Received: 5 February 2018 | Revised: 5 March 2018 | Accepted: 5 March 2018

DOI: 10.1111/jfd.12808

ORIGINAL ARTICLE

WILEY Journal of Fish Diseases

Rapid visual detection of cyprinid herpesvirus 2 by recombinase polymerase amplification combined with a lateral flow dipstick

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Funding information
General Financial Grant from the China Postdoctoral Science Foundation, Grant/Award Number: 2017M611534; Earmarked Fund for China Agriculture Research System, Grant/Award Number: CARS-45-19; Doctoral Fund from Shanghai Ocean University, Grant/Award Number: A2-0203-17-100303

Abstract

Herpesviral haematopoietic necrosis (HVHN), caused by cyprinid herpesvirus 2 (CyHV-2), causes significant losses in crucian carp (*Carassius carassius*) aquaculture. Rapid and convenient DNA assay detection of CyHV-2 is useful for field diagnosis. Recombinase polymerase amplification (RPA) is a novel isothermal DNA amplification and detection technology that can amplify DNA within 30 min at ~37°C by simulating in vivo DNA recombination. Herein, a rapid and convenient detection assay based on RPA with a lateral flow dipstick (LFD) was developed for detecting CyHV-2. The highly conserved *ORF72* of CyHV-2 was targeted by specific and sensitive primers and probes. The optimized assay takes only 15 min at 38°C using a water bath, with analysis of products by 2% agarose gel electrophoresis within 30 min. A simple lateral flow strip based on the unique probe in reaction buffer was developed for visualization. The entire RPA-LFD assay takes 50 min less than the routine PCR method, is 100 times more sensitive and displays no cross-reaction with other aquatic viruses. The combined isothermal RPA and lateral flow assay (RPA-LFD) provides a simple, rapid, reliable method that could improve field diagnosis of CyHV-2 when resources are limited.

KEYWORDS
crucian carp, CyHV-2, herpesviral haematopoietic necrosis, isothermal detection, lateral flow dipstick, recombinase polymerase amplification

1 | INTRODUCTION

Crucian carp, *Carassius auratus* (L.), a member of the family Cyprinidae, has been cultivated in freshwater aquaculture systems in Asia and Europe for many years and is particularly popular in China, where annual production is over 2 million tons (FAO) (Pang et al., 2017). Unfortunately, in 2011 and 2012, a new viral disease emerged and spread to most crucian carp aquaculture sites in China, resulting in huge economic losses (Xu et al., 2013). Cyprinid herpesvirus 2 (CyHV-2) was confirmed as the pathogen responsible (Wang et al., 2012) and represents the first CyHV-2 disease in this species in China. In 1995, herpesviral haematopoietic necrosis virus (HVHNV) and goldfish haematopoietic necrosis virus (GFHNV) were isolated from ornamental goldfish in Japan and found to be the same virus (Jung & Miyazaki, 1995; Wu et al., 2013). This virus has since spread quickly to other areas including the USA (Goodwin, Khoo et al., 2006), Taiwan (Rangel, Rockemann, Hetrick, & Samal, 1999), Australia (Stephens, Raidal, & Jones, 2004) and China. Although many research groups have tried to develop vaccines to protect against CyHV-2, no effective prevention or treatment methods for this disease have been reported.

CyHV-2, also known as GFHNV and HVHNV in goldfish, is a member of the genus *Cyprinivirus* in the family *Herpesviridae* that includes carp pox virus (CyHV-1) and koi herpesvirus (CyHV-3) (Goodwin,

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Sadler, Merry, & Marecaux, 2009). The CyHV-2 virion is hexagonal in shape, measuring 110–120 nm in diameter with a 170–200 nm envelope (Xu et al., 2013). This virus infects gill, kidney and spleen tissue, and can multiply in both the nucleus and cytoplasm of leucocytes and hematopoietic cells (Wu et al., 2013). At present, only two CyHV-2 isolates have been completely sequenced by different research groups, namely SY-C1 (Li et al., 2015) and ST-J1 (Davison et al., 2013). The complete genome of strain CyHV-2-SY-C1 from China consists of 290,304 bp and encodes ~150 genes, and the full-length genome of SY-C1 shares 98.8% homology with that of ST-J1 isolated from Japan.

