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CRISPR-Cas fluorescent cleavage assay coupled with recombinase polymerase amplification for sensitive and specific detection of *Enterocytozoon hepatopenaei*

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ABSTRACT

Enterocytozoon hepatopenaei (EHP) is a parasite that infects pacific whiteleg shrimp, *Penaeus vannamei*, causing growth retardation and uneven size distributions that lead to severe losses in shrimp productivity. Routine monitoring is crucial to timely prevention and management of EHP, but field-deployable diagnostic kits for EHP are still scarce. Here, we proposed the use of recombinase polymerase amplification (RPA) and CRISPR-Cas12a fluorescence assay, henceforth RPA-Cas12a, for detection of EHP. Targeting *ptp2* gene, RPA-Cas12a could detect as few as 50 copies of DNA and showed no reactivity with closely related microsporidia. The entire procedure could be performed at a temperature close to 37 °C within 1 h. Naked eye visualization was possible with UV/blue-light excitation or lateral flow detection. Thus, RPA-Cas12a is a rapid, sensitive and specific detection platform that requires no sophisticated equipment and shows promise for on-site surveillance of EHP.

1. Introduction

Enterocytozoon hepatopenaei (EHP) is a microsporidian parasite in the family *Enterocytozoonidae*, primarily infecting the hepatopancreas of shrimp species including *Penaeus vannamei*, *Penaeus monodon*, and *Penaeus japonicus* [1–5]. Infection with EHP causes hepatopancreatic microsporidiosis (HPM), a disease characterized by severe growth retardation [6]. While HPM does not normally result in mortality, diminished average body weight and high size variations of shrimp lead to poor pond performance and eventually severe economic losses [7,8]. Recently, the high prevalence of EHP has become a major threat to the shrimp aquaculture sector in Asia [8,9]. For example, it has been estimated that, in Thailand alone, economic losses due to EHP could be as high as US$ 232 million per year [10].

As shrimp infected with EHP do not exhibit outward symptoms until a few months into cultivation, regular surveillance is essential in ensuring that the animals that appear normal are truly free from EHP [6]. In addition, early discovery of EHP in asymptomatic shrimp can prompt timely intervention, such as regular changing of pond water to remove feces and free EHP spores, which may allow shrimp to continue growing without symptoms until harvest. Thus far, several detection methods have been developed for EHP, including loop-mediated isothermal amplification (LAMP), nested polymerase chain reaction (nested PCR), and single-step PCR coupled with lateral-flow detection (PCR-LFD) [5,11–13]. Each of these methods has strengths and limitations. For example, PCR-LFD is moderately sensitive and produces signal visible to the eye, but the requirement of an expensive thermal cycler precludes its adoption in resource-limited settings [13]. On the other hand, LAMP is highly sensitive and isothermal, requiring only a water bath as the heat source, but the technique occasionally produces non-specific amplicons [11,14]. Nested PCR is 1000-fold more sensitive than its one-step counterpart in EHP detection, but, in addition to requiring a thermocycler, an inappropriate choice of

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated protein; RPA, recombinase polymerase amplification; RPA-Cas12a, RPA coupled with Cas12a cleavage assay; EHP, *Enterocytozoon hepatopenaei*; *Eca*, *Enterospora canceri*; *Her*, *Hepatospora eriocheir*; WSSV, white spot syndrome virus; IHHNV, infectious hypodermal and hematopoietic necrosis virus; LFD, lateral flow dipstick; PAM, protospacer adjacent motif; FQ, fluorescent-quencher reporter; FB, FAM-ssDNA-Biotin reporter; *swp*, spore wall protein; SWP-PCR, nested PCR targeting *swp*; *ptp2*, polar tube protein 2; NTC, no-template control.

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target reportedly yielded false positive results with closely-related microsporidia [12]. Therefore, a rapid, field-deployable diagnostic that also offers high sensitivity and specificity is still needed.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has emerged as a powerful tool for genome editing of organisms across all domains of life [15,16]. Evolved as an adaptive immune system in bacteria and archaea, CRISPR in its native context employs a family of protein called Cas endonucleases to cleave foreign nucleic acids or the genome of invading pathogens [17,18]. While homologues of Cas endonuclease differ in their substrate preferences and mechanism of target recognition, they generally cleave sequences that meet the following requirements: 1) that a short nucleic acid sequence called protospacer adjacent motif (PAM) is present near the target site; 2) that the 20–28 bp sequence located next to PAM is complementary to "guide RNA," a short RNA that is bound to Cas protein and plays a key role in target recognition [16,19]. Therefore, by including an appropriate guide RNA, Cas endonuclease can be programmed to bind and cleave any target nucleotide sequences with minimal constraints.

Recently, CRISPR applications have been extended to encompass nucleic acid detection, exploiting a distinct Cas homologue called Cas12a whose activity can be coupled to fluorescent emission [20–24]. Briefly, Cas12a, upon cleaving the target double-stranded DNA (dsDNA), will proceed to cleave single-stranded DNA (ssDNA) in a non-specific fashion, the so-called "trans cleavage" activity. By including a fluorophore-quencher pair linked by ssDNA ("FQ reporter"), trans cleavage events will free the fluorophore from its quencher, in effect activating fluorescence that can be measured with a microplate reader or by eye [20,25] (Fig. 1). Cas12a detection has been demonstrated to be exceptionally sequence-specific, capable of distinguishing targets with only 1-bp difference [21]. Although Cas12a on its own is theoretically not sensitive enough to detect low levels of nucleic acids, an upstream amplification step could dramatically boost the sensitivity of the assay. For this purpose, recombinase polymerase amplification (RPA) has been the amplification technique of choice because it can be performed isothermally at temperature between 37–42 °C, close to the optimal temperature for the Cas12a cleavage assay (37 °C) [26]. In our previous report, pre-amplification of the target region in white spot syndrome virus (WSSV) with RPA enhanced the detection limit from 10^9 to 200 copies per reaction — a near 5 million-fold improvement [27]. Taken together, the advantages of the RPA-coupled Cas12a platform (RPA) could potentially fill the critical gaps in current EHP diagnostic needs.

Here, we report the application of Cas detection in rapid, sensitive, and specific EHP diagnosis. The polar tube protein 2 (*ptp2*) gene was selected as the target because its protein product constitutes an essential component of EHP host-invasion apparatus, the polar tube, and it shares low nucleotide sequence similarity with homologues from other microsporidia. Different RPA primers and Cas12a guide RNAs were tested for their efficiency, and the optimized RPA-Cas12a workflow was then evaluated for sensitivity and specificity. Visualization platforms, including UV excitation, blue light excitation, and lateral flow detection were also assessed for their compatibility with the assay. Finally, the performance of RPA-Cas12a was compared with nested PCR in a blind test, in detection of EHP in shrimp challenged by laboratory cohabitation, and in evaluation of field samples from commercial farms in Thailand.

