Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 251 (2018) 92–98

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Real-time isothermal detection of Abalone herpes-like virus and red-spotted grouper nervous necrosis virus using recombinase polymerase amplification

Fang Gao[a,b], Jing-Zhe Jiang[a,*], Jiang-Yong Wang[a,*], Hong-Ying Wei[a,b]

[a] Key Laboratory of Aquatic Product Processing, Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China

[b] Shanghai Ocean University, Shanghai, 201306, China

ARTICLE INFO

Keywords:
AbHV
RGNNV
qRPA
Aquaculture
Pathogen detection

ABSTRACT

Abalone herpes-like virus (AbHV) and Red-spotted grouper nervous necrosis virus (RGNNV) are two serious viruses that infect animal populations in aquaculture. Both viruses cause diseases associated with high mortality rates, resulting in dramatic economic losses in the aquaculture industry. There are currently no effective treatments for either of these two viral diseases. Thus, early, rapid, and accurate diagnosis plays a fundamental role in disease prevention and control in aquaculture. Traditional methods of diagnosis, such as virus culture, enzyme-linked immunoassay, and polymerase chain reaction (PCR), are either time consuming or require sophisticated temperature control devices. In this study, one sets of specific primers and probes were designed for the real-time quantitative recombinase polymerase amplification (qRPA) detection of AbHV and RGNNV separately. The sensitivity and specificity of detection were evaluated by comparison with detection by conventional PCR and quantitative PCR. The optimal reaction temperature and time for virus detection is 37 °C for 20 min. The detection limit is 100 copies per reaction, making this approach faster and more sensitive than qPCR in this study. In a field application, the detection percentage of qRPA was higher than that of qPCR for both AbHV and NNV. Additionally, good correlation was found between qRPA and qPCR detection ($R^2 > 0.8$). The methods presented here can be used as alternatives to qPCR for quick and quantitative detection of pathogens infecting aquaculture species.

1. Introduction

Abalone herpes-like virus (AbHV) is a major pathogen that affects the nervous system of abalones (Corbeil et al., 2012b). The pathogen is highly virulent for *Haliotis diversicolor*, but does not have any apparent effects on other abalone species (Corbeil et al., 2012a). Abalones can be infected at all life-stages and suffer a high mortality rate. Once symptoms occur, the entire pool of abalone can die within three days. Between 1999 and 2003, the yields of cultured abalone in China were severely affected by an epidemic of continuous outbreaks of this fatal disease. In addition to AbHV, the pathogen viral nervous necrosis virus (VNNV) can cause severe disease in fish populations. The disease caused by VNNV is known as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) (Doan et al., 2016; Grotmol et al., 2000; Toubanaki et al., 2015a). It is a major cause of mortality in larvae and juveniles of farmed marine fish, occurring frequently in adult fish in recent years (Chi et al., 2005; Su et al., 2015; Toubanaki et al., 2015a,b). With a high mortality rate (80–100%), the disease can rapidly wipe out a local fish population in a week. Data from fish populations in South Korea, Japan, and China reveal that RGNNV is causing a major epidemic in East and West Asia, with strains specific to each region (Gomez et al., 2008, 2004; Ma et al., 2015). Over the past decade, RGNNV infection has caused severe losses to seedling production in the large-scale culture of groupers in the coastal regions of China.

Polymerase chain reaction (PCR) is the most common diagnostic method for detection of AbHV infection. The TaqMan® PCR assay developed by Corbeil et al. can detect 30 copies of recombinant plasmid DNA per reaction (Corbeil et al., 2010). There are several methods for the detection of NNV, such as enzyme-linked immunoassays (ELLSA) (Arimoto et al., 1992; Huang et al., 2001; Nunez-Ortiz et al., 2016), immuno-fluorescence antibody test (IFAT) (Nguyen et al., 1996), in situ hybridization (Comps et al., 1996), reverse transcription PCR (RT-PCR) (Gomez et al., 2008; Grotmol et al., 2000; Nishizawa et al., 1995), reverse transcription quantitative PCR (RT-qPCR) (Dalla Valle et al., 2005; Hodneland et al., 2011; Kuo et al., 2011; Mazelet et al., 2011), reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Mekata et al., 2014; Sung and Lu, 2009; Wang et al., 2011), and

* Corresponding authors.
E-mail addresses: tianchengyinuo@163.com (F. Gao), jingzhejiang@gmail.com (J.-Z. Jiang), wjy104@163.com (J.-Y. Wang), hongyingwei0104@163.com (H.-Y. Wei).

http://dx.doi.org/10.1016/j.jviromet.2017.09.024
Received 16 May 2017; Received in revised form 19 September 2017; Accepted 19 September 2017
Available online 28 September 2017

molecular beacon (Su et al., 2015). Although these methods have their own advantages, drawbacks such as complex and time-consuming procedures, lower sensitivity, and the requirement of well-trained personnel and precise equipment, severely hinder their application in the field and popularization among farmers.

