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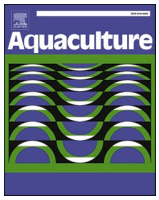

Development of a real-time enzymatic recombinase amplification assay (RT-ERA) and an ERA combined with lateral flow dipsticks (LFD) assay (ERA-LFD) for rapid detection of acute hepatopancreatic necrosis disease (AHPND) in shrimp *Penaeus vannamei*

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) is a major bacterial acute disease of shrimp caused by *Vibrio* infection, which can lead to high mortality and cause huge economic losses to the shrimp farming industry. Therefore, the development of early, rapid, and accurate AHPND diagnostic methods plays an important role in disease prevention in shrimp aquaculture. In this study, a real-time enzymatic recombinase amplification assay (RT-ERA) and an ERA combined with lateral flow dipsticks (LFD) assay (ERA-LFD) based on the conserved sequence of the pirA and pirB gene was developed and evaluated. Both the two methods can complete the detection within 30 min at a constant temperature of 37–42 °C. Using plasmid standards as template, the detection limits of the both methods are 10^1 copies/μL, which is 10 times more sensitive than those of nested PCR and RT-PCR. Taking DNA extracted from the hepatopancreas of diseased shrimp as template, the detection limit of the both methods is 1 pg/μL, which is as the same as nested PCR and 1000 times more sensitive than that of RT-PCR. The specificities of both assays were tested and no cross-reaction with white spot syndrome virus (WSSV), *Enterocytozoon hepatopenaei* (EHP), infectious hypodermitis and hematopoietic necrosis virus (IHHNV) and healthy shrimp was exhibited. The practical applicability of the both assays were evaluated using 24 field samples. The coincidence rates of RT-ERA, ERA-LFD and nested PCR on samples collected from shrimp farms were all 100%, which were higher than RT-PCR. Taken together, the developed RT-ERA and ERA-LFD assays are simple, rapid, sensitive, and affordable on-site diagnostic methods for AHPND infection, with great potential to help control AHPND infection and reduce economic losses in the shrimp industry.

1. Introduction

Penaeus (*Litopenaeus*) *vannamei* is one of the most important aquatic species in the world. Since 2009, the emergence of a bacterial disease called acute hepatopancreatic necrosis disease (AHPND) has led to a decline in shrimp production and severe economic losses (Hong et al., 2016). AHPND is characterized by sudden mass mortality (up to 100%), and usually breaks out at 30–35 days after juveniles (de la Peña et al., 2015). AHPND-affected shrimp exhibited lethargy, anorexia, slow growth, empty digestive tract and pale to white hepatopancreas. In 2013, the causative agent of AHPND was identified as *Vibrio parahaemolyticus* (*Vp*AHPND) (Tran et al., 2013). The pathogen carries the avirulent pVA1 plasmid, a virulence plasmid of approximately 69 k bp in size, with characteristics of insects related to the Photorhabdus (Pir) toxin, a binary toxin gene similar to PirA and PirB (Lee et al., 2015; L Tran et al., 2013). PirA and pirB toxin proteins are the main pathogenic

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factors causing AHPND, to some extent, through gastric sieves to the hepatopancreas (Lai et al., 2015). In addition to these toxin genes, plasmids also encode conjugative transfer genes and transposons, suggesting the potential for plasmid transfer into other strains or species (Lee et al., 2015). Recent information indicates that pVA1 plasmids and variants are present in many *V. parahaemolyticus* serotypes, as well as in other *Vibrio* species, such as *Vibrio cambei, Vibrio harvey* and *Vibrio erwinia* (Dong et al., 2017; Kondo et al., 2015; Liu et al., 2015; Prachumwat et al., 2019; Restrepo et al., 2018). The bacteria that cause AHPND initially colonize the stomach of infected shrimp, wihle in the hepatopancreas, this toxin induces shedding of tubular epithelial cells and causes blanching of the hepatopancreas (Kumar et al., 2021; Loc Tran et al., 2013). The first outbreak of AHPND was reported in China. Since then, outbreaks of the disease have been reported in shrimp production in Mexico, Malaysia, Thailand, Philippines, Vietnam, Bangladesh, and the United States, all causing severe economic losses (de la Peña et al., 2015; Dhar et al., 2019; Flegel, 2012; Kongrueng et al., 2015a, 2015b; Nunan et al., 2014). In crustaceans, regular vaccinations don't work because they don't have acquired immune system. Treatment methods for AHPND mainly include application of antibiotics, disinfectants or phages, and the supplementation of immunostimulants, probiotics, recombinant protein, gold nanoparticles or plant-based compounds. (Boonchuen et al., 2018; Campa-Córdova et al., 2017; Jun et al., 2018; Junprung et al., 2019; Visetnan et al., 2017). Among these potential treatments, antibiotics might cause the problem of pathogen resistance, disinfectants or phages would lead to environmental pollution, and the supplementation of immunostimulants, probiotics, recombinant protein, gold nanoparticles or plant-based compounds have shown good effects in the laboratory, but there is still a large gap between them and the actual application (Zhou et al., 2020). Therefore, the development of rapid and sensitive methods for the detection of AHPND is necessary for the prevention and control of this disease.

Initially, the diagnosis of AHPND was confirmed by clinical signs and histopathology of shrimp. Later, the PCR techniques with specific primers and probes were developed to detect plasmids or PirAB toxins. PCR methods based on AP1, AP2 and TUMSAT-Vp3 could specifically detect targeted plasmid sequences (Tinwongger et al., 2014). AP3 method only amplify the gene sequence of known proteins in pathogenic strains of AHPND (Sirikharin et al., 2015). To avoid false positive results, a nested two-step PCR method (AP4) was developed based on the method recommended by the World Organization for Animal Health (OIE) to detect lower levels of AHPND-causing bacteria (Dangtip et al., 2015). AP1, AP2, TUMSAT-Vp3, AP3 and AP4 are the names of published PCR techniques for the detection of AHPND. Real-time PCR (RT-PCR) methods such as AHPND-specific TaqMan RT-PCR and SYBR Green RT-PCR methods were also developed later (Cruz-Flores et al., 2019; Han et al., 2015). In addition to PCR methods, several new detection methods to detect AHPND have been also investigated, such as monoclonal antibody assays, biosensors and enzyme-linked immunosorbent assay (ELISA) (Mai et al., 2020; Rizan et al., 2018; Wangman et al., 2020). However, these techniques all require complex detection procedures, expensive instruments and trained staff, and require extended periods of time in popular field environments. In recent years, isothermal amplification techniques have also been widely used for the detection of AHPND, such as loop-mediated isothermal amplification (LAMP) (Arunrut et al., 2016; Koiwai et al., 2016; Kongrueng et al., 2015a, 2015b), recombinase polymerase amplification (RPA) (Mai et al., 2021; Zhou et al., 2020) and enzymatic recombination amplification (ERA). LAMP targeting sequence is 6 or 8 regions within a small fragment, therefore, primer design is subject to many restrictions and faces a high risk of contamination. Therefore, it is necessary to develop a rapid and sensitive method for the detection of AHPND for the prevention and control of this disease.