PCR (Goodwin, Merry, & Sadler, 2006), real-time PCR (Goodwin et al., 2009), LAMP (Park et al., 2018), and immunohistochemical and blood smear methods (Kong et al., 2017) have been reported for diagnosis of CyHV-2, but a more convenient method is still required. Herein, we developed a rapid and efficient assay for the detection of CyHV-2 using recombinase polymerase amplification (RPA), a type of isothermal amplification, to allow DNA amplification and detection using simple equipment (Jaroenram & Owens, 2014). The products of RPA reaction with a specific probe can be visualized simply by a lateral flow dipstick (LFD) assay (Milenia Biotec, Giessen, Germany). The RPA amplicons are complexed with invisible gold-labelled anti-FAM antibodies coated on the sample pad, then travel in a buffer stream to be trapped at the test line by biotin-ligands, resulting in an appearance of red-pink colour indicative of a positive result. Non-captured gold particles move through the test line to be fixed at the control line by anti-rabbit antibodies, and then produce colour serving as a flow control for the strip (Jaroenram & Owens, 2014). When combined with a lateral flow disc (LFD), the developed RPA-LFD assay could facilitate detection and visualization at 38°C within 50 min, representing a significant improvement on the conventional PCR assay.

2 | MATERIALS AND METHODS

2.1 | Fish and pathogens

Crucian carp was obtained from the Shanghai Ocean University fish breeding farm. According to the previous method (Xu, Podok, Xie, & Lu, 2014), the CyHV-2 virus (Wang, Xu, & Lu, 2016) was isolated from diseased crucian carp kidney, spleen and liver using sucrose gradient centrifugation and stored at −80°C.

2.2 | Total DNA extraction

Diseased crucian carp tissues were processed into cell suspensions and total DNA from the serum samples of CyHV-2 was extracted using a TIANamp Genomic DNA Kit (Tiangen, China) according to the manufacturer's instructions. Samples were stored at −20°C until needed.

2.3 | RPA primer and probe design

According to the principle for RPA primer design (Jaroenram & Owens, 2014) (http://www.twistdx.co.uk/images/uploads/docs/Appendix.pdf), RPA primer length should be 30–35 bp, and the amplification efficiency is high when the amplification product is within 500 bp. Thus, a series of gradient candidate primers (Table 1) were designed based in the conserved region, and the best primers were selected for RPA gel detection. DNA probes for LFD assays were designed based on the sequence between RPA primers and consist of an upstream stretch (30 nt) carrying a 5′-FAM antigenic marker linked by a THF spacer to an adjacent downstream oligonucleotide (15 nt) carrying a C3-spacer (polymerase extension blocking group) at its 3′ end, cactctggcgacgcgtttgtggttgaaccgcca(BHQ1-dt)c(THF)g(FAM-dT)ggaggcttcaaaggc(C3-spacer). Primers and probes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

2.4 | RPA conditions and optimization

RPA reaction mixtures containing forward primer (10 μM, 2.4 μl), reverse primer (10 μM, 2.4 μl), 1 × rehydration buffer (29.5 μl), DNase-free water (11.2 μl), DNA template (2 μl) and 47.5 μl of rehydration solution were transferred into a reaction pellet with lyophilized enzyme preparation (TwistAmp Basic kit, TwistDX, Cambridge, UK). Next, 2.5 μl of 280 mM magnesium acetate was added to the reaction tube to initiate the reaction. After mixing well, the tube was immediately incubated under the indicated conditions. To define the optimal amplification temperature, RPA was performed at 32, 32.5, 33.4, 35.2, 37.1, 38.3, 39.3 and 40°C for 40 min. To determine the optimal amplification time, reactions were performed at the most suitable temperature for 5, 10, 20, 30, 40 and 50 min. Products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) then analysed by 2% agarose gel electrophoresis (AGE).

2.5 | Lateral flow dipstick (LFD) assay

The RPA-LFD reaction system was as described above, except 10 μM upstream and downstream primer (2.1 μl) and 10 μM fluorescent probe (0.6 μl) was added. Reactions were carried out at the optimum temperature for the optimum duration. To detect RPA

