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# A COLORIMETRIC MULTIPLEX RPA APPROACH FOR ON-SITE DUAL MONITORING OF *VIBRIO PARAHAEMOLYTICUS* AND WHITE SPOT SYNDROME VIRUS IN PACIFIC WHITELEG SHRIMP

**Diem Hong Tran[1], Vo Ngoc Truc Giang[2], Huong Thi Thu Phung[1,✉], Le Minh Bui[2,✉]**

[1]*NTT Hi-tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Viet Nam*

[2]*Department of Biotechnology, NTT Hi-tech Institute, Nguyen Tat Thanh University, Ho Chi Minh, Viet Nam*

✉To whom correspondence should be addressed. E-mail: ptthuong@ntt.edu.vn, and blminh@ntt.edu.vn

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## ABSTRACT

White spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* represent the most prevalent and serious pathogens in Viet Nam's shrimp industry, annually inflicting major production losses. With no existing therapeutic measures, timely and accurate diagnosis is imperative for curbing outbreaks and limiting economic impacts through isolation and culling protocols. However, conventional techniques like polymerase chain reaction (PCR) and quantitative PCR require advanced laboratory infrastructure that is incompatible with rapid on-site pathogen surveillance. This work details the development of a multiplex recombinase polymerase amplification (RPA) assay for the simultaneous detection of WSSV and *V. parahaemolyticus* using a visual colorimetric readout amenable to field deployment. Primers targeting unique WSSV and *V. parahaemolyticus* sequences were designed for selective amplification. Specificity screening verified exclusive pathogen detection against common shrimp microbiota and human-handling contaminants with no cross-reactivity. Singleplex reactions identified 39 °C for 35 minutes as optimal conditions; hence, these parameters were subsequently applied in multiplex format. Incorporation of the $Mg^{2+}$-sensitive eriochrome black T (EBT) dye enabled clear discrimination between positive blue and negative violet reactions by the naked eye. The multiplex assay demonstrated high analytical sensitivity, down to 1 copy of DNA template per reaction. Clinical validation of the multiplex RPA method using shrimp samples versus quantitative PCR showed 100% agreement. By coupling rapid isothermal amplification with visual indicator-based detection in a multiplexed format, this assay provides simple, robust, and user-friendly identification of major shrimp pathogens in resource-limited settings in less than one hour to facilitate prompt on-site disease control decisions. The field-suitable platform aims to mitigate outbreak magnitude and economic consequences through timely response.

**Keywords:** colorimetric, multiplex RPA, WSSV, *V. parahaemolyticus*.

## INTRODUCTION

The shrimp aquaculture industry plays a vital economic role in Viet Nam. With long-standing development in agriculture, forestry, and fisheries, shrimp farming has grown continuously. According to 2023 statistics from the Viet Nam Association of Seafood Exporters and Producers (VASEP), the production volume of Pacific whiteleg shrimp (*Litopenaeus vannamei*) reached 750,000 tons, accounting for 75% of nationwide shrimp output. However, infectious diseases pose a persistent threat, especially acute hepatopancreatic necrosis disease (AHPND) and white spot syndrome (WSS) in farmed shrimp, which can result in substantial mortality (Xupeng *et al.*, 2016; Joyanta *et al.*, 2017; Lo *et al.*, 1996).

AHPND, caused by several *Vibrio parahaemolyticus* strains, has driven widespread epidemics, resulting in huge financial losses in Viet Nam's *L. vannamei* shrimp industry (Xupeng *et al.*, 2016). WSS, induced by the white spot syndrome virus (WSSV), also produces high morbidity and mortality, spreading through horizontal transmission from other crustacean reservoirs and vertical transmission from broodstock to larvae (Joyanta *et al.*, 2017; Lo *et al.*, 1996). Infected shrimp ponds can experience 100% losses within one week after rapid WSSV proliferation (Lo *et al.*, 1996). There are presently no viable treatment options for either WSS or AHPND. Therefore, rigorous biosecurity protocols and expeditious diagnostic testing are imperative for early, precise WSSV detection, outbreak prevention, and the protection of shrimp aquaculture.