Recombinase polymerase amplification (RPA), developed by Piepenburg et al., can rapidly amplify nucleic acid at a constant temperature (Piepenburg et al., 2006). The entire reaction is performed between 37 and 39 °C for 20–40 min without the requirement of a heating and cooling system. Therefore, it may be more suitable for field detection than PCR. RPA has been widely used in the life sciences, such as for food testing and clinical diagnosis, as well as in other fields (Babu et al., 2017; Boyle et al., 2014; Hill-Cawthorne et al., 2014; Mekuria et al., 2014; Miles et al., 2015; Murinda et al., 2014; Xia et al., 2014; Yang et al., 2016a,b). Kersting et al. developed RPA chips that can simultaneously amplify and detect three pathogens, including *Neisseriagonorrhoeae*, *Salmonella enterica* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Kersting et al., 2014a). Using the chip, the assay can complete detection of these three pathogens, including control plasmids, within 20 min. Abd El Wahed et al. developed a portable RT-RPA device that can identify Middle East respiratory syndrome coronavirus (MERS-CoV), a recently emerged zoonotic virus, within 3–7 min with a detection limit of 10 RNA molecules, making it as sensitive as real-time RT-PCR (Abd El Wahed et al., 2013). Kersting et al. combined the RPA TwistAmp® nfo probe and primers with a lateral flow dipstick to achieve high sensitivity and rapid detection of the 18S rRNA gene for the parasite *Plasmodium falciparum* (Kersting et al., 2014b).

In the present study, a real-time quantitative RPA method for rapid detection of AbHV and RGNNV was developed. The sensitivity and specificity of detection were evaluated by comparison with detection by conventional PCR. To the best of our knowledge, this is the first study to use RPA technology to detect both viruses, and it lays the foundation for further development of field detection methods.

2. Materials and methods

2.1. Sample collection

H. diversicolor samples infected with AbHV were collected during 1999 and 2005 from the coast of South China Sea and stored at −70 °C until use. In the 50 selected samples, 10 were collected from 2003 in Shenzhen, 10 were collected in 2005 from Shenzhen, 10 were collected in 2009 from Guangdong Luzhou island, 10 were collected from Guangdong Xuwen, and 10 were collected in 2009 from Guangdong Shanwei. *Epinephelus akaara* samples infected with RGNNV were purchased from the Hainan Wanning area, where mass mortality occurred due to viral infection. Samples of sick fish show significant symptoms of NNV infection, such as black body, spiraling, whirling, or being belly-up at rest. In order to facilitate the preservation and retention of the sample, the brain tissues of the fish were collected. All animal work have been conducted according to relevant national and international guidelines. South China Sea Fisheries Research Institute Academic Committee approved this research.

2.2. Viral nucleic acid preparation

DNA and RNA were extracted from the muscle tissue of *H. diversicolor* and the brain tissue of *E. akaara*, respectively. DNA extraction was completed using a TIANamp Marine Animals DNA kit (Tiangen Biotech, Beijing, China). Grouper Tissue used the HiPure Universal RNA kit (Magen, Suzhou, China). cDNA synthesis was performed using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). Specific protocols followed the instructions designated in each kit. The extracted nucleic acid concentrations were determined by using a NanoDrop ND-2000 ultramicro spectro-photometer. After division into portions of equal concentration, the samples were stored at −20 °C.

2.3. Design of primers and probe for RPA

The primers and probes (Table 1) were designed using Primer 5.0 software based on the gene sequence of AbHV ORF38 (GenBank accession number JX453331.1) and RGNNV RNA2 (GenBank accession number AY744705.01). Corbeil et al. (2010) had reported that the primer set ORF 38, also known as ORF 49 in OsHV, yielded the best results with regard to specificity and sensitivity and was, therefore, selected for further development of the diagnostic TaqMan assay. RNA2 of NNV encodes a capsid protein, which is a relatively conserved gene (Kuo et al., 2011).

2.4. Specificity of RPA detection

To evaluate the specificity of the AbHV-RPA and RGNNV-RPA systems, we included additional templates as controls: *Penaeus vannamei* genomic DNA infected with white spot syndrome virus (WSSV) (provided by Dr. Guo Zhixun),*Aeromonas hydrophila*genomic DNA (provided by Dr. Su Youlu), *Trachinotus ovatus* genomic DNA (provided by Mr. Xu Liwen), *Babylonia areolata* genomic DNA, *Crassostrea gigas*genomic DNA, and microbes genomic DNA from 500 mL water of abalone ponds which filtered onto a 0.22 m filtrate. Unless designated, DNA for the above samples were extracted in our laboratory. RPA specificity was tested using a TwistAmp® Basic kit (TwistDx, UK). Each reaction

Table 1
The primer and probe used in this study.

