) such as quantitative PCR and nested PCR[7,26,29,35,45,51], each offering different levels of sensitivity, specificity, cost-effectiveness and practical utility. The choice of diagnostic method often depends on available resources, the scale of the operation and the required level of accuracy[32,44]. In many cases, a combination of these methods is employed to ensure comprehensive detection and monitoring of EHP in aquaculture settings [7,11,26,45,46].

Although PCR techniques are considered the gold standard for molecular diagnostics, they require sophisticated equipment's such as thermocyclers, electrophoresis apparatus and gel documentation systems, in addition to highly skilled personnel. This complexity makes PCR impractical for on-site application in the aquaculture field[29]. Isothermal amplification, which amplifies DNA or RNA at a constant temperature without the need for thermal cycling equipment, represents a significant advance in molecular biology[10,16,47]. Recent research on isothermal amplification techniques for detecting EHP in shrimp has yielded promising results.

Sathish Kumar et al[34] developed a loop-mediated isothermal amplification (LAMP) assay with high specificity and sensitivity, enabling early diagnosis and effective management of EHP infections. Enhancements to this approach include combining LAMP with colorimetric nanogold detection, making the process visually interpretable and accessible for field applications[2]. Another isothermal technique used for EHP detection is the recombinase polymerase amplification (RPA) assay which allows for rapid and visual detection with a lateral flow dipstick within 20 minutes, suitable for on-site testing[30,49]. However, these methods still require purified DNA, leading to extended experiment duration, increased costs and added complexity in sample testing. These challenges limit their application in resource-limited areas and hinder the development of rapid on-site detection methods.

Polymerase Spiral Reaction (PSR)[24], a newer isothermal PCR approach, offers several advantages over techniques

like LAMP and RPA. One key benefit is its simpler primer design, reducing complexity and cost compared to the multiple primers required for LAMP[9,14,19,20]. The ease of use associated with PSR, including its straightforward primer design and amplification process, makes it particularly suitable for resource-limited settings and users with less technical expertise[9]. The unique spiral amplification mechanism of PSR also lowers the risk of non-specific amplification compared to RPA, simplifying the process and reducing the need for meticulous optimization[22]. The cost-effectiveness of PSR, due to fewer reagents and basic design requirements, further enhances its appeal[9,14,50,52]. These attributes allow PSR to be readily adapted for various applications including field diagnostics and point-of-care testing, where rapid and reliable results are crucial.

PSR's simplicity, speed, specificity, ease of use and cost-effectiveness make it a compelling alternative to LAMP in many diagnostic scenarios[14,20]. Utilizing PSR with specific primers that target DNA sequences, enables rapid and sensitive detection of pathogens[8,9,13,15,22,25,42,48].

This study introduces a novel colorimetric PSR assay that enhances the speed, accuracy and cost-efficiency of on-site EHP detection. By implementing the direct EHP-PSR approach this method simplifies the diagnostic process by eliminating the need for conventional DNA extraction. The assay demonstrates high sensitivity and specificity compared to PCR, enabling the detection of minimal amounts of the parasite in host populations. This innovative colorimetric PSR assay represents significant progress in diagnosing EHP, offering a valuable tool for managing diseases in prawn farming.

Material and Methods

Sample collection and preparation: *P. vannamei* shrimps, aged 40–91 days and ranging from juveniles to sub-adults, were collected from aquaculture operations in Tien Giang and Ben Tre provinces, Vietnam. These farms were experiencing issues with inconsistent shrimp sizes, growth delays and white feces syndrome during this stage of cultivation. Live shrimp were collected and transported to the laboratory, preserved on ice to maintain their condition. Concurrently, water samples alongside regular and white fecal strings from the same ponds were gathered and kept cool during transit. Each 25-miligram sample of intestine and hepatopancreas was homogenized with nuclease-free water to serve as the crude specimen. Genomic DNA was extracted from the crude specimen using the Monarch® Genomic DNA purification kit (NEB, UK) and analyzed using a qPCR assay.