2. Materials and methods

2.1. Construction of ptp2 plasmid

The predicted open reading frame (855 base pairs) of *ptp2* was previously annotated to produce a hypothetical protein in the draft genome of EHP (locus_tag: EHP00_803; MNPJ01000011.1 (39992−39138)) [28]. However, our subsequent analysis of its 284 amino acid sequence showed a highly conserved N-terminal signal peptide (15 residues, positions 1–15) and a conserved polar tube protein 2 family domain (186 residues, positions 65–250; InterPro: IPR031507; Pfam: PF17022), similar to the protein domain architecture of other microsporidian PTP2 proteins [29]. To the best of our knowledge, only a single copy of *ptp2* is present in EHP according to both nucleotide and protein BLAST searches in the EHP draft genome. In addition, the orthologous group of this of *ptp2* gene, both inside and outside of the family *Enterocytozoonidae*, reportedly contains only a single copy of the gene in all other available microsporidian genomes [28–30].

This *ptp2* coding sequence was amplified from EHP genomic DNA using Phusion-HF polymerase with primers PTP2-GG-F and PTP2-GG-R (Table 1). To a PCR microtube was added 10 ng of the template DNA, Phusion GC buffer, 10 μM of forward and reverse primers each, 0.2 mM dNTPs, and 1 unit of Phusion-HF polymerase. Total volume of the reaction was adjusted to 50 μl with nuclease-free water (Appsalagen). Then, the reaction tube was incubated in thermocycler (T100, BioRad) using the following temperature program: 30 s at 98 °C for initial denaturation, followed by 35 cycles of 10 s at 98 °C, 20 s at 60 °C and 20 s at 72 °C; and 10 min at 72 °C for final extension. The amplicon was verified with agarose gel electrophoresis and gel-purified with NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel). Concentration of the purified amplicon was measured by Nanodrop™ One UV–vis spectrophotometer (Thermo). Finally, Golden Gate Assembly (GG) was employed to insert the amplified *ptp2* fragment into the GG destination vector, following the protocol and condition outlined in Chaijarasphong et al. [27].

2.2. Preparation of Cas12a guide RNA

The guide RNA for Cas12a consists of 24-bp direct repeats (DR) and 20-bp spacer. The nucleotide sequence of DR is characteristic of Cas12a and is unchanged regardless of the target sequence, whereas spacer sequence must be modified to complement the

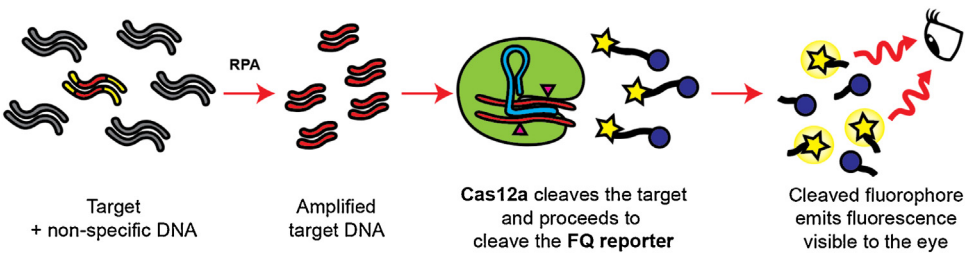

Fig. 1. Schematic of EHP detection with RPA-Cas12a assay. Steps include: 1) amplification of target DNA with RPA 2) cleavage of target DNA with Cas12a, followed by trans cleavage of the fluorophore-quencher reporter 3) visualization of the unleashed fluorescence signal by exposure to UV or blue light. During the development of the assay, a microplate reader was also used for signal quantitation.

Table 1
Primers and oligonucleotides used in this study.

Name	Sequence	Description	Reference
EHP-F1	CACTCAAGGAATGGCTCAAGGGTTCAAAAT	Candidates for RPA primers that were screened in this study. EHP-F1/EHP-R1 pair was used for all other experiments	This study
EHP-F2	ACTGCTACACAAAGGCAGCACTCAAGGAAT		
EHP-F3	AACCAGGCAACCAAAAACTGCTACACAAAG		
EHP-R1	ACCCTGTGAATTGATCATATCTCCTGCCCT		
EHP-R2	CTTTTCGTTAGGCTTACCCTGTGAATTGAT		
EHP-R3	CAATGTCCTTCTTTTCGTTAGGCTTACCCT		
PTP2-GG-F	CACACCAGGTCTCAGTCCATGAGTCTTTATAATGCACTGG	Primers for amplifying *ptp2* coding sequence from EHP genomic DNA. BsaI restriction sites for Golden Gate Assembly are underlined.	This study
PTP2-GG-R	CACACCAGGTCTCACGCTTTATTCGTTGGATGTTAATGTTTCA		
T7-DR	CACCAAAGCAATACGACTCACTATAGGGTAATTTCTACTAAGT GTAGAT	DNA oligos for overlap extension PCR to synthesize templates for in vitro transcription. Template for Guide 1 was assembled from T7-DR and Spacer-EHP-1. Template for Guide 2 was assembled from T7-DR and Spacer-EHP-2.	This study
Spacer-EHP-1	ACCTTCATATAAGATGGACGATCTACACTTAGTAGAAATTACC CTATAGTGAG		
Spacer-EHP-2	ACTACGTTCTTAAACACATCATCTACACTTAGTAGAAATTACC CTATAGTGAG		
Guide 1	GUAAUUUCUACUAAGUGUAGAUCGUCCAUCUUAUAUGAAGGU	Expected sequences of guide RNA obtained from in vitro transcription. Spacers are underlined.	This study
Guide 2	GUAAUUUCUACUAAGUGUAGAUGAUGUGUUUAAGAACGUAGU		
FQ	/5HEX/TTATT/3IABkFQ/	Trans cleavage reporter for Cas12a	[25]
SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT	First step primers for nested PCR targeting *swp*	[12]
SWP_1R	CACGATGTGTCTTTGCAATTTTC		
SWP_2F	TTGGCGGCACAATTCTCAAACA	Second step primers for nested PCR targeting *swp*	
SWP_2R	GCTGTTTGTCTCCAACTGTATTTGA		

DNA target ("protospacer"). To qualify as a protospacer, the sequence must also be preceded on the 5′-end with protospacer adjacent motif (PAM), which has the nucleotide sequence 5′-TTTN-3′, in which N can be any nucleotide.