TABLE 1 Primers and probes used in this study

Name	Sequence(5′–3′)
RPA01F	CAATCAGGGTCAGTGGACGAGACTGGCGTTGT
	5′Biotin-CAATCAGGGTCAGTGGACGAGACTGG CGTTGT (LFD)
RPA01R	CCTCCCAGAGCCATGTTACCCGGTCTGAAGGA
RPA02F	CCCAGGAGACCAGCAGACTGTTGAACCCGTAC
RPA02R	GTATCCGCCTCGTCCATCATAGAGCCGAAACC
RPA03F	AGACCAGCAGACTGTTGAACCCGTAC
RPA03R	GTATCCGCCTCGTCCATCATAGAGCC
RPA-LFA Probe	(FAM)cactctggcgacgcgtttgtggttgaaccgccaTc(THF)
	gTggaggcttcaaaggc(C3spacer)
Routine PCR-F	CTTTAGCGTCAGGTCCATAGAGG
Routine PCR-R	CGTCAGTCCCTGGCAGAAATAAG

amplicons by LFD, 10 μl of RPA product was added to 100 μl of HybriDetect Assay Buffer, and the LFD strip from the TwistAmp Basic kit (TwistDX, Cambridge, UK) was placed into the solution and incubated for 5 min, then observed immediately. The entire LFD assay was carried out at 38°C.

2.6 | Specificity of AGE and RPA-LFD

The specificity of RPA primers and probes was tested using virus CyHV-2 positive DNA, infectious hypodermal and hematopoietic necrosis virus (IHHNV) positive DNA, white spot syndrome baculovirus (WSSV) positive DNA, grass carp reovirus genotype I (GCRV-JX01) cDNA, grass carp reovirus genotype II (GCRV-JX02) cDNA, grass carp reovirus genotype III (GCRV-104) cDNA, spring viraemia carp virus (SVC), koi herpesvirus (KHV) and negative controls (water) under optimal conditions. Resulting products were analysed by AGE and RPA-LFD, and the results of the two methods were compared.

2.7 | Sensitivity of AGE and RPA-LFD

Serial dilutions of total DNA extracted from crucian carp infected with CyHV-2 (1 ng, 100 pg, 10 pg, 5 pg, 1 pg, 500 fg, 100 fg, or 10 fg) were used as templates for RPA assays. The original DNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Products from AGE and LFD were compared. A standard PCR protocol for the detection of infectious CyHV-2 (Wang, Xu & Lu 2016) was also performed using specific primer pair CyHV-2F (CCTCCGTATCTTTGGGGACT) and CyHV-2R (ATGATGCCCTCAAAGGTGTC); Table 1. PCR amplification conditions were as follows: initial denaturation at 98°C for 1 min, followed by 35 cycles of 10 s at 98°C, 10 s at 55°C and 60 s at 72°C, and a final extension at 72°C for 10 min.

2.8 | Applicability of the RPA assay

Crucian carps with or without CyHV-2 were used to test the performance of the RPA and routine PCR assays. DNA extraction was performed using the TIANamp Virus DNA/RNA Kit (Tiangen, Beijing, China). The assays were carried out as described previously.

3 | RESULTS

3.1 | Screening of CyHV-2 RPA primers and reaction conditions

In the primer screening test, all three primer pairs (Table 1) successfully amplified the CyHV-2 target, but primer pair 1 was yielded the clearest product on the gel (Figure 1a). Hence, primer pair 1 was chosen for subsequent evaluation (Table 1). To obtain the optimal amplification temperature for the RPA assay, a temperature range of 32–40°C was assessed in the 40 min reactions. As shown in Figure 1b, the test line was weak at 32°C, and there were no significant differences in amplification between 32.5 and 40°C. Thus, 38°C was selected as the standard CyHV-2 RPA-LFD assay temperature. The RPA assay could be performed in less than 10 min (Figure 1c), since the LFD signal was detected after only 10 min of the amplification reaction at 38°C followed by a 5 min of incubation (Figure 2).

3.2 | Specificity of AGE and RPA-LFD

The specificity of the RPA assay was investigated using DNA samples extracted from pathogens infecting various aquatic animals. As shown in Figure 3, the RPA assay did not detect genomic DNA from IHHNV positive DNA, WSSV positive DNA, GCRV-JX01 cDNA, GCRV-JX02 cDNA, GCRV-104 cDNA, SVC, KHV or negative control (water) samples; only CyHV-2 genomic DNA yielded a positive result by AGE (Figure 3a) and lateral flow strip (Figure 3b). These results indicate that the primers and probes were specific for CyHV-2 detection.