PSR primer design: The design of specific primers was based on the nucleotide sequence of the small subunit ribosomal RNA (SSU rRNA) gene, obtained from the GenBank database (Accession no. MT539781.1). Following the principles of PSR-specific primer design previously described[21], a forward primer (F) and a reverse primer (B) were developed, incorporating Nr and N sequences derived from a botanical gene. These spiral primers, designated EHP-Ft and EHP-Bt, specifically target the SSU rRNA gene sequence, as illustrated in figure 1. To enhance the efficiency of the reaction, the IDT PrimerQuest Tool was used to design two additional rapid primers, EHP-IF and EHP-IB. The sequences of all primers used in this study are detailed in table 1.

Figure 1: Diagram illustrating the position of primers in the PSR assay.

Table 1
Oligonucleotides used in this study

Name	Sequence 5'-3'
EHP-IF	ACGGACCCTTTAACTGCAGCA
EHP-IB	AGTAGCGGAACGGATAGGGAGC
EHP-Ft	acgattcgtacatagaagtatagTGGAGGGCAAGTTTTGGTG
EHP-Bt	gatatgaagatacatgcttagcaGAGCATCGCTTTCGCCTC
EHP-510F	GCCTGAGAGATGGCTCCCACG
EHP-510R	GCGTACTATCCCCAGAGCCCG
EHP-gblock	AGAAGGGTCGAGTGTAAAAACCTTGACGTGAAGCAATTGGAGGGCAAGTTTTG GTGCCAGCAGCCGCGGTAATTCCAACTCCAAGAGTGTCTATGGTGGATGCTGCA GTTAAAGGGTCCGTAGTCGTAGATGCAATTAAAAGGTGGCGTTAAAAGCCATTG AGTTTGTTGAGAGTAGCGGAACGGATAGGGAGCATGGTATAGGTGGGCAAAGA ATGAAATCTCAAGACCCCACCTGGACCAACGGAGGCGAAAGCGATGCTCTTAG ACGTATCTGGGGATCAAGGACGAAGGCTAGAG

Colorimetric PSR assay: The colorimetric PSR assay was performed using a 15 µL reaction mixture which included 7.5 µL of WarmStart Colorimetric LAMP 2X Master Mix (DNA and RNA) (NEB, UK), 1.6 µM each of EHP-Ft and EHP-Bt primers, 0.2 µM each of EHP-IF and EHP-IB primers, 5 µL of template and nuclease-free water. The PSR amplification was conducted in a Benchmark Dry Bath (USA) at 65°C for 40 minutes. Amplification products were visualized by a color change from red to yellow in the test reaction, using phenol red as a pH-sensitive indicator, as recommended by the manufacturer. The positive control sample contained 10^2 copies per reaction of synthetic EHP DNA (EHP-gblock in table 1). The no-template control used nuclease-free water to check for contamination while the negative control sample contained DNA extracted from healthy shrimp (10 ng/reaction) confirming EHP-negative by qPCR to ensure specificity.

Limit of detection (LOD) and primer specificity of PSR assay: To determine the assay's limit of detection (LOD95), serial dilutions of synthetic EHP DNA were prepared, ranging from 0 to 10^2 copies per reaction. Results were calculated using PoDLoD software[52]. Primer specificity was assessed using common bacterial strains and closely related parasites with FastPCR software. Additionally, DNA samples extracted using Exgene™ Clinic SV (GeneAll, Korea) from different bacterial species (Table 2) were tested as templates in a direct PSR assay, with each strain used at a concentration of 1 ng/reaction.

qPCR assay: The qPCR reactions were performed using a total volume of 15 µl per reaction. This included the use of 2X Luna® Universal qPCR Master Mix (NEB, UK), 0.4 µmol of each primer (EHP-510F and EHP-510R) (Table 1) specified in the Vietnamese standard for EHP detection in shrimp (TCVN 8710-12:2019) and 5 µl of the DNA template. The qPCR reactions were carried out on a Prime Pro 48 system (Techne, UK). The thermal cycling conditions included an initial denaturation step at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds and a combined annealing and extension phase at 60°C for 20 seconds.