Timely pathogen detection is critical for controlling disease outbreaks in Vietnamese shrimp aquaculture. However, conventional diagnostic approaches have limitations that constrain rapid response. AHPND and WSS are primarily identified through symptomology, histopathology, microbial culture, and polymerase chain reaction (PCR). Nevertheless, these techniques may overlook early-stage infections, introduce observational bias, require days of incubation, or necessitate advanced laboratory infrastructure (Beatriz *et al.*, 2021). Though PCR-based methods remain the gold standard for sensitivity and specificity, the need for thermocycling equipment, skilled technicians, and dedicated facilities creates barriers to widespread, real-time deployment, especially in resource-limited areas (Beatriz *et al.*, 2021).

Isothermal nucleic acid amplification techniques (iNAATs) now offer alternative PCR-like methods that use a single optimal temperature instead of thermocycling (Beatriz *et al.*, 2021). Recombinase polymerase amplification (RPA) has shown particular promise through its convenience and accuracy (Ivan *et al.*, 2018). RPA utilizes a strand-displacing DNA polymerase and single-stranded DNA-binding proteins to prevent double-stranded DNA reannealing during primer-mediated sequence replacement. A recombinase enzyme facilitates this targeted displacement process (Ivan *et al.*, 2018). Coupling RPA with colorimetric indicator dyes that shift in response to changing pH or ions enables visual, equipment-free monitoring of reactions, which is ideal for in field diagnosis (Meiying *et al.*, 2022). Together, these features make RPA a potentially valuable tool for on-site shrimp pathogen surveillance if assays can be multiplexed for dual WSSV and AHPND screening. Previously, attempts have been made to

detect *Vibrio* spp. in the *Panaeus vannamei* shrimp using RPA (Mai *et al.*, 2021) or real-time RPA (Yu *et al.*, 2021). RPA-based WSSV detection assays were also developed for monitoring shrimp cultures (Zhang *et al.*, 2022). Even though simultaneous detection of pathogens has been demonstrated to be cost-effective for disease management in aquaculture (Leal *et al.*, 2014), duplex or multiplex diagnostic methods for the concurrent detection of *Vibrio* and WSSV have been scarcely reported, and only qPCR was applied (Caipang *et al.*, 2010; Huyen *et al.*, 2022).

In this study, we developed a multiplex recombinase polymerase amplification (RPA) assay for the rapid, simultaneous detection of *V. parahaemolyticus* and the WSSV. The isothermal RPA reaction proceeds at 39 °C, enabling visual interpretation without thermocycling equipment. We incorporated the $Mg^{2+}$-sensitive ionic indicator eriochrome black T (EBT) for colorimetric reaction monitoring. Upon amplified target detection, EBT elicits a visible color shift that is detectable by the naked eye. By combining multiplex DNA amplification with a simple visual readout, this field-deployable approach provides a viable option for dual *V. parahaemolyticus* and WSSV screening in resource-limited settings frequently encountered in Viet Nam. The assay's simplicity and speed aim to facilitate early intervention, isolated infected stock removal, and disease outbreak prevention to mitigate economic losses.

## MATERIALS AND METHOD

### Microbial strains, DNA extraction and quantification

The microorganisms used are listed in Table 1. *V. parahaemolyticus* was cultured in TSB+ medium (Tryptic Soy Broth TSB supplemented with 1.0% NaCl) at 32 °C in a shaking incubator (150 rpm) and collected at the log phase. The genomic DNAs (gDNAs) were extracted by the Cetyltrimethylammonium bromide (CTAB) method and stored at -80 °C until used. The extracted DNA of *V. parahaemolyticus* was used as the standard template for RPA reactions.

**Table 1.** Microorganisms used in this study.

| Microorganisms | Source |
|---|---|
| *Salmonella enterica* ATCC 14028 | ATCC |
| *Staphylococcus aureus* ATCC 29213 | ATCC |
| *Pseudomonas aeruginosa* ATCC 9027 | ATCC |
| *Vibrio vulnificus* ATCC 27562 | ATCC |
| *Vibrio parahaemolyticus* | XN9 (Ngo *et al.*, 2017) |
| *Listeria monocytogenes* | Lab collection |
| *Vibrio haveyii* | Lab collection |
| *Escherichia coli* | Lab collection |
| *Bacillus cereus* | Lab collection |
| *Enterocytozoon hepatopenaei* (EHP) | Lab collection |