ERA is an improved RPA method, which optimizes the molecular structure and function of specific recombinase, exonuclease, DNA polymerase and other enzymes in bacteria, viruses, phages, so as to obtain a better system to complete the amplification. Under the constant temperature of 37–42 °C, the specific fragments of trace DNA/RNA can be amplified billions of times in a few minutes. In recent years, ERA has emerged as a promising molecular biology technique for rapid diagnosis of various pathogens, such as detection of Streptomycin-resistant mutations in mycobacterium, cancer gene mutations, porcine epidemic diarrhea virus (PEDV), and porcine circovirus type 3 (PCV3) (Liu et al., 2022, 2021; Yang et al., 2021; Zhang et al., 2021). In this study, we aimed to develop two rapid and sensitive assay, real-time ERA assay (RT-ERA) and ERA combined with lateral flow dipsticks (LFD) assay (ERA-LFD), for the on-site detection of AHPND. Targeting the pirA and pirB gene of AHPND, we designed ERA primers and probes for the detection of AHPND, optimized reaction conditions, assessed sensitivity and specificity, and determined utility through clinical sample testing.

2. Materials and methods

2.1. Shrimp and pathogen samples

P. vannamei (average body weight 30 g, average length 10 cm) were taken from an aquaculture farm in Wenchang, Hainan, China and domesticated at 26 ± 1 °C before use. The pathogenic *V. parahaemolyticus* was kindly provided by Prof. Lei Wang, the Institute of Oceanology, Chinese Academy of Sciences. Other viruses and bacteria are kept in our laboratory.

2.2. DNA extraction

The prawn genomic DNA and raw DNA of *Vp*AHPND was extracted from the hepatopancreas of infected *P. vannamei* were extracted using the TIANamp Marine Animals DNA Kit (Tiangen, China), and then stored at −20 °C until use.

2.3. The construction of a standard recombinant plasmid

PCR was performed in a total volume of 10 μL containing 5 μL 2 × Taq PCR Master Mix, 2 μL ddH_2O, 1 μL forward primer *Vp*AHPND-F (10 μM), 1 μL reverse primer *Vp*AHPND-R (10 μM) and 1 μL of *V. parahaemolyticus* DNA (10 ng/μL). PCR products were purified using a gel extraction kit (DP209, Tiangen, China) and cloned into pEASY-Blunt Zero vector (CB501–01, TransGen, China). Three to five independent clones of each amplicon were sequenced. Positive plasmids were extracted using the EasyPure HiPure Plasmid MiniPrep Kit (EM121–01, TransGen, China) following the manufacturer's protocol. The concentration and purity of the recombinant plasmids were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA), and stored at −20 °C. The primers for universal PCR in this study are shown in Table 1.

2.4. Primer and probe design

Partial of pirA and pirB gene sequences (GenBank Accession number: KM067908) were selected as target. Primer Premier 5.0 was used to design primers for ERA reactions. And RT-ERA probes and ERA-LFD probes were designed based on sequences between ERA primers. The primer length of ERA is 28–35 bases to avoid unusual sequences in the primer, such as a long sequence consisting entirely of one particular nucleotide or a short sequence with many repeats. GC content should be between 40%–60% to avoid the formation of primer dimer and hairpin structure. Tm is between 50 °C and 70 °C. The length of RT-ERA probes should be 46–52 nucleotides, of which at least 30 are located at the 5 'end of the THF site and at least 15 are located at the 3' end. THF residues, quenchers, and fluorophores replace the bases in the target amplified sequence, rather than inserting them additionally. ERA-LFD probes add a fluorophore at the 5′ end, a C3 spacer at the 3′ end, and a THF residue in the middle. It should be noted that the reverse primer

Table 1
Primers and probes for universal PCR, nested-PCR, RT-PCR and ERA this study.

Primer name	Sequence (5′-3′)	Product size (bp)
VpAHPND-F	AGTAACAATATAAAACATGAAACTGACTATTC	1665
VpAHPND-R	CTACTTTTCTGTACCAAATTCATCGG	
AP4-F1	ATGAGTAACAATATAAAACATGAA	1269
AP4-R1	ACGATTTCGACGTTCCCCAA	
AP4-F2	TTGAGAATACGGGACGTGGG	230
AP4-R2	GTTAGTCATGTGAGCACCTTC	
VpPirA-F	TTGGACTGTCGAACCAAACG	
VpPirA-R	GCACCCCATTGGTATTGAATG	
VpPirA-probe	/i6FAMdT/ AGACAGCAAACATACACCTATCATCCCGGA/ iTAMRA/	135
pirAB-F1	ATGGCGCTAGTCGTGGTTTCTGTACAAT	
pirAB-F2	GTACAATCTATTACCACTAAGAAGGTGCTC	
pirAB-F3	CGTGATGAAACTTACCATTTACAACGCCCT	
pirAB-R1	ATTCCATAACGTTGATAAAAAACCACCC	
pirAB-R2	TTGAATTTTATCGCGTGTCTCTTTGATTTT	
pirAB-R3	CGTGATGAAACTTACCATTTACAACGCCCT	
pirAB-R1-LFD	/Biotin/ATTCCATAACGTTGATAAAAAACCACCC	
pirAB-R2-LFD	/Biotin/TTGAATTTTATCGCGTGTCTCTTTGATTTT	
pirAB-R3-LFD	/Biotin/CGTGATGAAACTTACCATTTACAACGCCCT	
Probe1	CTCACATGACTAACGAATACGTTGTAACAA/ i6FAMdT//THF//iBHQ1dT/ CATCTTTGACGGAATTT/iSpC3/	
Probe2	/i6FAMdT/ CTCACATGACTAACGAATACGTTGTAACAAT/THF/ TCATCTTTGACGGAATTT/iSpC3/	

has another antigenic marker at its 5′ end. The primers and probes of ERA are shown in Table 1.