Direct colorimetric EHP PSR assay with shrimp samples: To determine the appropriate dilution factor of the crude specimen for the PSR reaction, extracted EHP DNA (Ct = 18.67 by qPCR) was spiked into homogenized shrimp samples at a ratio of 1:10. The spiked sample was then serially diluted by 5–100 fold in nuclease-free water. Subsequently, 5 µL of each diluted sample was directly added to the PSR reaction mixtures. Reactions were incubated at 65°C for 40 minutes. To evaluate the sensitivity and specificity of the Direct Colorimetric EHP PSR assay, sixty shrimp samples, both symptomatic and asymptomatic, were obtained and examined using both PSR and qPCR.

Results

Optimization of EHP PSR colorimetric reactions: To assess the general amplification capability and accuracy of the primers. the synthesized EHP DNA at a concentration of 10^2 copies/reaction was utilized as the template to carry out the colorimetric PSR reaction in the presence of the pH-sensitive indicator. The PSR reactions demonstrated a visible change in color from pink to yellow, which occurred after a 45-minute incubation period at 65°C (Figure 2).

Conversely, the no-template control reaction exhibited no change in color, confirming the absence of non-specific amplification or contamination.

The colorimetric PSR reaction for detecting EHP was optimized by adjusting incubation temperature and duration. As regards temperature, amplicons were generated between 60°C and 65°C, with the most pronounced color changes observed between 63°C and 65°C (Figure 3A). At 70°C, the effectiveness of the PSR primers decreased, resulting in a diminished color shift. Consequently, 65°C was selected for the PSR assay. To determine the most effective reaction duration, PSR reactions were conducted at 65°C for various intervals ranging from 5 to 60 minutes. No color shift was observed at intervals of 5 to 25 minutes. However, the color intensity increased significantly between 30 and 40 minutes, indicating a correlation between reaction time and the amount of amplicons produced. Beyond 40 minutes, the color intensity remained constant, suggesting that extending the reaction time did not further enhance the signal.

Table 2
Bacteria used in this study

Bacteria	Source
Vibrio parahaemolyticus XN9	Nhatrang University[43]
Samonella enterica	ATCC 14028
Staphylococus aureus	ATCC 29213
Pseudomonas aeruginosa	ATCC 9027
Vibrio alginolyticus	Lab collection
Vibrio vulnificus	ATCC 27562
Vibrio haveyii	Lab collection
Vibrio cholera	Lab collection
Enterococcus faecalis	Lab collection
Escherichia coli	ATCC 25922

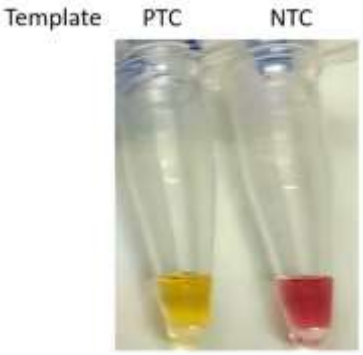

Figure 2: Colorimetric PSR reaction for EHP detection. The amplified product was visualized by the color change from red to yellow. The reactions were incubated at 65 ℃ for 45 minutes. Abbreviation, PTC: synthetic EHP DNA template (10^2 copies/reaction) and NTC: nuclease-free water