**RPA primer design for detection of V. parahaemolyticus and WSSV**

The *pirA* (*V. parahaemolyticus*) and *rr1* (WSSV) genes were selected as target sequences for RPA-based detection due to their stability and specificity according to previous research (Shin-Jen *et al.*, 2017; Yu-Ling *et al.*, 2023; Nazmul *et al.*, 2021). The RPA primer sets were designed using the PrimerQuest Tool (www.idtdna.com). Two primer sets for each target were selected and checked by *in silico* PCR using FastPCR software. The designed primers are shown in Table 2 and synthesized by Phu Sa Genomics (Can Tho, Viet Nam). The synthesized target DNA of WSSV (WSSV-gblock) was used as the standard template for RPA reactions.

**Table 2.** RPA primers used in this study.

| Name | Sequencing 5′ - 3′ | Primer set name |
|---|---|---|
| VP-RPA-pirA-F1 | ACATTGAGAATACGGGACGTGGGGAGCTTA | DP1 |
| VP-RPA-pirA-R1 | CGTTAGTCATGTGAGCACCTTCTTAGTGGT | |
| WSSV-RPA-167-F1 | GGATTACAGATATGTCGTTGAGAAGCCCCT | |
| WSSV-RPA-167-R1 | AGGCAGGCTGTTTCTATACTAAAAAGAACT | |
| VP-RPA-pirA-F1 | ACATTGAGAATACGGGACGTGGGGAGCTTA | DP2 |
| VP-RPA-pirA-R1 | CGTTAGTCATGTGAGCACCTTCTTAGTGGT | |
| WSSV-RPA-167-F2 | CATATTCTCTGGCATACGATCAGTCGAGTG | |
| WSSV-RPA-167-R2 | GTACCTCCAAATGAAGAGGTGACAAATTGT | |
| VP-RPA-pirA-F2 | AAACGGAGGCGTCACAGAAGTAGACAGCAAA | DP3 |
| VP-RPA-pirA-R2 | TTAGTGGTAATAGATTGTACAGAAACCACGACTAG | |
| WSSV-RPA-167-F1 | GGATTACAGATATGTCGTTGAGAAGCCCCT | |
| WSSV-RPA-167-R1 | AGGCAGGCTGTTTCTATACTAAAAAGAACT | |
| VP-RPA-pirA-F2 | AAACGGAGGCGTCACAGAAGTAGACAGCAAA | DP4 |
| VP-RPA-pirA-R2 | TTAGTGGTAATAGATTGTACAGAAACCACGACTAG | |
| WSSV-RPA-167-F2 | CATATTCTCTGGCATACGATCAGTCGAGTG | |
| WSSV-RPA-167-R2 | GTACCTCCAAATGAAGAGGTGACAAATTGT | |
| WSSV-gblock | TTAAGTAATGGACTTTAACCCTATTCTGAAACCTCTCCCTGG TGTTGTGGATTACAGATATGTCGTTGAGAAGCCCCTTCATA TTCTCTGGCATACGATCAGTCGAGTGCCAGTTGGCTAGCG CTAGAAGAACCTTGCCCTCCGGAGACTCTGGGTTCCATCCT CGAATCTTCTTTTCGGGAGTTCTTTTTAGTATAGAAACAGCC TGCCTACAATTTGTCACCTCTTCATTTGGAGGTACAAAATTT GACGCCAAGAAACCGCAGGACAATCTCATGGGCCATTTAT GGAGTCCGGGAC | Template DNA |

**RPA reaction condition**

The RPA reactions (singleplex and multiplex) were performed using the TwistAmp® Liquid DNA Amplification Kit (TwistDx Inc., UK). The 15 µL multiplex RPA reaction consisted of 7.5 µL of 2× Reaction Buffer, 2.06 µL of dNTPs (10 mM), 1.5 µL of 10× Basic E-mix, 0.48 mM each F/R primer, 0.75 µL of 20× Core Reaction Mix, 0.75 µL of MgOAc (280 mM), 1 µL of template and nuclease-free water. The reaction was incubated on a Biosan Bio TDB-100 dry thermostat at 39 °C for 35 minutes. The change in reaction color was observed by adding 0.08 mM EBT (working concentration). When needed, the reaction results were analyzed on a 2.5% agarose gel electrophoresis.