2.5. Development of ERA assays

Basic ERA nucleic acid amplification kit, fluorescent ERA nucleic acid amplification kit, test strip ERA nucleic acid amplification kit and lateral flow test strips (KS101, KS103, KS105, and TS101, GenDx, China) dipsticks were used for standard ERA, RT-ERA and ERA-LFD, respectively. Both the RT-ERA and ERA-LFD systems are mixed in an Eppendorf tube equipped with white powder. The 50 μL system consists of 2 μL forward primer (10 μM), 2 μL reverse primer (10 μM), 0.6 μL probe (10 μM), 2 μL DNA template, 20 μL lysate, 2 μL Mg^{2+} buffer (280 mM), and 21.4 μL ddH_2O. The cycle parameter of RT-ERA is 40 cycles, 30 s per cycle. In the experiment to optimize reaction temperature of RT-ERA, amplification was performed at 37 °C, 38 °C, 39 °C, 40 °C, 41 °C, 42 °C and 43 °C for 20 min. In the experiment to optimize the reaction temperature of ERA-LFD, the amplification was performed at 20 °C 25 °C, 30 °C, 35 °C, 38 °C, 39 °C 40 °C and 41 °C for 30 min. Then the samples were diluted with ddH_2O at 1:40, the dipsticks were put into the diluted samples, and the results could be read in 2 min. In the experiment to optimize the reaction time of ERA-LFD, the amplification was performed at the most suitable temperature for 5 min, 10 min, 15 min, 20 min, 25 min, 30 min and 35 min, then diluted with ddH_2O at 1:40 and tested with dipsticks.

2.6. Specificity analysis

The specificity of the AHPND-ERA assay was evaluated by detecting a group of pathogens including WSSV, EHP, and IHHNV and healthy shrimp. The positive control is DNA extracted from an AHPND positive validation sample. The response without template was used as a negative control.

2.7. Sensitivity analysis

To confirm the detection line of AHPND-ERA, two sets of sensitivity tests with different units were developed. One is to dilute the recombinant plasmid to the range of 10^8–10^0 copies/μL, and the other is to dilute the DNA extracted from the diseased shrimp to the range of 10 ng/μL-10 fg/μL. Two templates were used for nested PCR, RT-PCR, RT-ERA and ERA-LFD amplification. The detection results of the four methods were compared to evaluate the sensitivity of the four methods. The primers for nested-PCR and RT-PCR in this study are shown in Table 1.

2.8. Practical application test

Twenty-four Shrimp samples including 9 AHPND infected shrimps and 15 specific pathogen free (SPF) shrimps from a shrimp farm in Wenchang were randomly collected. Virological testing was performed by RT-ERA, ERA-LFD, RT-PCR (Han et al., 2015) and nested-PCR (Dangtip et al., 2015) to detect AHPND in these samples.

3. Results

3.1. Evaluation of AHPND-ERA primers

The relative performances of the candidate primer sets were evaluated and compared. Patrial of pirA and pirB gene sequence were selected as the target region of primer and probe design. The designed 3 pairs of forward primers and 3 pairs of reverse primers were arranged and combined to conduct ERA detection. In the basic ERA experiment, the three pairs of primers PirAB-F1/R3, PirAB-F2/R3 and PirAB-F3/R2 were abandoned when the template was at 10^5 copies/μL because of the appearance of hybrid bands and the lightness of the bands (Fig. 1A). When the template was at 10^4 copies/μL, the primers of PirAB-F2/R1 and PirAB-F2/R1 were abandoned because of the lighter bands (Fig. 1B). The remaining four pairs of primers were selected for RT-ERA and ERA-LFD experiments. In RT-ERA experiments, the combination of PirAB-F1 and PirAB-R1 was optimal based on the least Ct and the highest fluorescence value (Fig. 1C and D). In ERA-LFD experiments, the test band was the brightest at combination of PirAB-F1 and PirAB-R1 (Fig. 1E). Therefore, PirAB-F1 and PirAB-R1were selected as optimal primers for ERA assay.

3.2. Optimization of the AHPND-ERA assay

In the experiment to optimize reaction temperature of RT-ERA, the RT-ERA reaction could be performed in a wide temperature range of 37–43 °C, and the amplification curve had the smallest Ct and higher fluorescence value at 42 °C. Therefore, 42 °C was chosen as the optimal reaction temperature for RT-ERA (Fig. 2). In the experiment to optimize the reaction temperature of ERA-LFD, the ERA-LFD reactions could be performed in a wide temperature range of 20–41 °C, and the test band was the brightest at 39 °C (Fig. 3A). In addition, in the experiment to optimize the reaction time of ERA-LFD, the weak band could be seen in the test area when the reaction time was 10 min, and the test band was the brightest at 30 and 35 min (Fig. 3B). Thus, 39 °C was selected as the optimal reaction temperature and 30 min was selected as the optimal reaction time of ERA-LFD.

3.3. Specificity analysis

The specificity of ERA was studied using a variety of pathogens. No detectable amplification products from DNA templates from WSSV, EHP, and IHHNV-affected shrimp and healthy shrimp were observed using *Vp*AHPND targeted RT-ERA and ERA-LFD analysis (Fig. 4A and B).