Each DNA template for in vitro transcription was prepared via overlap extension PCR. Briefly, two short DNA oligos, one containing T7 promoter ("T7-DR") and the other containing the spacer ("Spacer-EHP-1" for Guide 1 and "Spacer-EHP-2" for Guide 2), were designed (Table 1). Direct repeats were included in the overlapping region present in both oligos. Both oligos were then used to establish a 50-μl PCR reaction, which contained GC buffer, 0.2 mM dNTPs, 2 μM of each oligo, and Phusion DNA polymerase. The reaction was incubated in a thermal cycler with the following temperature program: 30 s at 98 °C for initial denaturation, followed by 35 cycles of 10 s at 98 °C, 10 s at 64 °C and 10 s at 72 °C; and 5 min at 72 °C for final extension. The amplified product was directly used in in vitro transcription.

In vitro transcription was performed with HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England Biolabs), following the kit's instructions. The reaction was incubated at 37 °C for 16 h, followed by treatment with enzyme DNase I (Thermo Fisher Scientific) at 37 °C for 30 min to remove DNA template, and purification with RNA Clean and Concentrator™-25 kit (Zymo Research). The concentration was measured by NanoDrop One™ spectrophotometer (Thermo Fisher Scientific). The expected nucleotide sequences of all guide RNA used in this study are given in Table 1 (Guide 1 and 2).

2.3. Screening of RPA primers

After the guide RNA had been experimentally verified to function properly in the Cas12a step, three forward primers and three reverse primers flanking the chosen protospacer were designed (Table 1 and Supplementary Fig. S1). Each primer was 30-bp long and amplicons resulting from all primer combinations were between 221- and 280-bp long. Then, following the methods outlined in section 2.4 and 2.5, the RPA-Cas12a assay was performed on 9 pairwise combinations of the primers and fixed concentration of *ptp2* template (500 copies per reaction).

2.4. Recombinase polymerase amplification (RPA)

RPA reactions were carried out with TwistAmp Liquid Basic kit (TwistDx), following the manufacturer's instructions. Briefly, in a PCR microtube, the master mix was assembled from TwistAmp RPA buffer, 40 mM dNTPs, 240 nM of forward and reverse primers, Core Reaction Mix, Basic E-mix and Nuclease-free water. To each aliquot of the master mix was then added 2 μl of template and 14 mM of $Mg(OAc)_2$ to attain the final reaction volume of 12.5 μl. The vial was immediately mixed by vigorous inversion and then incubated at 42 °C for 20 min in a thermal cycler, after which the amplicon was immediately detected with the Cas12a cleavage assay (Section 2.5). An experiment was also repeated with TwistAmp Basic, the solid counterpart of the Liquid Basic kit, to ensure that the results were independent of the form of RPA reagents used.

2.5. Cas12a detection method

Cas12a from *Lachnospiraceae* ND2006 (Lba Cas12a) was purchased from New England Biolabs. Guide RNA was synthesized following the method outlined in Section 2.2. Fluorophore-quencher reporter (FQ reporter) was obtained from Integrated DNA Technology (IDT), of which the sequence was provided in Table 1. DNaseAlert™ was purchased from Integrated DNA Technology (IDT).

Each Cas12a reaction contained 50 nM Lba Cas12a, 62.5 nM guide RNA, 500 nM FQ reporter in NEBuffer 2.1 in a PCR microtube. (In the experiment to evaluate the commercially available reporter, FQ reporter was substituted with 500 nM DNaseAlert.) Total volume was adjusted to 18 μL with nuclease-free water. Then, 2 μL of the target DNA solution, either plasmid or unpurified RPA product, was mixed with the reagents, and the tube was incubated at 37 °C for 30 min. To quantify fluorescence, the solution was transferred to 384-well black polystyrene microplate (Perkin-Elmer) and analyzed with SpectraMax iD3 microplate reader (Molecular Devices) at excitation and emission wavelengths of 526 and 566 nm, respectively.

For direct visualization under UV, PCR microtubes containing Cas reactions were placed on the trans-illuminating platform and imaged with an Omega Fluor™ Gel Documentation System (Gel

Company). To visualize fluorescence under blue light, BluPAD Dual LED Blue /White Light Transilluminator (Bio-Helix) was used as the light source. After the samples were arranged on the transilluminator, a piece of orange translucent acrylic was placed on top to filter out blue light and enhance contrast. A photo hood was in turn placed above the acrylic to minimize stray light. The image was taken with a smartphone camera, through the peephole on top of the photo hood.

2.6. Lateral flow detection

To detect trans-cleavage activity with lateral flow dipsticks (HybriDetect 1, Milenia), the Cas assay was modified as follows: 1) Concentrations of Cas12a protein and guide RNA were increased to 250 nM and 312.5 nM, respectively; 2) FAM-ssDNA-biotin reporter (125 nM) was used in lieu of FQ reporter. Then, 7.5 μL of the reaction was applied to the sample pad at the end of each strip, which was subsequently immersed in 100 μl HybriDetect assay buffer. After 10 min of incubation, strips were removed for inspection, and images were captured with a smartphone camera.

2.7. Preparation of Her, Eca, and EHP genomic DNA

Genomic DNA from EHP and two aquatic microsporidia, *Enterospora canceri* (*Eca*) and *Hepatospora eriocheir* (*Her*) had been available in our laboratory, originally extracted from their host aquatic organisms — EHP from *P. vannamei*, *Eca* from the European edible crab (*Cancer paragus)* and *Her* from Chinese mitten crab. Total DNA concentrations were quantified with Qubit™ dsDNA BR assay kit. As quality control, EHP, *Eca* and *Her* DNA were subjected to nested PCR using primers targeting the microsporidian SSU-rRNA gene, following the primer sequences and protocol described in Jaroenlak et al. [12].

EHP spore DNA, previously prepared following the method by Aldama-Cano et al. [31], was also available in our laboratory. The copy number of EHP was determined by quantitative PCR (qPCR). DNA extracts from cohabitation-challenged shrimp had been prepared following the challenge protocol reported in Salachan et al., 2017 [32].

2.8. Quantification of EHP DNA with qPCR

The amplification was performed in reactions of 20 μL, each containing 1X SYBR Green PCR Master Mix (KAPA SYBR FAST qPCR kit master mix, Kapa Biosystems, USA), 0.2 μM of each SWP-1 F (TTGCAGAGTGTTGTTAAGGGTTT) and SWP-2R (GCTGTTTGT CTCCAAC TGTATTTGA) primers, 1X Roxbow and 5 μL of a 100-fold dilution of the DNA extracted from the purified EHP spores. For the construction of the standard curve, an extra set of reactions was prepared to contain different concentrations of the plasmid harboring *swp* (10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 EHP-copies/5 μL). Incubation and measurement were carried out using an Applied Biosystem 7500 Real-Time PCR system (AB Applied Biosystems, Foster City, CA, USA) with the following temperature program: 95 °C for 15 s; followed by 40 cycles of 95 °C for 15 s, 64 °C for 30 s and 72 °C for 30 s. This was followed by the dissociation stage (i.e., for construction of a melting curve) of 95 °C for 15 s, 60 °C for 1 min, then gradually increased to 95 °C. After the qPCR amplification, the melting curve was evaluated to ensure that there was no non-specific amplification, and then the baseline and threshold were assigned by using the ABI Prism 7500 Sequence detection software.