**EBT concentration optimization**

The RPA reaction produces a large number of pyrophosphate ions as a by-product; these ions react with $Mg^{2+}$ to form the insoluble product, magnesium pyrophosphate (Saurabh *et al.*, 2022). Consequently, in the presence of EBT, the positive reaction maintains the blue color of EBT while the negative reaction changes to violet because $Mg^{2+}$ binds to EBT. The use of EBT was optimized in the range of 0.04 - 0.12 mM (working concentration) by adding to the reaction at the end of the incubation period. The result was then examined by observing the shift in the reaction color.

**Optimization of multiplex RPA conditions**

Optimization of the multiplex RPA assay was conducted using $10^4$ copies per reaction of standard DNA templates for *V. parahaemolyticus* and WSSV. All primer pairs were included at equal working concentrations of 0.48 mM. To determine the optimal temperature, reactions were incubated at 35-42 °C for 35 minutes, with 1 °C increments. For optimal incubation time assessment, reactions were performed at the ideal temperature for 5-45 minutes, with each interval being 5 minutes apart. Successful amplification was evaluated through both colorimetric and electrophoretic detection methods. A visible color shift of the $Mg^{2+}$-sensitive EBT dye provided visual confirmation, while DNA band analysis using 2.5% agarose gel electrophoresis allowed sensitive reaction verification.

**Evaluation of the limit of detection (LOD) and primer specificity of multiplex RPA**

Different amounts of the standard DNA templates, at concentrations ranging from $10^5$ to $10^{-3}$ copies/µL for each target (1:1 molar ratio), were used to identify the LOD and primer specificity of the multiplex RPA assay. The LOD was determined as the lowest copy number reliably generating a positive result. Primer selectivity was examined by performing RPA reactions using the gDNA extracted from bacterial strains that are prevalent in aquaculture settings or potentially present as contaminants from human handling. Test strains included both shrimp pathogens and human microbiota to ensure precise detection and avoid inaccurate identification in real-world shrimp farm usage.

**Multiplex RPA and qPCR for diagnosing infected shrimp**

Twenty shrimp samples were obtained from the local farms. Ten samples were collected from a farm in Can Gio district, Ho Chi Minh

city, in June and July 2022. The other ten samples were obtained from a farm in Tien Giang province in August and September 2022. All selected shrimp specimens were initially diagnosed based on the clinical symptoms of AHPND and WSSV. The harvested shrimp were thoroughly rinsed with saline solution, followed by the careful removal of their internal organs using precise surgical tools. Subsequently, the samples were immersed in water and coarsely ground for analysis. The DNA samples were extracted using the CTAB method, verified using quantitative polymerase chain reaction (qPCR) analysis and subsequently utilized as a template for the multiplex RPA assay developed. For the qPCR assay, a reaction volume of 10 µl was prepared, consisting of 0.25 µL F/R primers at a concentration of 10 µM, 1 µL of the DNA template, 5 µL of Luna Universal qPCR Master Mix (New England Biolabs), and nuclease-free water. The thermal cycling conditions were set as follows: 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds, 65 °C for 30 seconds, and 72 °C for 15 seconds. The cycle threshold (Ct) values observed for the five WSSV-positive samples were 12.47, 20.12, 28.32, 20.24, and 21.48, respectively. Meanwhile, the *V. parahaemolyticus*-positive samples were detected at Ct values of 24.98, 22.12, 16.12, 18.32, and 25.24, respectively.

## RESULTS AND DISCUSSION

### Primer selection for multiplex RPA assay

RPA primers specifically designed for *V. parahaemolyticus* and WSSV detections were initially validated via simplex RPA reactions. Two distinct primer sets targeting unique regions were tested for each pathogen. The results indicated that all the primer combinations, VP-F1-R1/VP-F2-R2 and WS-F1-R1/WS-F2-R2, successfully amplified their respective target sequences (Figure 1). Specifically, positive reactions were indicated by a clear color shift from violet to blue upon addition of the $Mg^{2+}$-sensitive EBT dye, and the formation of RPA amplicons was further verified by agarose gel electrophoresis analysis. Both visual colorimetric inspection and electrophoretic band pattern confirmation reliably distinguished the difference between positive blue reactions that had the expected ~200 bp products and negative violet reactions that did not have amplified DNA (Figure 1, lower panel). These consistent simplex RPA results supported the primer sets' utility for subsequent multiplex assay development.