3.3 | Sensitivity of AGE and RPA-LFD

The RPA-LFD assay was performed using a dilution series of purified DNA from CyHV-2 samples containing 1 ng to 10 fg of DNA per reaction, and the limit of detection was 5 pg (Figure 4c). This was comparable to the detection limit for RPA reactions without the lateral flow probe following subsequent visualization by AGE (Figure 4b). As shown in Figure 4, the RPA-LFD assay was 100 times

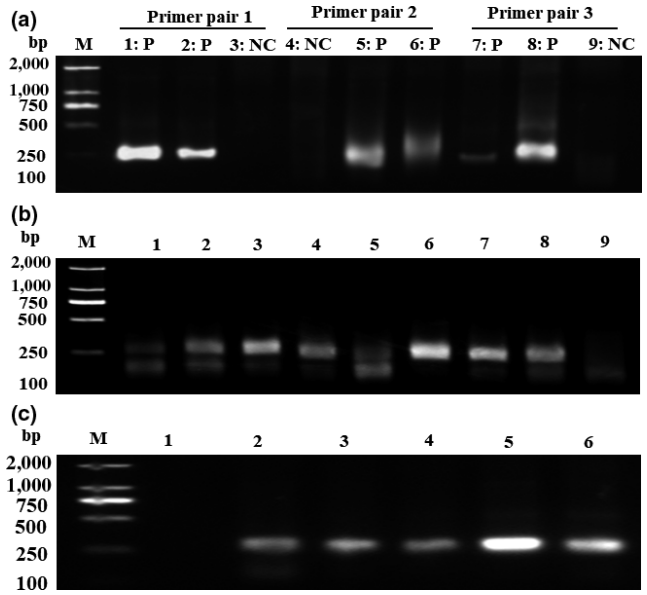

FIGURE 1 Screening of cyprinid herpesvirus 2 (CyHV-2) recombinase polymerase amplification (RPA) primer and reaction conditions. a, RPA reaction products using different pair primers detected by agarose gel electrophoresis (AGE). b, Amplification performance of RPA-LFD assays conducted at 32, 32.5, 33.4, 35.2, 37.1, 38.3, 39.3 and 40°C. c, Determination of optimal reaction duration (5, 10, 20, 30, 40 and 50 min)

more sensitive than the routine PCR method (Figure 4a), and unlike the specific band generated using the routine PCR-based amplification method, the RPA assay products could be directly identified by AGE (Figure 4b). Furthermore, using a lateral flow dipstick without a specific instrument was even more sensitive than AGE-based detection (Figure 4c).

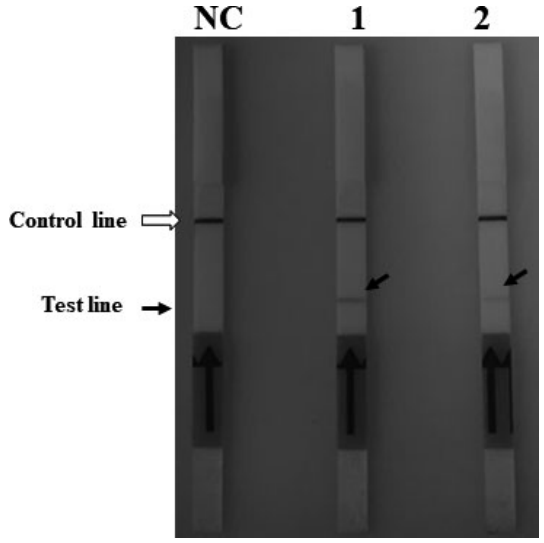

FIGURE 2 Combining CyHV-2 detection by RPA with an instrumentation-free read-out system. NC, negative control (water); 1 and 2, positive CyHV-2 infected tissues. Positive signals are indicated in the two reactions on the right (filled arrowheads). The control reaction only produces a flow-control signal

3.4 | Applicability of the RPA assay

The suitability of the assay to diagnose CyHV-2 was evaluated by comparing detection results of RPA assay on 20 crucian carp samples against routine PCR results on the same samples. Figure 5a, b shows the performance of the routine PCR amplification and RPA on detecting 20 crucian carp samples, respectively. RPA detected CyHV-2 DNA from eight infected samples, which also tested positive by the routine PCR. This result suggests that the developed RPA assay can feasibly be used as a diagnostic test of CyHV-2 infection.