2.9. Blind test comparing between nested PCR and RPA-Cas12a

Genomic DNA extracted from purified EHP spores was diluted in nuclease-free water to the final concentrations of 0, 10, 100, and 1000 copies per μl. Each dilution was spiked with 100 ng of background shrimp DNA and aliquoted to either two or three PCR microtubes. The set, consisting of nine samples, was further divided into two identical subsets, which were relabeled to conceal their identities. After the key was prepared, the samples were passed along to two technicians, one of whom analyzed the samples with nested PCR with primers targeting *swp* ("SWP-PCR"), using the method formerly described in Jaroenlak et al. [12], while the other performed detection with RPA-Cas12a. Sequences of SWP-PCR primers are given in Table 1. The results were then officially compared with the key. Before the comparison, all personnel involved were prohibited from discussing about sample preparation and outcome.

2.10. Evaluation of field samples

Hepatopancreatic tissues of shrimp samples had been recently obtained from commercial shrimp farms in March 2020 (n = 20). Five shrimps were sampled per farm. The farms are located in Surat Thani, Phetchaburi, Suphan Buri, and Chantaburi provinces in Southern, Western, Central and Eastern Thailand, respectively. Tissue lysis were carried out with TF lysis buffer [50 mM Tris−HCl (pH 9.0), 100 mM EDTA, 50 mM NaCl, 2% SDS and 10 μg/mL proteinase K], followed by purification with a QIAmp DNA mini kit (QIAGEN).

RPA-Cas12a and SWP-PCR were performed as described in previous sections. For each assay, 100 ng of total DNA extract was used. To evaluate the severity of infection using SWP-PCR, we inspected the number of amplicon bands on agarose gel. To be considered heavily infected, a sample must display both 148- and 514-bp bands, whereas a sample was considered lightly infected if only one band (148 bp) was present.

2.11. Ethics statement

All protocols that involved shrimp and their DNA extracts were approved by The National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand) Animal Care and Use Committee (Protocol Number: BT - Animal 36/2562) and the Faculty of Science, Mahidol University (Thailand) Animal Care and Use Committee (Protocol Number: MUSC62−040-504).

3. Results

3.1. Cas12a trans-cleavage assay can detect ptp2 gene from EHP

Previously, EHP nucleic acid detection assays have been primarily developed to detect two targets: SSU rRNA and spore wall protein (*swp*) genes. As sequences of SSU rRNA are highly conserved, nested PCR that targets this gene was shown to be particularly susceptible to cross-reactivity with other closely related microsporidia, whereas this problem was mitigated when the target was changed to *swp* [12]. In this work, we chose the gene encoding polar tube protein 2 (*ptp2*) as the target due to its low similarity to other microsporidian orthologs. Pairwise nucleotide sequence alignments of EHP *ptp2* with orthologs from three other closely-related microsporidia — *Enterospora canceri* (*Eca*), *Hepatospora eriocheir* (*Her*) and *Enterocytozoon bieneusi* (*Ebi*) — revealed the pairwise identities between 46–56% (Genbank Accession Numbers: LWDP01000024.1, LVKB01000002.1 and CH991540.1 for *Eca*, *Her* and *Ebi,* respectively). In comparison, a previous study that aligned *swp* orthologs from the same set of microsporidia reported the pairwise identities in the range of 60–66% [12].Therefore, like *swp*, an RPA-Cas12a assay designed to target *ptp2* should also be capable of distinguishing EHP from other closely-related microsporidia.

The efficiency of Cas12a cleavage was reported to be strongly influenced by the sequence of the guide RNA, but the precise factors have yet to come to light [33]. For this reason, we tested two different guide RNAs, Guide 1 and Guide 2, on the plasmid carrying *ptp2*. When the trans-cleavage fluorescent signal was measured, we found that Guide 2 significantly outperformed Guide 1 by nearly three folds, while both candidates produced comparable background fluorescence (Supplementary Fig. S2). Therefore, Guide 2 was employed for the rest of the study.

Recent work on CRISPR diagnostics has reported that, without a preceding nucleic acid amplification step, Cas12a trans-cleavage assay is not sensitive enough to detect a small quantity of target [20,27]. To confirm whether this held true for our system, the Cas12a assay was applied to a ten-fold dilution series of *ptp2* plasmid (1 fM -10 nM). Measurement with fluorescence microplate reader revealed that the lowest concentration of the plasmid that yielded significant signal above background was 100 pM, equivalent to roughly 3×10^9 copies per reaction ($p < 0.001$) (Fig. 2). This suggests that the Cas12a assay alone was not capable of detecting low level of target DNA and nucleic acid amplification was thus required.

3.2. RPA-Cas12a detects ptp2 with high sensitivity

Similar to guide RNA, the lack of tools to predict the efficiency of RPA primers *a priori* necessitates a screening effort [34,35]. To this end, three forward and reverse primers were designed as described in Section 2.4, and nine pairwise combinations were tested in an RPA-Cas12a assay with a fixed concentration of the *ptp2* plasmid. When measured by microplate reader, all primer combinations produced signal that was at least 40-fold higher than the background (Supplementary Fig. S1), with only slight differences among primer pairs (Figs. 1 and 2).

We picked one of the primer combinations, EHP-F1/EHP-R1, and set out to evaluate its sensitivity. RPA-Cas12a was performed on a 10-fold dilution series of *ptp2* plasmid (5 – 5×10^5 copies/reaction). The detection limit was found to be 50 copies per reaction (**Fig. 3A**). This level of sensitivity is comparable to that of the RPA-gel electrophoresis approach, consistent with our previous finding [27] (Supplementary Fig. S3). Between 50–5×10^5 copies, the fluorescence intensity showed no correlation with target quantity. Hence, by coupling with RPA, the sensitivity of Cas12a was boosted by approximately 60 million folds and reached the level demanded for detection of early EHP infections.

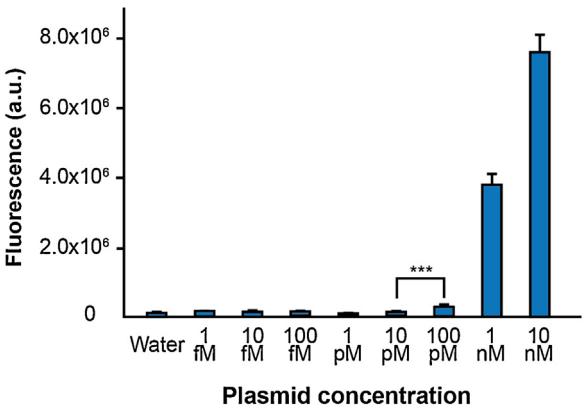

Fig. 2. Cas12a detection of *ptp2* plasmid at various concentrations without nucleic acid amplification. Fluorescence emission at 566 nm was measured with a microplate reader. The lowest concentration of plasmid that yielded a signal above background level was 100 pM, corresponding to approximately 3×10^9 copies per reaction. Results are displayed as mean ± SD (n = 3); ***$p < 0.001$.