The multiplex RPA assay incorporated four primer set combinations, outlined in Table 2, with two distinct sets targeting each of the *V. parahaemolyticus* and WSSV templates. As shown in Figure 2, the DP1 and DP2 mixtures successfully co-amplified both target sequences simultaneously. However, reactions using DP3 and DP4 sets failed to produce the correct multiplex results. Non-optimal dimer formation or secondary structures arising between mismatched primers likely hindered assay performance. Consequently, the DP1 and DP2 combinations that exhibited reliable dual target detection were used for further analytical evaluation.

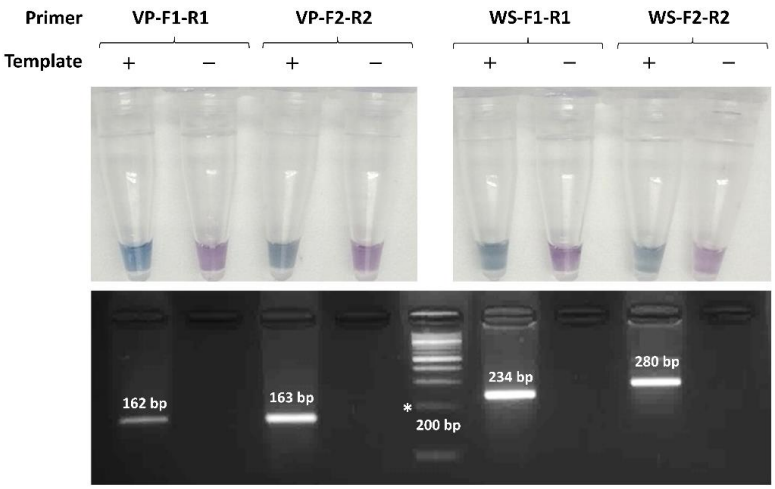


**Figure 1.** Evaluation of primer performance using singleplex RPA. *V. parahaemolyticus* (VP) DNA sample extracted (~$10^4$ copies/reaction) and WSSV synthetic target DNA ($10^4$ copies/reaction) were used in the reaction. The reaction was incubated at 39 °C for 35 minutes. The results were analyzed using 0.08 mM EBT or 2.5% agarose gel electrophoresis.

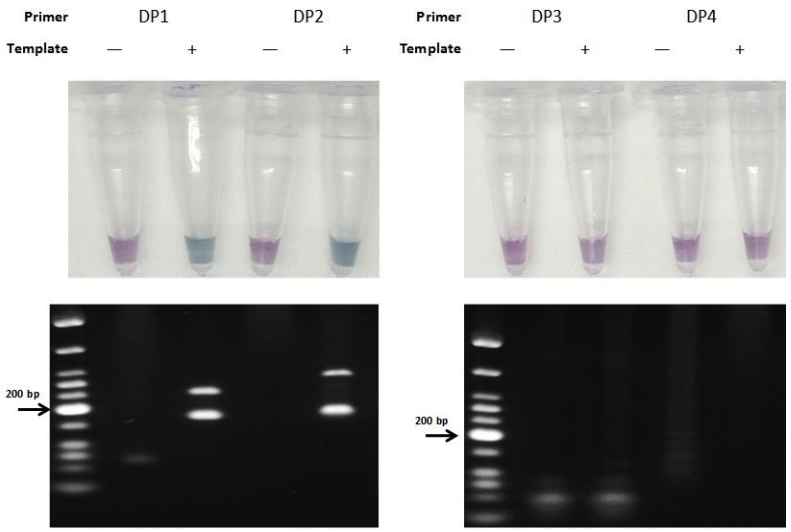


**Figure 2.** Primer set selection for multiplex RPA reactions. The reaction was incubated for 35 minutes at 39 °C. *V. parahaemolyticus* DNA sample extracted ($10^4$ copies/reaction) and WSSV synthetic target DNAs ($10^4$ copies/reaction) were used in the reaction. The reaction was incubated at 39 °C for 35 minutes. The results were analyzed using 0.08 mM EBT and 2.5% agarose gel electrophoresis. (+) and (-) represent the positive and negative samples, respectively.