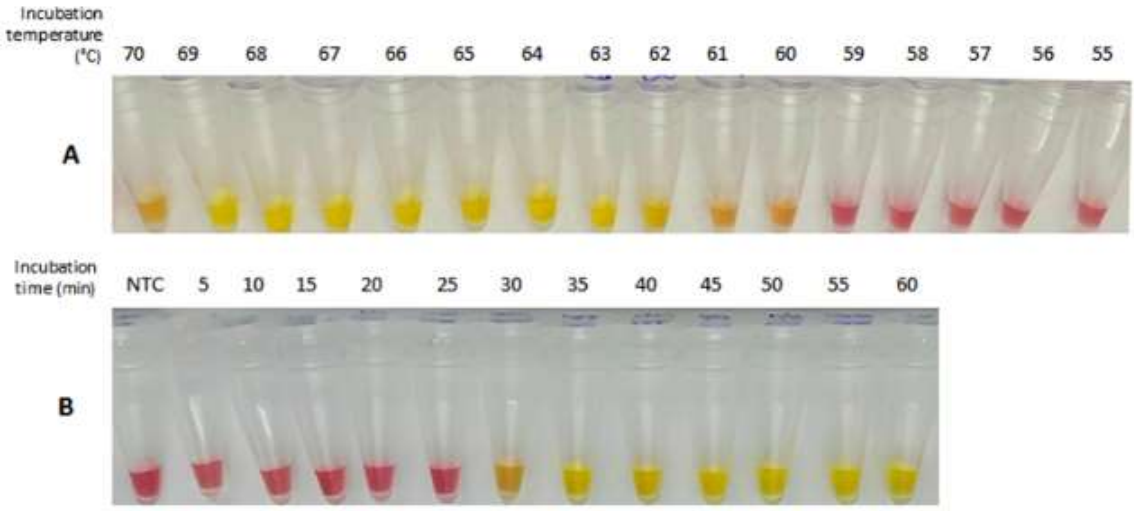

Figure 3: Optimization of PSR reaction temperature and time. The synthesized EHP DNA at a concentration of 10^2 copies/reaction was utilized.
A) The reactions were incubated at different temperatures from 55 to 70 ℃ for 45 min.
B) The reactions were incubated from 5 to 60 min at 65 ℃.

Table 3
LOD_{95} of EHP-PSR assay calculated by PODLOD software ver 12[52].

EHP DNA (copies per reaction)	Ratio of positive tests to the total test number
0	0/3
1	1/3
2	1/3
5	3/3
10	3/3
100	3/3
LOD_{95}	7.142 (2.99-16.061)

Therefore, a reaction time of 40 minutes was established as sufficient for completing the PSR reaction, ensuring optimal signal outcomes (Figure 3B). Thus, the finalized optimal reaction conditions were set at 65°C for 40 minutes.

LOD and specificity of designed primers: The LOD_{95} for the colorimetric EHP PSR assay was determined by analyzing serial dilutions of standard EHP synthesis DNA templates, ranging from 1 to 10^2 copies/reaction. The analysis revealed that the detection limit was as low as 7.142 copies/reaction, with a confidence range of 2.99-16.061 (Table 3).

To verify the specificity of the designed primers, additional aquatic pathogens were used as templates for the PSR assay. The results showed that except for EHP, none of the other pathogen samples produced amplified products, indicating no cross-reactivity (Figure 4). This specificity test confirms

that the developed PSR assay does not exhibit cross-reactivity with non-target aquatic pathogens. Additionally, FastPCR software was used to conduct an *in-silico* PCR assay to evaluate the primer set's specificity. The findings indicated that the EHP-PSR primer set specifically amplified the targeted sequences for EHP, with no amplification of non-specific products from a variety of genetically similar parasite strains (Table 4). This demonstrates that the PSR primer set, developed primarily for detecting the EHP gene, is both specific and selective.

Table 4
***In-silico* PCR used EHP-PSR primer set designed**

Strain	Amplicon formation
CH991540.1 *Enterocytozoon bieneusi* H348 SC_2496 genomic scaffold, whole genome shotgun sequence	0
AF369029.2 White spot syndrome virus, complete genome	0
CM080476.1 *Macrobrachium rosenbergii* isolate ZJJX-2024 chromosome 1, whole genome shotgun sequence	0
NC_002190.2 Infectious hypodermal and hematopoietic necrosis virus, complete genome	0
NC_004603.1 *Vibrio parahaemolyticus* RIMD 2210633 chromosome 1, complete sequence	0
NZ_CP098033.1 *Vibrio alginolyticus* strain E110 chromosome 1, complete sequence	0
LK021130.1 *Vibrio anguillarum chromosome 1, strain NB10*, complete sequence	0
LC704899.1 Microsporidium seriolae SS2020 gene for SSU ribosomal RNA, partial sequence	0
NZ_CP016321.1 *Vibrio vulnificus* strain FORC_037 chromosome I, complete sequence	0
NC_048215.1 Yellow head virus isolate 20120706, complete genome	0
LWDP01000001.1 *Enterospora canceri* strain GB1 scaffold_1, whole genome shotgun sequence	0
LTAI01000001.1 *Hepatospora eriocheir* strain canceri scaffold_0, whole genome shotgun sequence	0
NC_017187.1 Aliarcobacter butzleri ED-1, complete sequence	0
AL111168.1 *Campylobacter jejuni* subsp. *jejuni* NCTC 11168 complete genome	0
CP007224.1 *Pseudomonas aeruginosa* PA96 genome	0
NZ_OU701459.1 *Campylobacter upsaliensis* strain *Campylobacter upsaliensis* 17-M197059 chromosome 1, complete sequence	0
NC_012039.1 *Campylobacter lari* RM2100, complete sequence	0
NC_000913.3 *Escherichia coli* str. K-12 substr. MG1655, complete genome	0
NZ_CP035288.1 *Staphylococcus epidermidis* strain ATCC 14990 chromosome, complete genome	0
CP125875.1 *Vibrio harveyi* strain SB1 chromosome I, complete sequence	0
CP014034.2 *Vibrio fluvialis* strain ATCC 33809 chromosome 1, complete sequence	0
MT539781.1 *Ecytonucleospora hepatopenaei* isolate Cq/2020/20-004 small subunit ribosomal RNA gene, partial sequence	1