Target Gene	Primer name	Sequence (5′-3′)	Product size (bp)	Sequence ID
AbHV ORF 38	RF	CTTTCTTACCGCTTTCAATCTGATCCGTGG	169	JX453331.1
	RR	GAACAGGGGTAATTGTATAGCAACTGCGTA		
	Probe	GCGTACAGTAAAACGAAAACCATGGCACA(dT-FAM)GC(THF)CA(dT-BHQ1)TGAAAACATCCAA GC(C3spacer)		
	F	AACCCACACCCAATTTTTGA	126	
	R	CCCAAGGCAAGTTTGTTGTT		
	Probe	6FAMCCGCTTTCAATCTGATCCGTGG-TAMRA		
RGNNV RNA2	RF	CATGACACAAGGTCCCCTGTACAACGATTC	158	AY744705.01
	RR	AACATCTCCAGTTCCAAGGCTGTAGTCAAT		
	Probe	CAAGGCTGTAGTCAATGGACAGCGGACGG(dT-FAM)CCA(THF)GC(dT-BHQ1)GGAAGACTGCTC C(C3spacer)		
	F	GACGCGCTTCAAGCAACTC	203	
	R	CGAACACTCCAGCGACACAGCA		

RF and RR stand for RPA forward and reverse primers used in qRPA assay.
F and R stand for PCR forward and reverse primers used in qPCR assay.

contained 14.8 μL rehydration buffer, 1.2 μL RF (10 μM), 1.2 μL RR (10 μM), 4.6 μL ddH_2O, 2 μL DNA (50–500 ng), and 1.2 μL MgAC (280 mM) for a total volume of 25 μL. Reactions were incubated at 37 °C for 35 min. The final amplification products were visualized using agarose gel electrophoresis Label not found for Float ElementLabel not found for Float Element.

2.5. Construction of recombinant plasmid of qRPA

Recombinant plasmids were synthesized by Invitrogen Trading (Shanghai) Co., Ltd. (Shanghai, China). Each plasmid contains a virus-specific gene fragment (see below) inside the pMD 18-T vector.

Gene fragments for each virus are as follows:

AbHV:5′-TGAACAGGGGTAATTGTATAGCAACTGCGTACAGTAAAACG AAAACCATGGCACATGCCATTGAAAACATCCAAGCAAACACCCAAGGC AAGTTTGTTGTTCCTTTATGCAAACAGATTCTCAAAACGACTAAAGAA ACCACGGATCAGATTGAAAGCGGTAAGAAAGA -3′.

RGNNV: 5′ -AACATCTCCAGTTCCAAGGCTGTAGTCAATGGACAGC GGACGGTCCAGCTGGAAGACTGCTCCATCAGGGGCAATATCCAGTGGT GTGGATCCTAGGAGGATGGACTTGAAGTCATTTGTGGAAAGGGAATC GTTGTACAGGGGACCTTGTGTCATGA -3′.

2.6. Sensitivity of qRPA detection

Serial 10-fold dilutions of AbHV and RGNNV plasmid standards (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10) were prepared in TE buffer. DNA was amplified using TwistAmp® Exo kit (TwistDx, UK) with the Thermal Cycler Real Time System (Eppendorf, Germany). Each reaction contained 14.7 μL rehydration buffer 1.1 μL RF (10 μM), 1.1 μL RR (10 μM), 0.3 μL Probe (10 μM), 4.6 μL ddH_2O, 2 μL DNA (50–500 ng), and 1.2 μL MgAC (280 mM) for a final volume of 25 μL. Reactions were incubated at 37 °C for 30 min. Three replicates were performed for each dilution.