## EBT concentration optimization of multiplex RPA assay

The EBT indicator was incorporated into the multiplex reactions to enable visual differentiation of positive and negative results through the change in $Mg^{2+}$ concentration. As shown in Figure 3, both DP1 and DP2 primer combinations produced a distinct color shift from violet to blue for

positive reactions containing amplified target DNA. Therefore, a 0.08 mM EBT concentration was selected to be utilized in all subsequent experiments. This optimal EBT concentration mirrors the 0.1 mM amount previously utilized for colorimetric monitoring of loop-mediated isothermal amplification (LAMP), validating its alignment with optimal visual indicator ranges for isothermal amplification (Yinhua *et al.*, 2022). This is also the first report that utilizes EBT in RPA reactions.

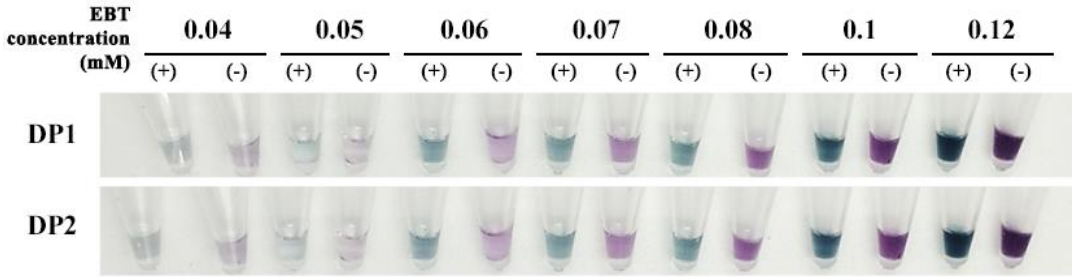


**Figure 3.** Optimization of EBT concentration. The reaction was incubated at 39 °C for 35 minutes. The indicated concentrations of EBT were used to analyze the reaction outcome. (+) and (-) represent the positive and negative control samples, respectively.

## Temperature and incubation time optimization of Multiplex RPA assay

Reaction temperature and incubation time are critical parameters for the optimal amplification yield. As shown in Figure 4A, the incubation temperature of 39 °C was demonstrated to be ideal for both DP1 and DP2 primer sets. Subsequent incubation time testing at this temperature showed amplicon formation within 15 minutes, as indicated by the electrophoresis result. However, distinct, robust colorimetric differentiation by EBT required at least 30 minutes of incubation (Figure 4B). While the product was detectable earlier, the most distinctive visible color shift emerged at longer durations. Similar kinetics were observed for both multiplex primer combinations, aligning with singleplex behavior. Consequently, 35 minutes was selected as the optimal incubation time to ensure consistent, unambiguous color-based discrimination while avoiding unnecessary extended incubation.

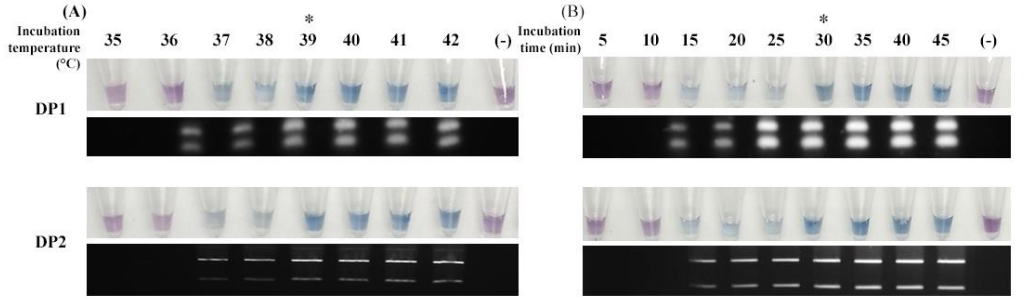


**Figure 4.** Temperature and time optimization for the multiplex RPA reaction. (A) The reaction was incubated at various temperatures between 35 and 42 °C for 35 minutes. (B) The reaction was incubated at 39 °C for 5-45 minutes. The asterisks indicate the value chosen as the optimal parameter for the multiplex RPA; (-) is the negative control.