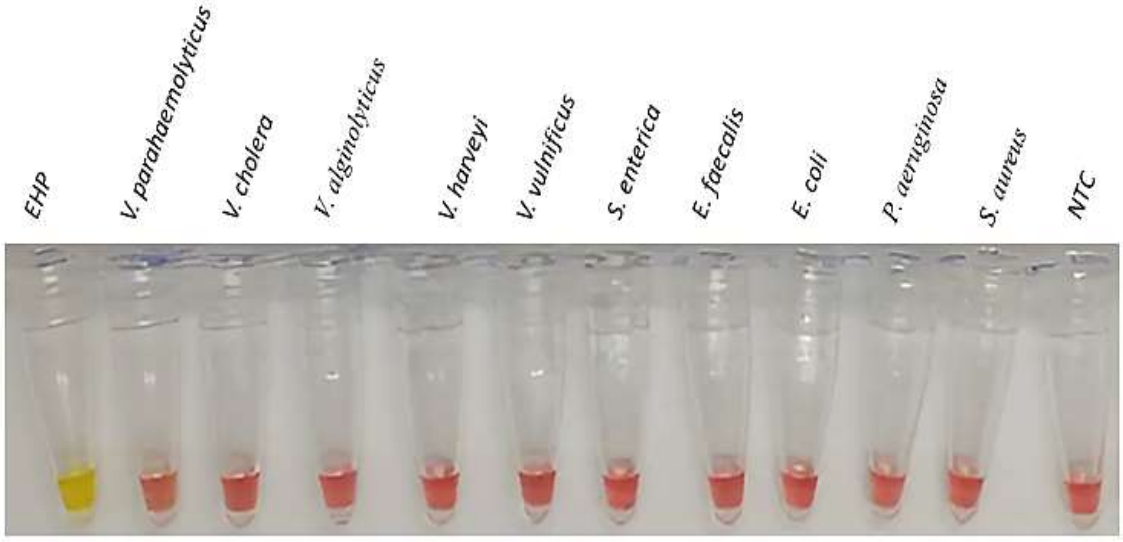

Figure 4: The specificity of the EHP-specific PSR primer. All reactions were incubated at 65°C for 40 minutes.

Table 5
Sensitivity and specificity of direct colorimetric EHP PSR assay

qPCR results	EHP-PSR		Total
	Positive	Negative	
Positive	30	0	30
Negative	0	30	30
Total	30	30	60
Sensitivity: 100% Specificity: 100%			

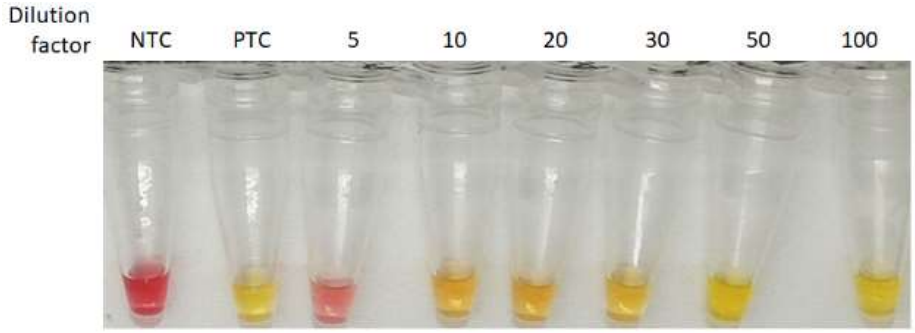

Figure 5: Titration of crude sample dilution factor for EHP PSR assay. Five µL of each diluted sample was added to the reaction mixtures. Reactions were incubated at 65°C for 40 minutes