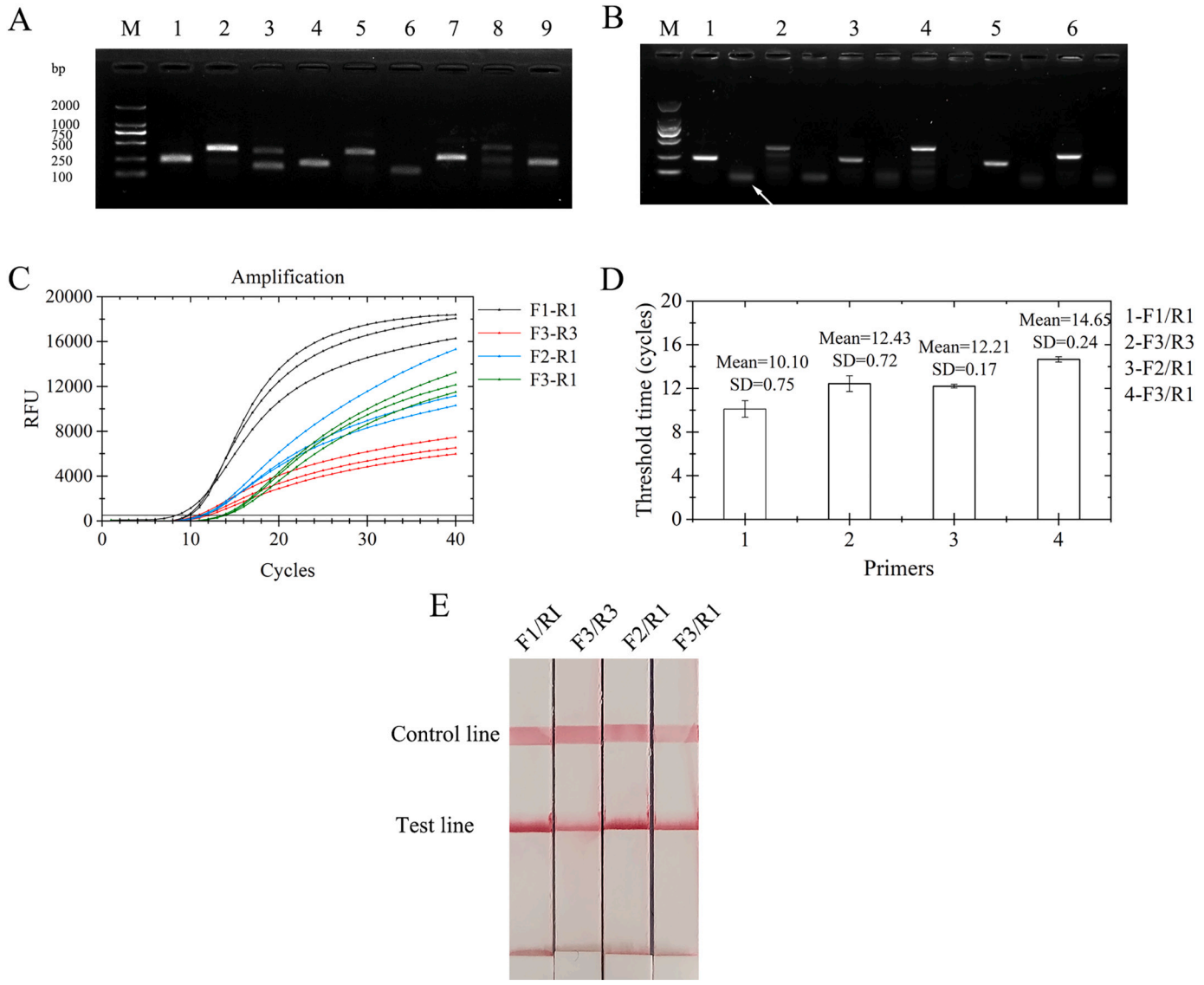

Fig. 1. Screening primers of enzymatic recombinase amplification (ERA) for acute hepatopancreatic necrosis disease (AHPND) detection. (A) Agarose gel electrophoresis of basic ERA products generated by nine sets of primers based on pirA and pirB sequence at 10^5 copies/μL template concentration, M: DNA 2000 Marker, 1–9:F1R1, F1R2, F1R3, F2R1, F2R2, F2R3, F3R1, F3R2, F3R3. (B) Agarose gel electrophoresis of the basic ERA product generated by the 6 sets of primers screened at 10^4 copies/μL template concentrations, 1–6: F1R1, F1R2, F2R1, F2R2, F3R1, F3R3. AHPND standard plasmid and negative samples water were used as templates in ERA, respectively, the arrowheads are primer dimers. (C) Amplification curves of fluorescent ERA products generated by the 4 sets of primers selected at 10^5 copies/μL template concentration. Experiments are tested in three replicates run independently. (D) Threshold times of the amplifications using different primer sets. SD: standard deviation. (E) The amplification products of ERA-LFD in 4 sets of primers selected were detected through lateral flow dipsticks (LFD) at 10^5 copies/μL template.

3.4. Sensitivity analysis

RT-ERA, ERA-LFD, nested PCR, and RT-PCR were performed using recombinant plasmids diluted by 10^8–10^0 copies/μL, and the results showed the minimum detectable concentration of nested PCR was 10^2 copies/μL (Fig. 5A and B), and that of RT-PCR was also 10^2 copies/μL (Fig. 5C and D). And the minimum detectable concentration of RT-ERA and ERA-LFD using the pirAB-F1/pirAB-R1 primers was 10^1 copies/μL (Fig. 5E, F and G). RT-ERA, ERA-LFD, nested PCR, and RT-PCR were performed using DNA extracted from the hepatopancreas of diseased shrimp diluted by 10 ng/μL-10 fg/μL, and the results showed that the lowest detectable concentration was 1 pg/μL for nested PCR (Fig. 6A and B), 1 ng/μL for RT-PCR (Fig. 6C and D), and 1 pg/μL for RT-ERA (Fig. 6E and F) and ERA-LFD (Fig. 6G). When the template is a standard plasmid, the sensitivity of RT-ERA and ERA-LFD are 10 times more sensitive than that of nested PCR and RT-PCR, while using DNA extracted from the hepatopancreas of diseased shrimp as template, the sensitivity of RT-ERA and EPA-LFD is the same as that of nested PCR and 1000 times more sensitive than that of RT-PCR.

3.5. Detection of samples collected from shrimp farms

AHPND detection by RT-ERA and ERA-LDF were compared with nested-PCR and RT-PCR using 24 shrimp samples, including 9 AHPND infected shrimps and 15 SPF shrimps. Among the 24 samples, 9 were tested positive in the nested-PCR (Fig. 7A, B, C and D), 5 were positive by RT-PCR (Fig. 7E), and 9 were tested positive in the RT-ERA (Fig. 7F) and ERA-LFD assays (Fig. 7G and H).

4. Discussion

Acute hepatopancreatic necrosis disease (AHPND) is an OIE-listed

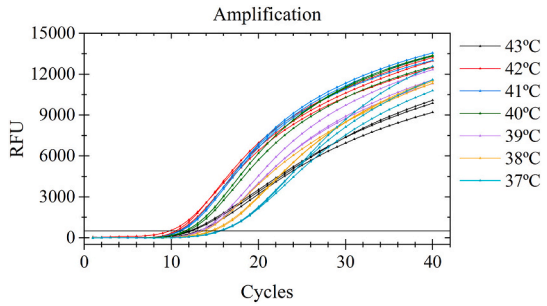

Fig. 2. Optimizing the reaction conditions for RT-ERA assays. Amplification curves of fluorescent ERA at different temperatures. The primersused for optimizing reaction conditions were the optimal AHPND primers and tested in three replicates run independently. The template is a standard plasmid with 10^5 copies/μL.

intestinal disease that has plagued the shrimp farming industry. Since it was first identified in China in 2009, severe cases of AHPND have been reported in Asia and Central and South America, resulting in significant economic losses to the global shrimp farming industry (Ahmed et al., 2021). The ERA assays reported in the current research is a fast and robust surrogate test for detecting AHPND.