**Primer specificity of RPA assay**

The specificity of the primers designed was evaluated using common bacteria in shrimp aquaculture and human-resided bacteria that may contaminate the sample. DNA extracts of tested bacteria were subjected to multiplex RPA reactions utilizing DP1 and DP2 primer sets. The results exhibited that the multiplex RPA reaction was only successful in the presence of *V. parahaemolyticus* and/or WSSV (Figure 5), demonstrating a high selectivity of the RPA primer sets designed for WSSV and *V. parahaemolyticus*.

The analytical specificity of the multiplex RPA assay was evaluated through reactions containing genomic DNA extracted from bacterial species commonly encountered in shrimp aquaculture environments or potentially present from human handling of the samples. As shown in Figure 5, DP1 and DP2 primer combinations only successfully amplified their *V. parahaemolyticus* and WSSV targets, without non-specific amplification of non-target templates. The assays' exclusivity for the desired pathogens despite phylogenetically related background flora exhibits robust specificity, preventing inaccurate detection within complex real-world shrimp pond ecosystems.

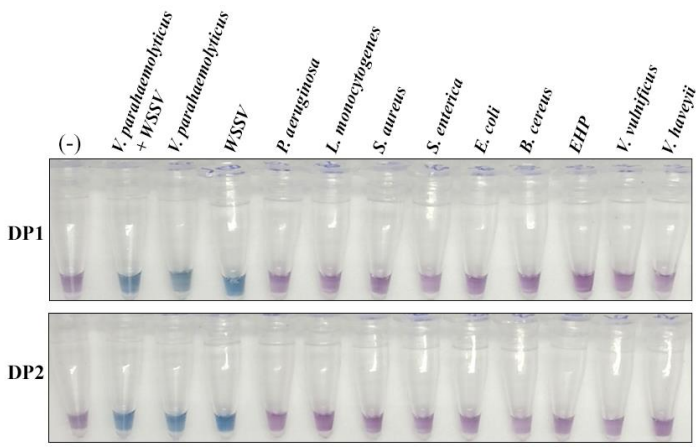


**Figure 5.** Specificity of DP1 and DP2 primer sets. Genomic DNA of bacteria tested (1 ng/reaction) was used. The reaction was incubated at 39 °C for 35 minutes. (-) indicates negative control.

**LOD of multiplex RPA assay**

In order to determine the limit of detection (LOD) of the assay, the template DNA concentration of a sample was measured by a spectrophotometer. Firstly, 5 mL of *V. parahaemolyticus* cells at the log phase were collected, and DNA was extracted using the CTAB method. The concentration of the extracted DNA sample or gblock DNA was determined using Nanodrop One C (ThermoFisher Scientific). DNA in the sample will be converted into DNA copy numbers using DNA Copy Number and Dilution Calculator software (ThermoFisher Scientific). The LOD of the multiplex RPA reaction was determined using a serial dilution of *V. parahaemolyticus* genomic DNA extracted from the culture medium and the WSSV synthetic DNA. The results demonstrated that the multiplex RPA reaction utilizing the DP1 primer set could identify the target sequence at a concentration as low as 1 copy/reaction,

while the LOD of the DP2 primer set was significantly higher (100 copies/reaction) (Figure 6). The primer set DP1 was thus chosen for the subsequent analysis.