4 | DISCUSSION

CyHV-2 has become a major pathogen in cultivated crucian carps. The disease affected all size of crucian carps, and the mortality was as high as 90%–100%. Rapid and convenient diagnosis of CyHV-2 is necessary to prevent further spread of this disease. Several isothermal nucleic acid amplification assays have been developed in recent years. Recombinase polymerase amplification (RPA) assay is a novel approach for rapid and specific DNA amplification. The RPA assay combined with a LFD uses two target-specific oligonucleotide primers, which are able to bind to the template DNA with the assistance of a recombinase in combination with strand-displacement DNA synthesis. The TwistAmp nfo reaction mechanism generates double-labelled reporter simultaneously with amplification, and only requires minimal post-amplification processing (Daher, Stewart,

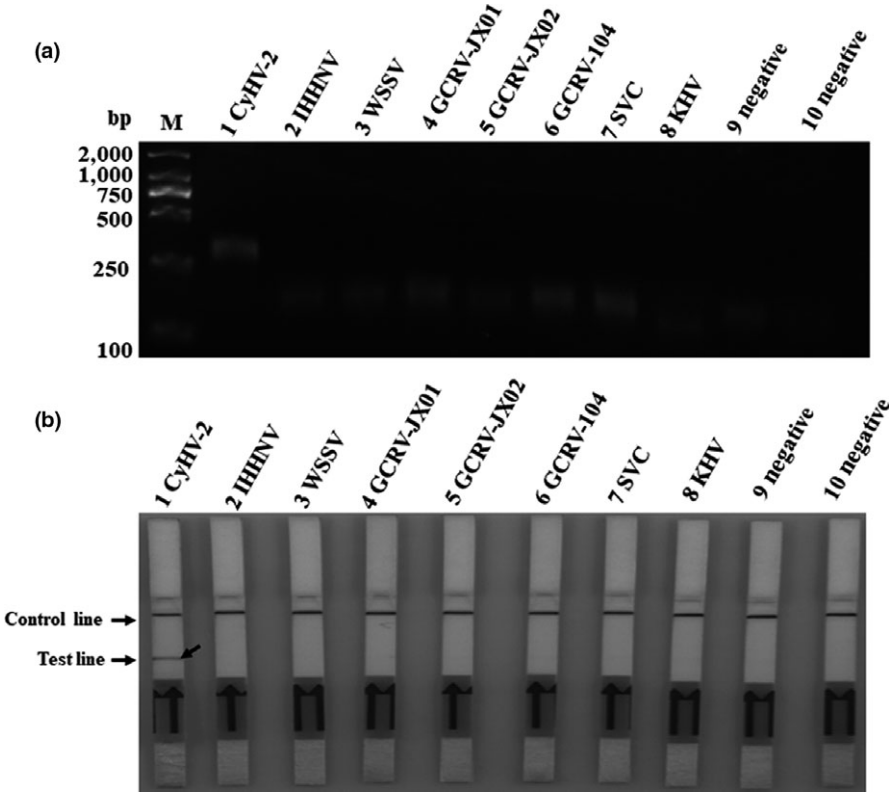

FIGURE 3 Specificity of the RPA assay. a, Product detection by AGE. b, Product detection using lateral flow dipsticks. The assay was tested against positive CyHV-2 infected tissues, infectious hypodermal and hematopoietic necrosis virus (IHHNV) positive DNA, white spot syndrome baculovirus (WSSV) positive DNA, grass carp reovirus genotype I (GCRV-JX01) cDNA, grass carp reovirus genotype II (GCRV-JX02) cDNA, grass carp reovirus genotype III (GCRV-104) cDNA, spring viraemia carp virus (SVC), koi herpesvirus (KHV) and negative controls (water)