The nucleotide sequences of pirA and pirB are highly conserved, while previous detection techniques are often used only pirA as target gene for detection (Han et al., 2015; Sirikharin et al., 2015). In this study, 9 ERA primer combinations were designed for the detection of AHPND, targeting the consensus fragments of pirA and pirB as target genes. Primer pairs pirAB-F1 and pirAB-R1 turned to be the most efficient for amplification, and was suitable for both RT-ERA and ERA-LFD systems, while the positions of the two probes are also the same, with the same sensitivity and specificity. The RT-ERA reaction can be carried out in a wide temperature range of 37–43 °C, and the amplification curve has the smallest Ct and a higher fluorescence value at 42 °C. Therefore, 42 °C was chosen as the optimal reaction temperature for RT-ERA. According to the brightness of the test band, 39 °C and 30 min were chosen as the optimal reaction temperature and time for ERA-ERA. The specificity of both RT-ERA and ERA-LFD assays are determined by testing for different pathogens. Only the standard plasmid for AHPND and DNA extracted from the hepatopancreas of diseased shrimp with AHPND produced positive results from the amplification curve and test band.

The performance of AHPND-ERA is highly comparable to published nested PCR (Dangtip et al., 2015) and RT-PCR (Han et al., 2015), with detection limits of 10^1 copies/μL (standard plasmid sample) or 1 fg/μL (DNA sample extracted from diseased shrimp), respectively. Using standard plasmid as template, the sensitivity of RT-ERA and ERA-LFD are 10 times more sensitive than that of nested PCR and RT-PCR. It is important to note that the presence of background shrimp DNA in the sample may affect the detection limits. To more comprehensively

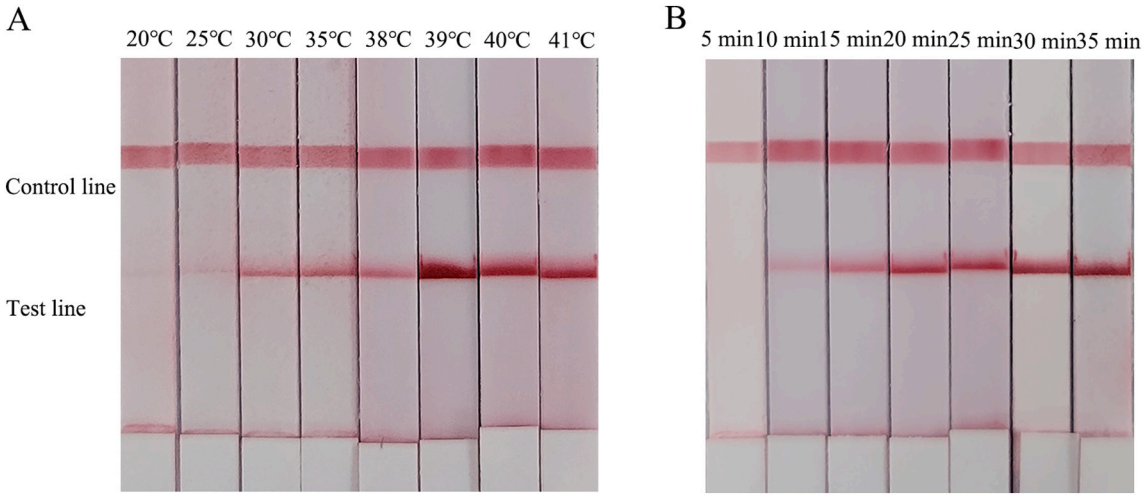

Fig. 3. Optimizing the reaction conditions for ERA-LFD assays. The amplification products of ERA-LFD in different temperatures (A) and time (B) were detected through lateral flow dipsticks (LFD). The primersused for optimizing reaction conditions were the optimal AHPND primers. The template is a standard plasmid with 10^5 copies/μL.

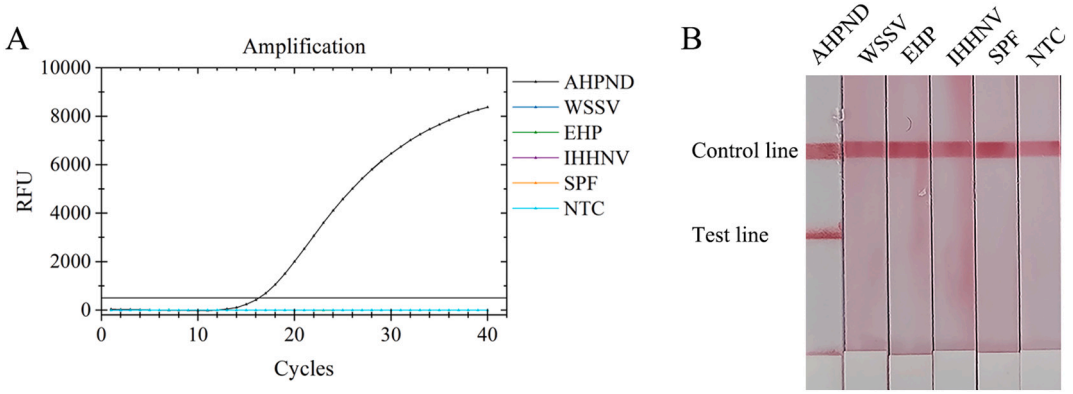

Fig. 4. Specificity of the RT-ERA (A) and ERA-LFD (B) assay using related templates. The positive templates were DNA extracted from the hepatopancreas of diseased shrimp. Control DNA templates were WSSV, EHP, IHHNV, healthy shrimp and negative control.