The field use of a fluorescence microplate reader would be impractical from cost and transportability standpoints, but recent studies have demonstrated that simple UV or blue light generators could also be used to excite the reporter in the Cas12a assay [27,36]. When we exposed the finished reaction vials on UV and blue light transilluminators, strong fluorescence emission could be observed by eye, with clear contrast between positive and negative results (Figs. 3**B-C**). The level of sensitivity was also 50 copies per reaction, equal to the detection limit achieved with a microplate reader.

As a large amount of shrimp genomic DNA is often present alongside the target DNA, we evaluated whether the unrelated DNA would compromise the sensitivity of RPA-Cas12a. We applied the assay to analytes containing 0 or 50 copies of *ptp2* plasmid, spiked with 100 ng of DNA extracted from naïve shrimp (i.e., shrimp not infected with EHP), and compared the results with the samples that did not possess background shrimp DNA. In all modes of measurement, fluorescence emission from the spiked samples was indistinguishable from the samples without excess shrimp DNA added (Figs. 3D-F).

All experiments up to this point had been performed with liquid RPA reagents (TwistAmp Basic Liquid) which are suitable for R&D applications but cumbersome for field use. On the other hand, the solid version of RPA, TwistAmp Basic, combines every reagent into a dry pellet and is therefore portable, easier for storage and convenient for field work. To ensure that both forms are interchangeable, we compared the performance between solid and liquid RPA and found that they offered the same level of sensitivity and fluorescence intensity under UV visualization (Supplementary Fig. S4). Additionally, the presence of 100 ng of shrimp background DNA in each solid RPA reaction did not alter the final fluorescent responses, across the entire range of *ptp2* concentrations tested.

Thus far in this work, we had used a custom-made FQ reporter, based on the design reported by Gootenberg et al., though a number of studies in this field opted for a commercially available reporter such as DNaseAlert™ (Integrated DNA Technologies; [20,37,38]. We compared the custom reporter and DNaseAlert in RPA-Cas12a detection of 50 and 50,000 copies of *ptp2* plasmid, and observed that DNaseAlert was superior to its custom-made counterpart at both low and high copies of template, although the signal differences were less than 30 % (Supplementary Fig. S5). In addition, the commercial reporter yielded an approximately 4-fold higher background signal compared to the custom FQ. Hence, due to the negligible difference in terms of sensitivity between the two reporters, we continued to use the custom FQ reporter for the rest of this study.

3.3. RPA-Cas12a is highly specific to EHP

In addition to the sensitivity, our choices of RPA primers and guide RNA were confirmed for their specificity to EHP. Through nucleotide sequence alignments, we found the protospacer and RPA primer annealing sites in EHP *ptp2* to be highly dissimilar to the corresponding regions in two aquatic microsporidians — *Her* and *Eca* — and a human microsporidian, *Ebi* (Fig. 4A). We also BLASTed the protospacer and primers against the draft genomes of these microsporidia and found no similarities to either other regions outside *ptp2* in EHP or any regions in the three closely-related microsporidia (Supplementary Table S1). As predicted, when we tested our RPA-Cas12a protocol with 10 ng genomic DNA extracts from EHP, *Her*, and *Eca*, only EHP produced intense fluorescence, whereas *Eca* and *Her* exhibited the baseline level of signal (Figs. 4B-D).

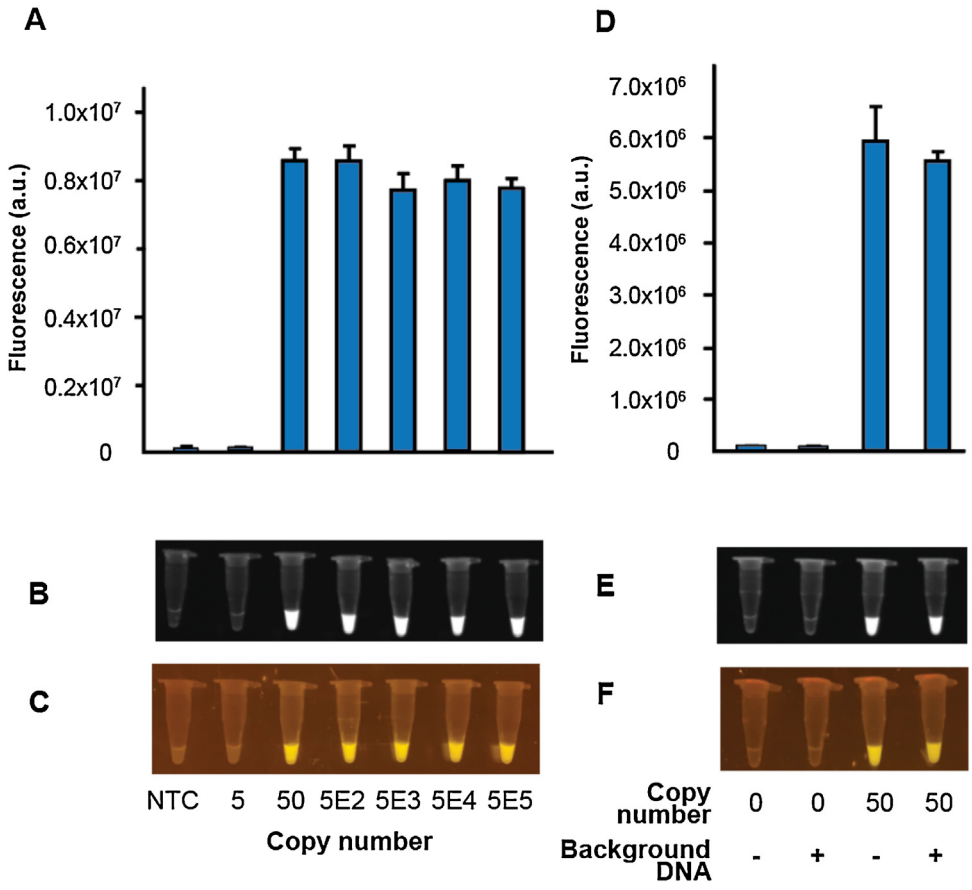

Fig. 3. Sensitivity of the RPA-Cas12a assay. (A) Fluorescence emission at 566 nm of the reactions containing between 0 – 5×10^5 copies of the *ptp2* plasmid. The measurement was performed with a microplate reader. (B and C) Exposure of reaction vials from (A) to UV (B) and blue light (C). (D) Fluorescence emission at 566 nm when the reactions contained 0 or 50 copies of *ptp2* plasmid, in presence or absence of 100 ng of EHP-free shrimp genomic DNA. The measurement was performed with a microplate reader. (E and F) Exposure of reaction vials from (D) to UV (E) and blue light (F). Results from measurements with the microplate reader are shown as mean ± SD (n = 3). One out of three replicates is shown for UV and blue light experiments.