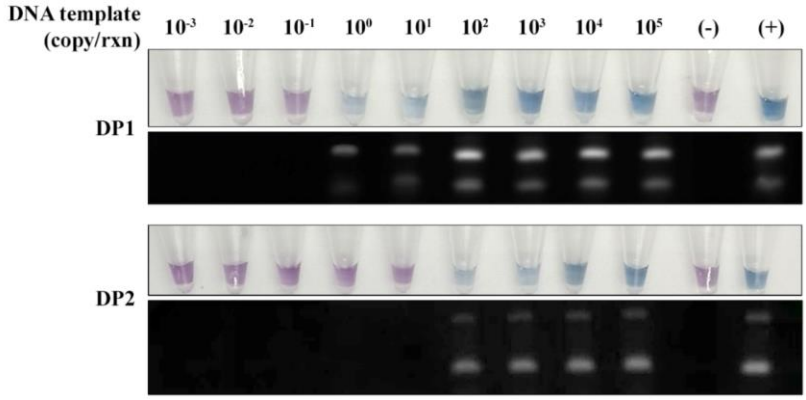


**Figure 6.** LOD of a multiplex RPA reaction. The templates were diluted at the indicated concentration and used for the multiplex RPA reaction. The 1:1 molar ratio of the genomic DNA of *V. parahaemolyticus* to the synthetic DNA fragment of WSSV and the copy number of each template used in the reaction are denoted on top of the figure. The reaction was incubated at 39 °C for 35 min. (+) and (-) mean positive and negative samples, respectively.

## Multiplex RPA to detect pathogenic DNA extracted from shrimp samples

Clinical validation employed shrimp samples (n = 20) with a known infection status predetermined through qPCR. Pathogen panels identified 5 specimens harboring *V. parahaemolyticus*, 5 containing WSSV, and 10 lacking either pathogen. Simplex and multiplex RPA assays demonstrated 100% diagnostic agreement with the reference qPCR method (Table 3). Concordant detection across both amplification platforms and all specimen subsets confirms the analytical and clinical accuracy of the developed multiplex RPA test for on-site dual shrimp pathogen identification.

**Table 3.** Agreement between qPCR and RPA assays.

| | **Number of negative samples** | | **Number of positive samples** | |
|---|---|---|---|---|
| | WSSV | VP | WSSV | VP |
| Singleplex qPCR | 15 | 15 | 5 | 5 |
| Singleplex RPA | 15 | 15 | 5 | 5 |
| Multiplex RPA | 10 | | 10 | |

Several PCR and iNAAT-based approaches have recently emerged for WSSV and *V. parahaemolyticus* screening. In 2019, a research team from the Industrial University of Ho Chi Minh used two pairs of PCR primers specific for 16S rRNA and *idh* genes to detect *V. parahaemolyticus* with a LOD of $3.5 \times 10^3$ CFU/ml (Thi-Huyen *et al.*, 2019). A modern commercial procedure, the IQ Plus™ AHPND/EMS Plasmid Kit (GeneReach Biotechnology, Taiwan), applies the principle of isothermal

amplification combined with the luminescence method of gold nanoparticles (AuNP) to identify pathogenic genes. As for white spot disease, nested-PCR methods and multiplex PCR (Huyen *et al., 2014)* have been proposed. Several real-time PCR or PCR kits are available, such as IQREAL WSSV, IQ2000 WSSV of GeneReach Biotechnology (Taiwan), and Topspec® WSSV qPCR Kit of ABT Company (Viet Nam). GeneReach Biotechnology also has a kit that uses isothermal amplification to detect WSSV (IQ Plus WSSV), but it requires an expensive readout and thermostat for field use. Nonetheless, assay multiplexing capacity remains limited, and few options exist for combined pathogen detection in a single reaction. Additionally, existing solutions either preclude visual inspection or rely on pH indicators prone to ambiguity from matrix variations. Field-deployable tools for dual WSSV and *V. parahaemolyticus* identification would facilitate on-site disease management and outbreak response. Even though the multiplex RPA assay developed in this study still requires validation using larger clinical samples, including those with co-infection of both pathogens, and might need more adjustments to the on-field testing conditions, it is a promising approach for the efficient surveillance of pathogens at shrimp farms.

## CONCLUSION

This work describes the development of a multiplex RPA assay using the $Mg^{2+}$-sensitive EBT dye for unambiguous colorimetric reaction monitoring. Coupling primers targeting unique WSSV and *V. parahaemolyticus* sequences with optimized isothermal amplification conditions and a simple visual readout generated sensitive on-site detection capability in less than 35 minutes without expensive, laboratory-grade equipment. The assay showed strong analytical sensitivity and specificity, with a 100% diagnostic accuracy relative to quantitative PCR across shrimps with known infection statuses. By enabling rapid dual pathogen identification in resource-limited settings, this multiplex RPA-EBT approach has the potential to assist aquaculture disease surveillance and containment worldwide.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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