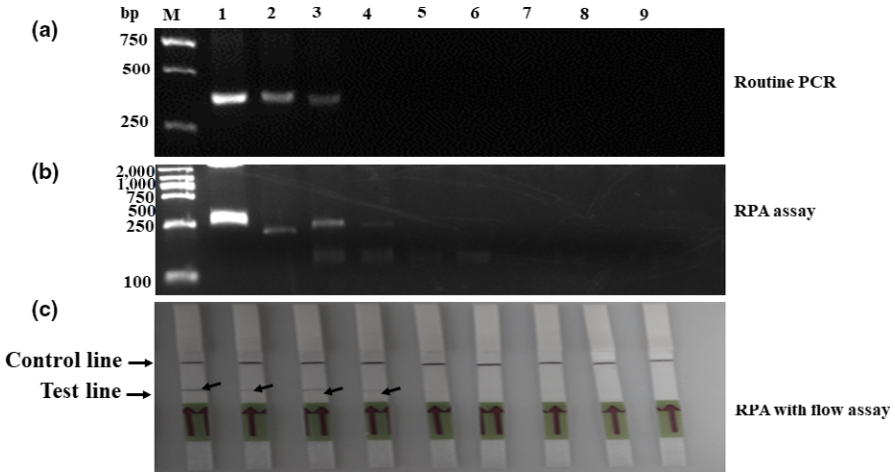

FIGURE 4 Comparison of the sensitivity of RPA-LFD and routine PCR methods. a, Routine PCR assay. b, RPA-AGE. c, RPA-LFD. A dilution series of purified DNA from CyHV-2 infected tissues containing between 1 ng and 10 fg DNA per reaction was screened. NO.1:1 ng, 2: 100 pg, 3: 10 pg, 4: 5 pg, 6: 1 pg, 7: 500 fg, 8: 100 fg, 9: 10 fg, 11: NTC

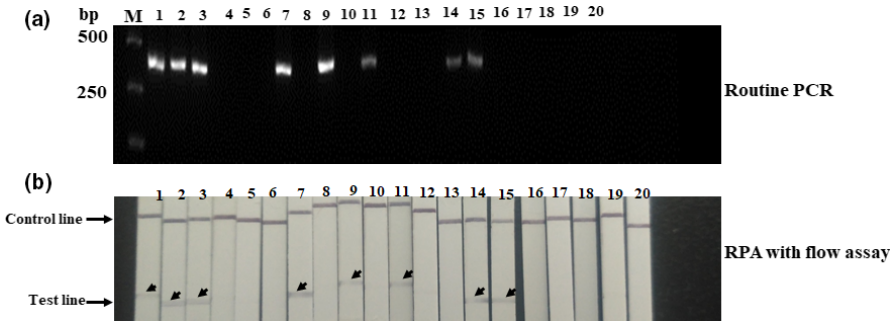

FIGURE 5 Applicability of the RPA assay with or without CyHV-2 infected. a, the routine PCR. b, the RPA assay with LFD. NO. 1, 2, 3, 7, 9, 11, 14 and 15 were the samples with CyHV-2 infected. NO. 4, 5, 6, 8, 10, 12, 13, 16, 17, 18, 19 and 20 were the samples without CyHV-2 infected

Boissinot, Boudreau, & Bergeron, 2015). At room temperature, an amplification of complex DNA targets can be achieved in less than 30 min. Unlike routine PCR and real-time PCR assays, the RPA-LFD assay does not require thermal or chemical melting, thereby dispensing with the need for an expensive thermocycler or other additional equipment or reagents. This convenient method for detecting aquatic pathogens without the need for expensive instruments could be useful for aquatic biosecurity.

In this study, a typical isothermal RPA method was combined with lateral flow detection. Optimization of the RPA reaction revealed no significant difference in amplification at 35.2, 37.1, 38.3, 39.3 or 40°C (Figure 1b). Thus, 38°C was selected as the standard assay temperature. For RPA reactions performed at 38°C between 5 and 50 min, a reaction time longer than 5 min was required for successful virus detection, hence 15 min was selected (Figure 1c). The specificity of the RPA assay was determined by testing against pathogens of various aquatic organisms. The red-purple line was only observed at the control line using LFD strips (Figure 3b), and this was confirmed by AGE (Figure 3a). These results suggest the CyHV-2 RPA-LFD assay is specific for the detection of CyHV-2. The sensitivity of the RPA assay was found to be comparable to the routine PCR protocol, and able to detect template at concentrations as low as 10 pg (Figure 4).

In summary, our combined RPA and LFD assay takes only 30 min, which four times faster than the RPA-AGE method, and five times faster than the routine PCR-AGE detection of CyHV-2. The RPA-LFD method is also 100 times more sensitive than the routine PCR-AGE detection method. The high sensitivity and specificity of this rapid and efficient method are key advantages over existing methods, as is amplicon confirmation by hybridization.

ACKNOWLEDGEMENTS

This work was supported by the General Financial Grant from the China Postdoctoral Science Foundation [grant number 2017M611534], the Doctoral Fund from Shanghai Ocean University [grant number A2-0203-17-100303], and the Earmarked Fund for China Agriculture Research System [grant number CARS-45-19].

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How to cite this article: Wang H, Sun M, Xu D, et al. Rapid visual detection of cyprinid herpesvirus 2 by recombinase polymerase amplification combined with a lateral flow dipstick. *J Fish Dis*. 2018;00:1–6. https://doi.org/10.1111/jfd.12808