3.4. RPA-Cas12a coupled with lateral flow detection allows for instrument-free visualization

Although the visualization of RPA-Cas12a results does not rely on expensive equipment other than a UV or blue light trans-illuminator, a detection technique that does not need battery- or electricity-powered devices altogether will be ideal for rapid field tests. Cas12a detection had been adapted for lateral flow detection (LFD), with an important alteration: the FQ reporter was replaced with FAM-ssDNA-Biotin reporter (FB reporter) [22,23,25,39]. Without trans cleavage, the FB reporter will remain intact and its biotinylated end can be captured by streptavidin at the control line. Anti-FAM antibody conjugated to gold nanoparticles can then bind the exposed FAM moiety, resulting in the color deposit on the control line. On the other hand, if the FB reporter has undergone trans cleavage by Cas12a, a portion of reporter molecules will contain biotin, but not FAM (Fig. 5A). As a result, fewer FAM ends will be present at the control line, and a higher amount of antibody-conjugated gold nanoparticles will be able to travel further to deposit on the test line, forming a pink-colored band. In short, if EHP is present, application of the completed RPA-Cas12a reaction to the strip will culminate in coloration of both control and test lines.

Following the described modification, we applied RPA-Cas12a-LFD to serially diluted *ptp2* plasmid. Samples with at least 50 copies of the template produced a band at the test line, indicating that LFD has the same sensitivity as the fluorescent assays (Fig. 5B). Band intensity was also comparable in all positive samples.

3.5. Comparison of RPA-Cas12a method with nested PCR

To compare the performance of RPA-Cas12a and SWP-PCR in a bias-free manner, we organized a blind test using EHP spore DNA at various concentrations as templates, as described in Section 2.9. Out of nine blind samples, SWP-PCR and RPA-Cas12a showed excellent agreement for seven samples (Table 2). Both methods successfully detected analytes that possessed between 100 – 1000 copies of EHP DNA and yielded no false positive results. Neither techniques could reliably detect samples with 10 copies of EHP: SWP-PCR returned a negative result for one of such samples, and RPA-Cas12a was unable to detect any of them.

Additionally, SWP-PCR and RPA-Cas12a were employed to detect EHP in shrimp that had undergone a laboratory cohabitation challenge, collected 3, 14, and 20 days post infection (dpi) [32]. Both techniques showed perfect agreement in all 17 samples analyzed, including 3 lightly infected samples that were detectable only in the second step of nested PCR (Figs. 6A–C). Neither methods could detect EHP before 3 dpi.

Finally, we compared the performance of RPA-Cas12a and SWP-PCR in detecting field samples, which included 10 shrimp from healthy ponds and 10 from EHP ponds. By SWP-PCR, 12 samples gave positive results — 2 from healthy ponds and 10 from EHP

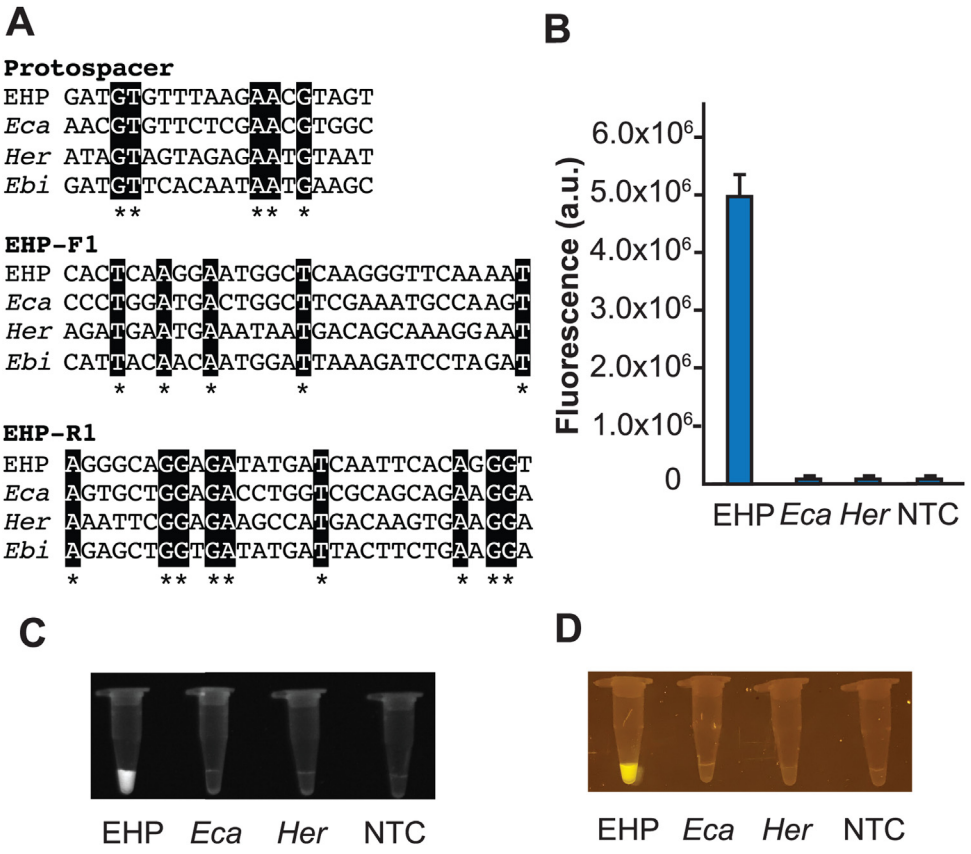

Fig. 4. Specificity of the RPA-Cas12a assay. (A) Multiple sequence alignments of the protospacer (the DNA sequence targeted by Cas12a) and RPA primers (EHP-F1 and EHP-R1) with homologous regions in *ptp2* from other microsporidia. (B) Fluorescence emission at 566 nm measured with a microplate reader. Each reaction contained 10 ng of DNA extracted from EHP, *Eca* or *Her*. A reaction with no microsporidian DNA was performed as a negative control. Results are displayed as mean ± SD (n = 3). (C and D) Visualization of reaction vials with UV (C) and blue light illumination (D). Only one out of three replicates is shown in (C) and (D).

ponds (Table 3). Based on the criteria described in Materials and Methods, two positive samples from the healthy ponds were considered lightly infected, while those from the EHP-affected ponds were heavily infected. Using RPA-Cas12a visualized with UV or LFD, samples S11-S20 gave positive results, consistent with SWP-PCR. However, samples S1-S10 were all tested negative.