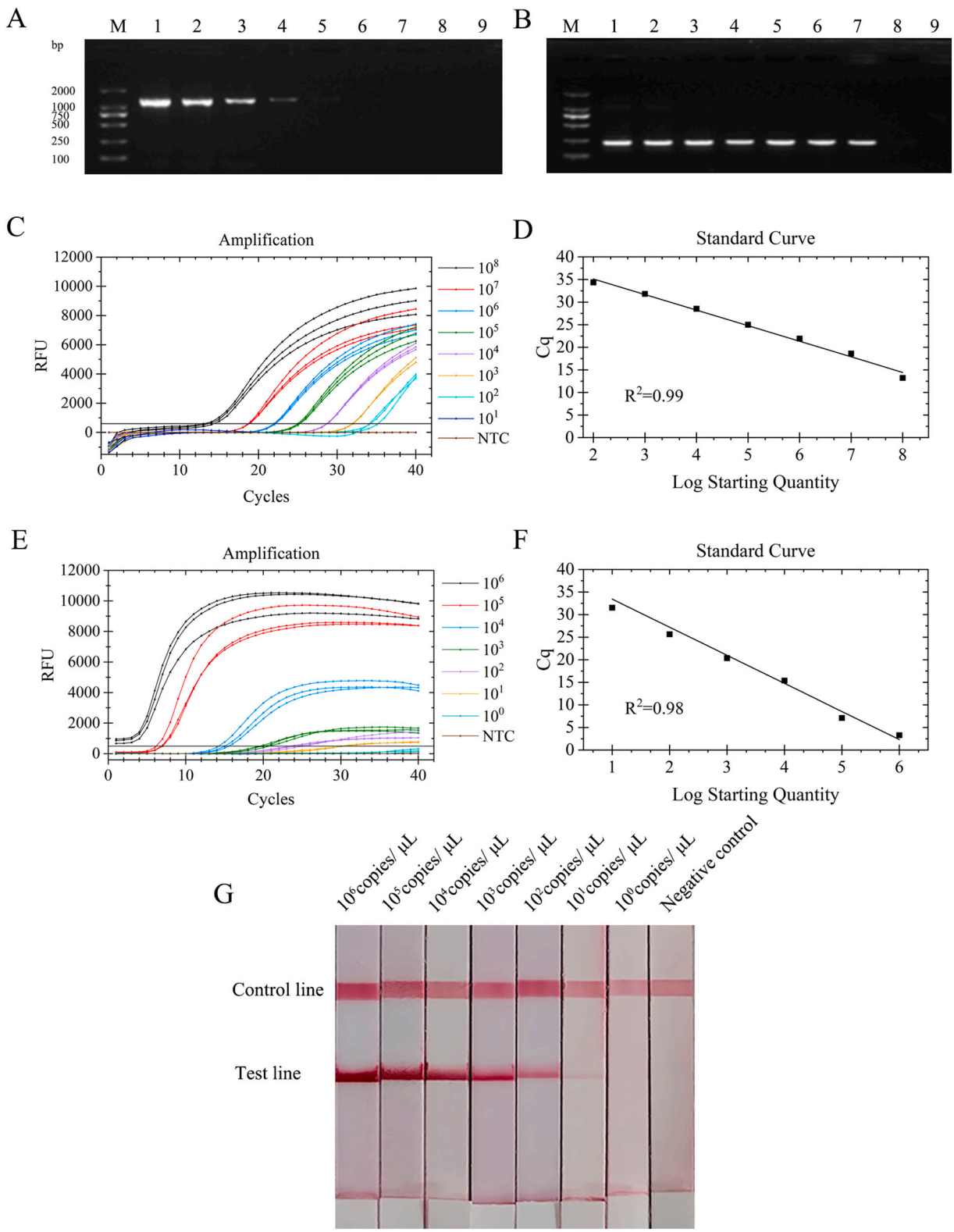

Fig. 5. Comparison of the sensitivity of nested PCR, RT-PCR, RT-ERA and ERA-LFD assays. Using 10^8–10^0 copies/μL pEASY-Blunt Zero plasmid standards as samples, the minimumdetectable concentration were determined by generic nested PCR (A, B), RT-PCR (C), RT-ERA(E) and ERA-LFD (G). (A, B): lanes 1–8, Bands from 10-fold serial dilutions (10^8–10^1 copies/μL) of AHPND plasmids standard; lane 9, negative control. (C): Amplification curve of RT-PCR in 10-fold serial dilutions (10^8–10^2 copies/μL) of AHPND plasmids standard. (D): The results of the threshold time analysis of RT- PCR for AHPND. A standard regression line was generated based on the mean of the 3 data sets, Cq: threshold cycle, Log Starting Quantity: logarithm of AHPND plasmid copy number; R^2: coefficient of determination. (E): Amplification curve of RT-ERA in 10-fold serial dilutions (10^6–10^0 copies/μL) of AHPND plasmids standard. Experiments are tested in three replicates run independently. (F): The results of the threshold time analysis of RT- ERA for AHPND. A standard regression line was generated based on the mean of the 3 data sets, Cq: threshold cycle, Log Starting Quantity: logarithm of AHPND plasmid copy number; R^2: coefficient of determination. (G): The amplification products of ERA-LFD in 10-fold serial dilutions (10^6–10^0 copies/μL) of AHPND plasmids standard were detected through lateral flow dipsticks (LFD).