Sensitivity and specificity of direct colorimetric EHP PSR assay: The newly developed colorimetric PSR assay for detecting EHP streamlines the diagnostic process by utilizing clinical samples directly, thereby bypassing the DNA extraction phase. However, our findings indicate that direct addition of samples to the PSR reaction mix can impede the synthesis of the desired product. To mitigate this, it was necessary to dilute the clinical samples to ensure that other substances within the samples did not interfere with the colorimetric PSR reactions. Preliminary experiments showed that crude samples required appropriate dilution to avoid false negative results due to insufficient template concentration.

It was observed that a 50-fold dilution of crushed intestine and hepatopancreas samples was necessary to detect EHP synthesis DNA effectively as indicated by a precise color change (Figure 5). This level of dilution ensured that the assay could accurately identify the presence of EHP in heavily mixed clinical samples. The direct colorimetric PSR assay was then compared to traditional qPCR to evaluate its performance in detecting EHP. The results demonstrated that the direct PSR assay achieved 100% sensitivity and specificity, matching the performance of qPCR. This was confirmed by accurate detection in all 30 positive and 30 negative samples (Table 5). These findings validate the optimized PSR assay as highly effective for field applications, significantly simplifying the process by eliminating the need for DNA extraction.

Discussion

Recently, EHP has rapidly emerged as a substantial threat to global shrimp farming industries, primarily due to the economic losses stemming from inhibited growth and reduced feed intake in infected shrimp[1,41]. Unlike other pathogens, EHP does not typically cause mass mortality but leads to considerable economic impacts through its sub-lethal effects[33]. The early and rapid diagnosis of EHP is critical for effective management and implementation of preventive measures at the farm level[33,36].

This study introduces a novel visual PSR assay specifically tailored for farm-level detection of EHP. The assay can detect as few as 7.142 copies of EHP DNA per reaction within 40 minutes at a constant temperature of 65°C. This sensitivity is competitive and in some cases surpasses other methods. LAMP has been widely employed for EHP detection, achieving a LOD of around 10 copies per reaction[2,38]. RPA has also been used for EHP detection, with a reported LOD of approximately 10 copies per reaction[17,18,49,51,52]. The rapid amplification at lower temperatures provided by RPA is advantageous, but the method requires additional reagents and proteins, adding complexity. qPCR methods achieve a LOD of about 1-5 copies per reaction[6,23,27], offering high sensitivity but necessitating thermal cycling. All the previous PSR methods for detecting EHP required DNA extraction, which can be time-consuming and require specialized equipment.

A key advancement presented in this study is the utilization of the PSR assay directly on samples without requiring prior DNA extraction. Conventional diagnostic techniques typically involve a DNA extraction phase, which is labor-intensive, expensive and prone to contamination risks. By eliminating this step, the direct PSR assay simplifies the diagnostic procedure, significantly reducing both the time and complexity needed for EHP detection. This improvement is especially advantageous for field diagnostics, where quick and accurate decisions are crucial. The primers were designed to target prevalent EHP strains

found in shrimp populations, enhancing the relevance and applicability of the assay in real-world scenarios.

Additionally, the ability to visually interpret the results through a color change from pink to yellow allows for immediate and straightforward assessment without advanced laboratory infrastructure. This streamlined workflow accelerates the detection process and minimizes the need for specialized equipment and technical expertise, making it highly suitable for use in resource-limited settings.

Conclusion

The direct colorimetric PSR method delineated in this research provides a robust, efficient and user-friendly tool for on-site EHP detection. Its cost-effectiveness, coupled with the lack of requirement for expensive equipment, positions this PSR assay as a potentially transformative approach for routine surveillance and early disease management in shrimp farming. This could significantly aid in establishing proactive strategies to mitigate the impact of EHP in aquaculture operations globally.

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