4. Discussions

Thus far, several nucleic acid-based detection platforms have been developed for EHP, including nested PCR, qPCR, one-step PCR coupled with LFD, and LAMP. Although these methods offer good sensitivity, they are limited by various constraints such as susceptibility to false positives and lack of compatibility with point-of-need applications. For example, SSU-PCR could detect 10 copies of EHP DNA per reaction, but also confers considerable false positive rates due to the high nucleotide sequence conservation among SSU rRNA homologues. While SWP-PCR successfully abolishes false positive results, it still needs a thermal cycler and visualization with agarose gel electrophoresis, hence more practical for laboratory settings than field work [12]. LAMP is characterized by a streamlined protocol and exemplary detection limit of 1 copy of EHP DNA per reaction, but spurious amplification can occasionally arise [14]. During the preparation of this manuscript, a research group reported the development of RPA targeting the SSU-rRNA gene of EHP, visualized by agarose gel electrophoresis but not coupled with Cas detection. The assay had the detection limit of 800 copies per reaction and was shown to exhibit no false positive results with shrimp pathogens such as IHHNV, WSSV, and *Vibrio parahaemolyticus* (AHPND strain), but the specificity was not tested against other microsporidia [40].

By comparison, RPA-Cas12a developed in this study had no cross-reactivity with *Her* and *Eca* and could consistently detect 50 copies of DNA per reaction. The entire operation takes only 1 h from sample to answer and requires minimal equipment, including a simple heating device for incubation and a cheap UV or blue light generator for naked eye visualization. In case that a heating instrument cannot be procured, it may even be possible to exploit body heat for incubation [41]. Furthermore, the modular nature of this assay also allows flexibility for modifications. By changing the type of reporter, the assay could be adapted into an LFD format that does not demand any visualization instrument. These advantages combined render RPA-Cas12a a strong option for routine surveillance in resource-limited settings.

With the detection limit of 50 copies per reaction, RPA-Cas12a is slightly less sensitive than SWP-PCR, which could explain the disagreement between two approaches at the lowest target concentration of EHP in the blind test and evaluation of field samples. Nonetheless, RPA-Cas12a developed in this work should still be sensitive enough to permit timely detection of EHP, as the negative correlation between average shrimp body weight and EHP load only manifested after EHP reached 1000 copies per 1 ng of hepatopancreas DNA [7].

In this study, we also evaluated the effects of several parameters on the performance of RPA-Cas12a. For example, we observed the large gap between the potency of Guide 1 and Guide 2, which could not have been predicted from their sequences, and this underscores the importance of testing more than one design of guide

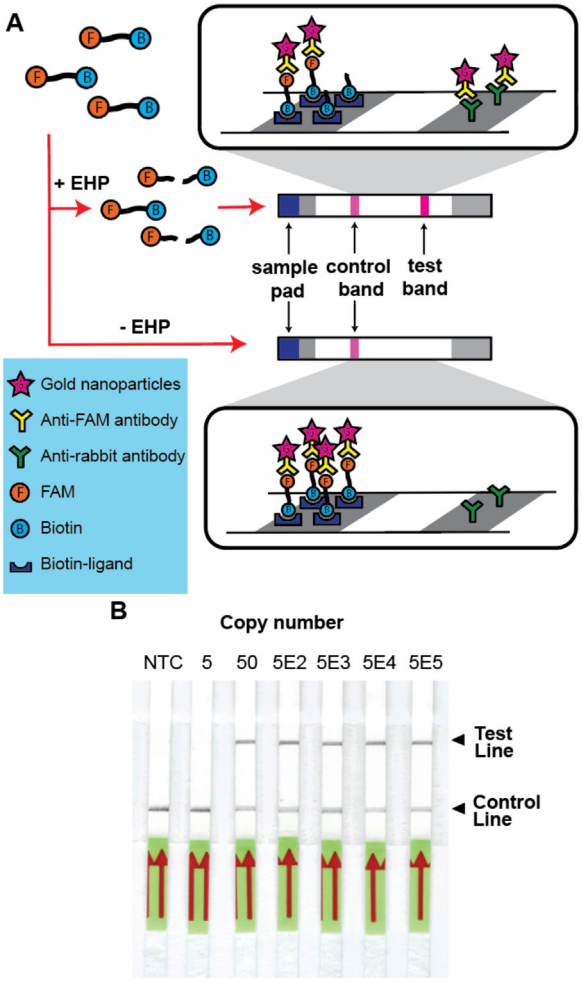

Fig. 5. Lateral-flow detection with RPA-Cas12a. (A) Schematic illustration describing how trans cleavage of FAM-biotin reporter leads to appearance of the test band. (B) Lateral-flow detection of serially diluted *ptp2* plasmid (5 – 50,000 copies/reaction). It should be noted that the locations of test and control lines are reversed compared to the conventional lateral flow dipsticks, in which the test line is located next to the sample pad.

Table 2
Comparison between blind test results from SWP-PCR and RPA-Cas12a. The results are sorted from low to high copy numbers and the order shown does not reflect how the blind samples were originally labeled.

Sample	Copy number	Result	
		SWP-PCR	RPA-Cas12a
1	0	–	–
2	0	–	–
3	10	+	–
4	10	+	–
5	10	–	–
6	100	+	+
7	100	+	+
8	1000	+	+
9	1000	+	+

RNA. On the other hand, changing the format of RPA reagents (liquid and solid) did not appear to affect sensitivity and overall fluorescence emission. In addition, we found that the commercial reporter, DNaseAlert, performed slightly better than the custom FQ reporter but the difference was not substantial. Nonetheless, future studies should assess other relevant parameters — such as stability during storage, susceptibility to inhibitors, and batch-to-batch