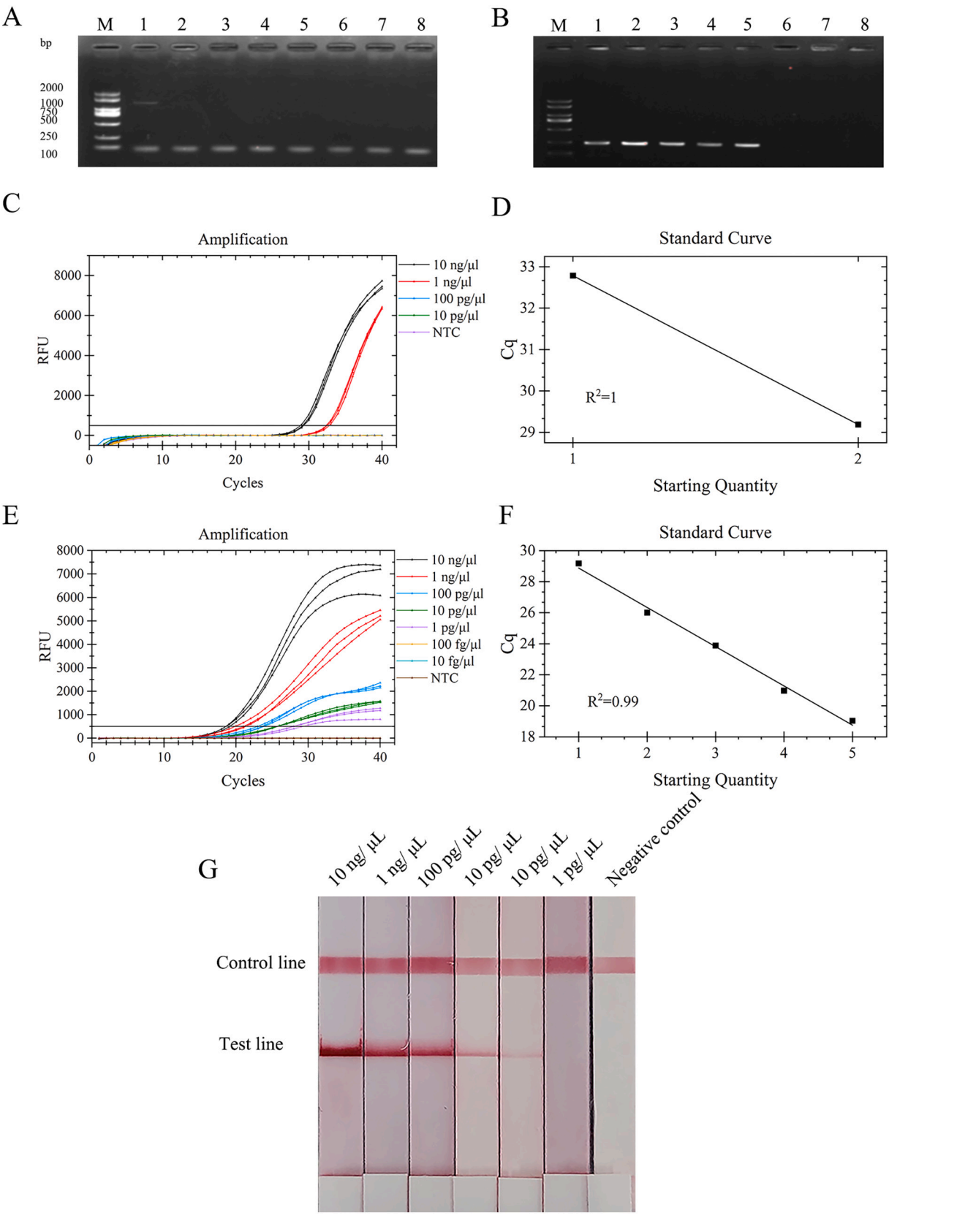

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Fig. 6. Comparison of the sensitivity of nested PCR, RT-PCR RT-ERA and ERA-LFD assays. Using10 ng/μL-10 fg/μL DNA extracted from the hepatopancreas of diseased shrimp as samples, the minimum detectable concentration were determined by generic nested PCR (A, B), RT-PCR (C), RT-ERA(E) and ERA-LFD (G). (A, B): lanes 1–7, Bands from 10-fold serial dilutions (10 ng/μL-10 fg/μL) of DNA extracted from hepatopancreas of diseased shrimp; lane 8, negative control. (C): Amplification curve of RT-PCR in 10-fold serial dilutions (10 ng/μL-10 pg/μL) of DNA extracted from hepatopancreas of diseased shrimp. (D): The results of the threshold time analysis of RT- PCR for AHPND. A standard regression line was generated based on the mean of the 3 data sets, Cq: threshold cycle, Log Starting Quantity: logarithm of AHPND plasmid copy number; R^2: coefficient of determination. (E): Amplification curve of RT-ERA in from 10-fold serial dilutions (10 ng/μL-10 fg/μL) of DNA extracted from hepatopancreas of diseased shrimp. Experiments are tested in three replicates run independently. (F): The results of the threshold time analysis of RT- ERA for AHPND. A standard regression line was generated based on the mean of the 3 data sets, Cq: threshold cycle, Log Starting Quantity: logarithm of AHPND plasmid copy number; R^2: coefficient of determination. (G): The amplification products of ERA-LFD in 10-fold serial dilutions (10 ng/μL-10 fg/μL) of DNA extracted from hepatopancreas of diseased shrimp were detected through lateral flow dipsticks (LFD).

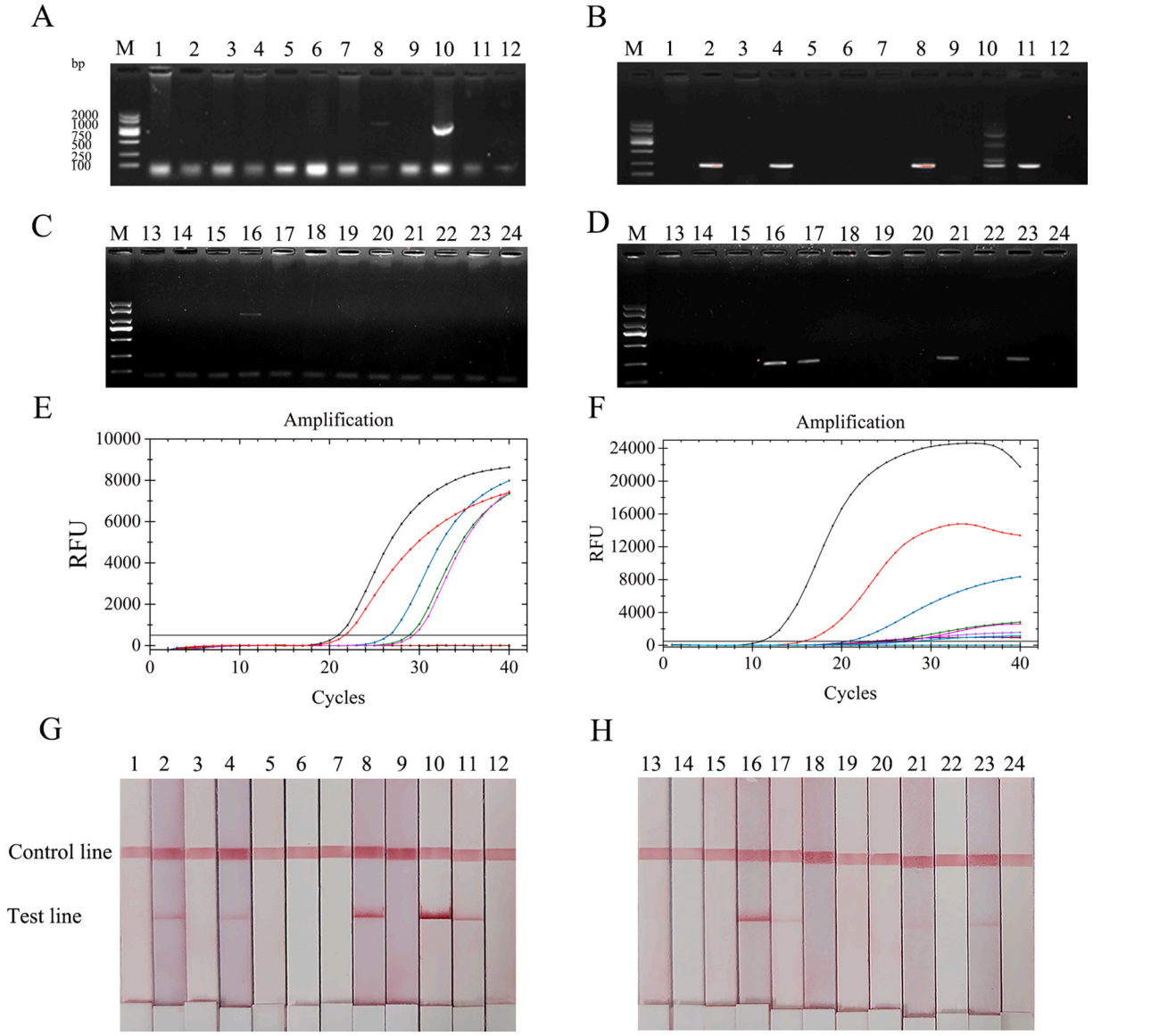

Fig. 7. Evaluation of the applicability of RT-ERA and ERA-LFD using the pirAB primers. 24 samples from aquaculture farms were detected by generic nested PCR (A, B, C, D), RT-PCR (E), RT-ERA (F) and ERA-LFD (G, H).

compare detection limits, we evaluated the performance of nested PCR, RT-PCR, RT-ERA, and ERA-LFD in the presence of 10 ng/μL-10 fg/μL shrimp hepatopancreas background DNA. While using DNA extracted from the hepatopancreas of diseased shrimp as template, the sensitivity of RT-ERA and ERA-LFD are the same as that of nested PCR and 1000 times more sensitive than that of RT-PCR. Furthermore, the nested PCR takes 150 min to complete the assay, the RT-PCR takes 60 min, while RT-ERA and ERA-LFD could get results within 20 min or 30 min. For high copy number plasmids, ERA can obtain results in as little as 10 min, giving it a great advantage over traditional PCR methods. A recent publication on the detection of AHPND has described an RPA assay that can be incubated at 39 °C for 30 min with a sensitivity of 5 copies/μL (Mai et al., 2021). However, this method analyzes the amplified signal by gel electrophoresis, which makes the total detection time up to 1 h and limits its application in the practical detection in shrimp farms. The ERA experiment involved in this study uses fluorescent signal reading and LFD to display amplified DNA rapidly. In the two methods, fluorescent probes with FAM fluorophores and test strip probes with FAM

fluorophores were added respectively, which makes the detection method has higher selectivity for mismatched DNA target sequences. The overall specificity is improved while further accelerating the time of ERA assays. Moreover, RT-ERA can utilize a portable tube scanner for real-time detection, ERA-LFD only requires a simple heating apparatus, and both methods eliminate the use of expensive quantitative machines associated with gel electrophoresis equipment. It makes DNA testing possible in a non-lab, point-of-care setting. LAMP based mothed was also developed for the detection of AHPND. The LAMP technique was used in combination with Loopamp real-time turbidimeter, agarose gel electrophoresis, and ss-DNA-labeled AuNP probes to visualize AHPND (Arunrut et al., 2016; Koiwai et al., 2016; Kongrueng et al., 2015a, 2015b). The assay lines were 53 CFU/mL, 1 pg of total genomic DNA and 100 CFU/mL. These three methods are qualitative or semi-quantitative detection methods, and cannot be accurately quantified. Moreover, LAMP technology needs to design three pairs of primers for four target sequences, and ERA only needs one pair of primers. LAMP reaction conditions are more severe, requiring a high temperature of 60 °C and a reaction time of 1 h, while ERA only needs 30 min at room temperature to complete the detection. Therefore, these findings ultimately benefit the use of ERA instead of LAMP for mobile isothermal detection of AHPND.

In the practical detection of 24 shrimp samples, the positive detection rates of RT-ERA and ERA-LFD were consistent with the nested PCR method and slightly better than RT-PCR. Although this sample size is small, it bodes well for the future development of this tool in the diagnosis of AHPND. The difference in the detection of pathogens between ERA and RT-PCR methods may be due to the possible presence of some PCR amplification inhibitors in the samples. ERA is highly resistant to primary samples and RT-PCR is more sensitive to inhibitors, so ERA is more favorable for the detection of crude clinical specimens. Similar phenomena have been reported in other studies (Gao et al., 2018; Prescott et al., 2016; Shahin et al., 2018). Comparison of the field assay results of RT-ERA and ERA-LFD with RT-PCR indicated that ERA is a sensitive assay for the detection of latent AHPND infection, the assays are capable of identifying AHPND in even lightly infected samples. ERA detection can greatly shorten the detection time and lower the reaction temperature, while maintaining a high detection sensitivity.

This study describes the development and evaluation of a visual ERA for the detection of AHPND infection in shrimp using fluorescent signal readouts and lateral flow dipstick (LFD). The main difference between the two methods is the presentation. RT-ERA can monitor results in real time using a portable fluorometer while ERA-LFD is easier to use because it does not require additional laboratory equipment and is similar to other strip-based testing methods such as pregnancy testing and pH value (Lobato and O'Sullivan, 2018). Since there are currently no effective treatments to prevent or reduce the spread of AHPND infection in the shrimp farming industry, early and rapid AHPND detection is a valuable strategy for monitoring AHPND outbreaks in shrimp farming facilities. We successfully developed two rapid ERA assays for the detection of AHPND with high sensitivity and specificity. What's more, compared to other methods, ERA assay has the advantages of high field adaptability, simple sample preparation requirements, lower inspection lines, faster inspection time, lower operating temperature and no need for complicated equipment. Therefore, the efficient ERA assay developed in this study should be very useful in the control of AHPND, especially in resource-limited settings.

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.

Data availability

Data will be made available on request.

Acknowledgments

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