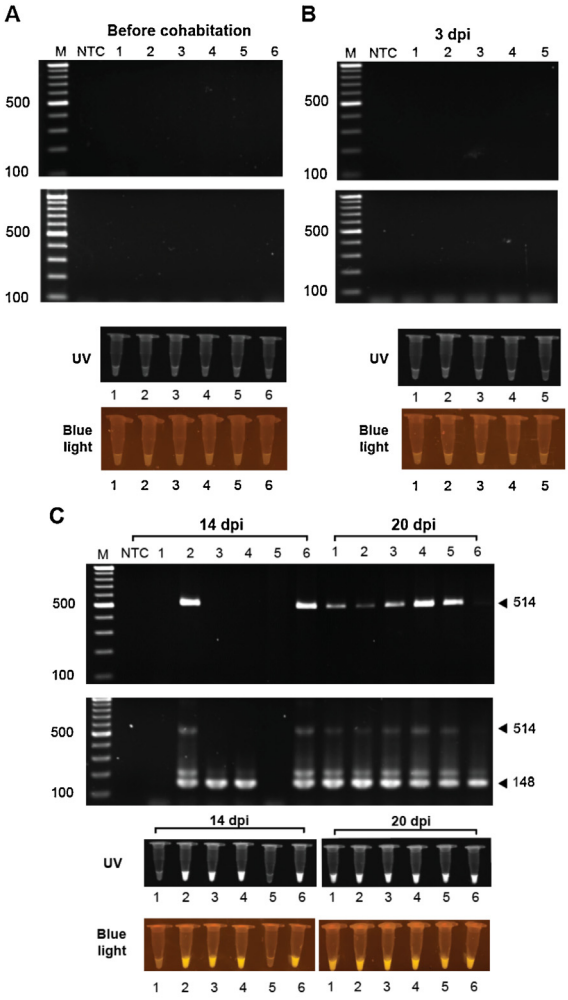

Fig. 6. Comparison between detection of EHP in cohabitation-challenged shrimp by SWP-PCR and by RPA-Cas12a. DNA was extracted from hepatopancreas collected before cohabitation and at 3, 14 and 20 dpi. (A) Data from before cohabitation; (B) data from 3 dpi; (C) data from 14 and 20 dpi. Each subpanel of the figure shows the following results (from top to bottom): agarose gel electrophoresis of the first step of SWP-PCR, second step of SWP-PCR, UV-exposed reaction vials, blue light-exposed reaction vials. The amplicons from the first and second steps of SWP-PCR are 514- and 148-bp long, respectively, as indicated by black arrows. SWP-PCR amplicons from 14 and 20 dpi were analyzed on the same agarose gel. Abbreviations above the agarose gel images: M = molecular weight marker; 1-6 corresponds to different shrimp from the same date.

consistency — to determine whether the custom reporter would be a truly practical alternative to its commercial counterpart.

In terms of costs, Gootenberg et al. estimated that each RPA-Cas reaction should cost less than $0.62, taking into account the costs of RPA-Cas reagents and devices [42]. However, this calculation had not considered the licensing fees in case of commercialization and the costs of DNA extraction kits. Thus far, the licensing fee of RPA-Cas12a technology has not been publicly disclosed and the actual number will depend largely on the licensing options and the agreements between the patent owner and individual patentees. On the other hand, the costs due to DNA extraction can be readily minimized. For example, in lieu of costly commercial DNA purification kits, the user could opt for cellulose-based DNA extraction methods that require only filter papers or paper dipsticks and other cheap consumables [43,44]. Therefore, while the final cost per assay for RPA-Cas12a remains subject to several

Table 3
Comparison between SWP-PCR and RPA-Cas12a in evaluation of field samples. For nested PCR targeting *swp* (SWP-PCR): -, +, ++ denote, negative result, light infection, and heavy infection, respectively. Infection severity was graded based on the number of PCR product bands, as explained in Materials and Methods. For RPA-Cas12a visualized with UV excitation and LFD, - and + denote negative and positive result, respectively. Since this method offered only binary responses, infection severity was not graded.

Sample number	Region	Result		
		SWP-PCR	RPA-Cas12a (UV)	RPA-Cas12a (LFD)
S1	Southern Thailand	–	–	–
S2		+	–	–
S3		+	–	–
S4		–	–	–
S5		–	–	–
S6	Western Thailand	–	–	–
S7		–	–	–
S8		–	–	–
S9		–	–	–
S10		–	–	–
S11	Central Thailand	++	+	+
S12		++	+	+
S13		++	+	+
S14		++	+	+
S15		++	+	+
S16	Eastern Thailand	++	+	+
S17		++	+	+
S18		++	+	+
S19		++	+	+
S20		++	+	+

factors, it should still fall within an affordable range for shrimp farmers in resource-limited locales.

The RPA-Cas12a method has demonstrated many strengths, but there are improvements that could be made to augment its efficiency and versatility even further. First, RPA-Cas12a is currently not quantitative. As it has been reported that the degree of growth retardation is correlated with EHP copy number, quantitative RPA-Cas12a could additionally inform the user about the stage of disease progression [7]. Although Cas12a alone displayed a linear relationship between fluorescence and target quantity when plasmid concentrations were between 100 pM to 10 nM, such linearity was no longer observed upon the introduction of RPA. This agrees with previous reports that RPA rapidly saturated even at a low concentration of template, and it follows that an effort to make RPA-Cas12a quantitative should primarily focus on the RPA step [25,27,42].

Moreover, while the assay in the current form has minimal liquid handling steps relative to PCR-based methods, further simplifications could still be made. For instance, it has recently been reported that RPA and Cas12a could be combined into a semi one-pot reaction with the detection limit of 10 aM (150 copies of DNA per reaction), but Cas12a has to be physically separated from RPA until several minutes into the reaction [36,45]. A true, efficient one-pot reaction in which RPA and Cas12a reagents can be combined into a master mix still remains to be developed. To enhance shelf-life and portability, a previous study reported that Cas13a, an RNA-targeting Cas homologue that has also been employed for nucleic acid detection, could be freeze-dried on a piece of filter paper without significant loss of activity, but such tolerance has not been validated in Cas12a [42]. Lastly, RPA was reported to be exhibit more tolerance to inhibitors from field samples than qPCR [46]. For this reason, as DNA extraction and purification are often constraints in field implementation, future work should consider adopting crude DNA isolation protocols that have been verified as compatible with RPA such as sodium hydroxide (NaOH)-based extraction or mechanical lysis followed by DNA enrichment with cellulose dipsticks [43,47]. Compatibility of such techniques with Cas12a must also be verified, although the effects from any potential inhibitors may have been partly attenuated due to fact that only 2 μl of RPA is applied to 20 μl of Cas12a reaction. Ultimately, a rapid low-cost DNA isolation technique and freeze-dried paper-based platform should be implemented together to achieve the most portable, accessible and practical form of RPA-Cas12a detection.

5. Conclusions

Herein, we have demonstrated that RPA-Cas12a could detect EHP from infected shrimp with high sensitivity and specificity, all while demanding one hour and relatively constant temperature to carry out. The method did not exhibit false positive results with other aquatic microsporidia, and was robust to the presence of abundant background DNA from shrimp. Various low-hassle options were available for visualization of results, including UV, blue light, and LFD. Therefore, RPA-Cas12a shows great promise for routine in-field applications, and is highly accessible to users without extensive experience in molecular diagnostics.

CRediT authorship contribution statement

Suthasinee Kanitchinda: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Jiraporn Srisala:** Data curation, Investigation, Resources, Validation, Writing - review & editing. **Rungkarn Suebsing:** Resources, Validation, Writing - review & editing. **Anuphap Prachumwat:** Resources, Writing - review & editing. **Thawatchai Chaijarasphong:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00485.

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