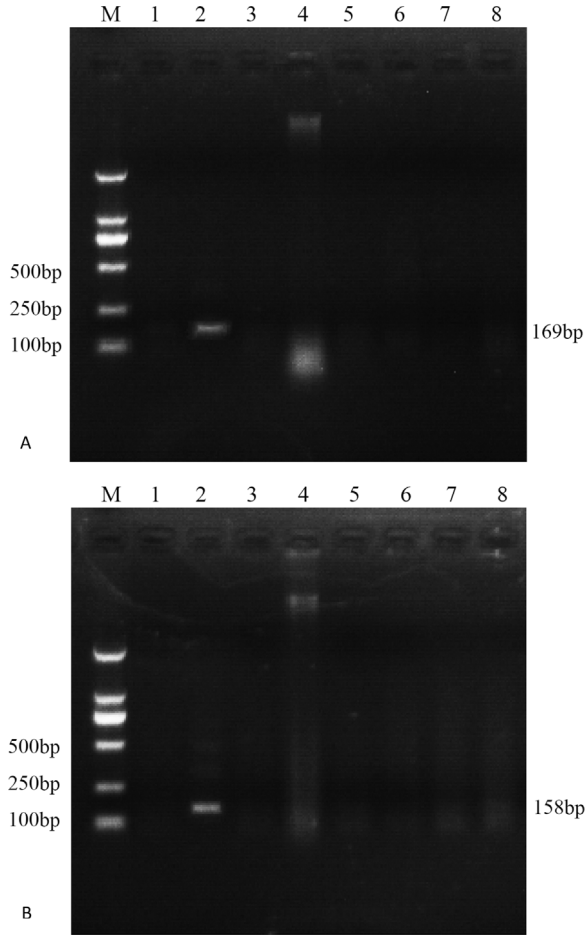

Fig. 1. Specificity assays of RPA detection. (A) Specificity results of AbHV. (B) Specificity results of RGNNV. M, marker DL2000 (Takara); lane 1, negative control; lane 2, positive control; lanes 3–8, genomic DNA of *Trachinotus ovatus*, *Crassostrea gigas*, *Penaeus vannamei* infected with WSSV, *Babylonia areolata*, total microbes in 500 mL water of abalone ponds which filtered onto a 0.22 m filtrate, and *Aeromonas hydrophila*.

2.7. Construction of the recombinant plasmid of qPCR

The primers of AbHV and RGNNV for qPCR were designed as described by Corbeil et al. (Corbeil et al., 2010) and Kuo et al. (Kuo et al., 2011). The specific sequences are shown in Table 1. The total reaction volume for AbHV was 50 μL, including 2 μL each of primer (10 μM), 25 μL PCR DSMix (Dongsheng, Guangdong, China), 20 μL ddH_2O, and 50–500 ng of DNA from *H. diversicolor* samples infected with AbHV. Reaction volumes were the same for RGNNV except that *E. akaara* genomic DNA infected with RGNNV was used as the template. PCR conditions were as follows: 94 °C for 5 min, followed by 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, with 30 cycles, and extension at 72 °C for 10 min. The amplified products were visualized using 1.5% agarose gel electrophoresis. Target bands were gel extracted and purified using a Universal DNA Purification kit (TIANGEN, Beijing, China), and subsequently ligated into the pMD® 18-T Vector (TaKaRa, Beijing, China). Plasmids were transformed into TOP 10 competent cells (TIANGEN, Beijing, China) following the instructions indicated in the kit. Finally, plasmids were prepared by plasmidTIANprep Mini Plasmid Kit (Tiangen Biotech, Beijing, China), before concentrations were

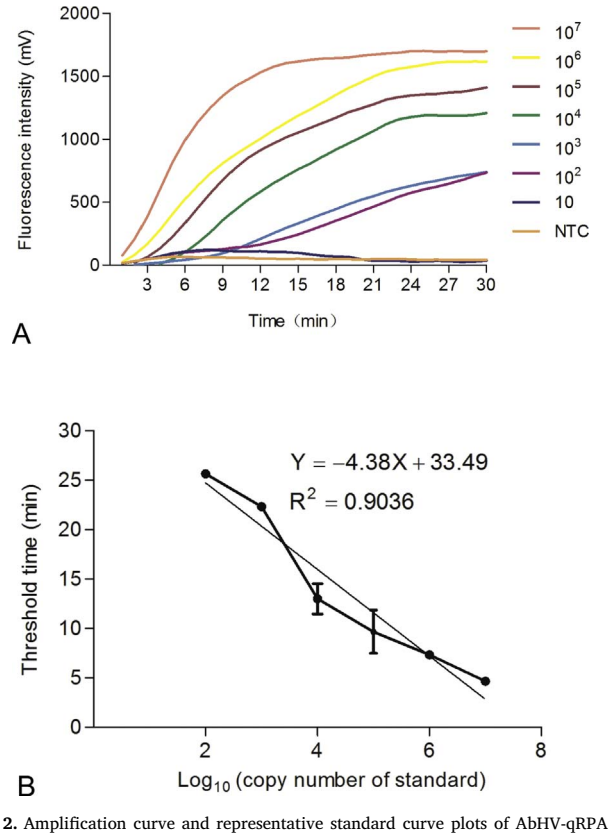

Fig. 2. Amplification curve and representative standard curve plots of AbHV-qRPA assays. (A) Fluorescence amplification curve with 10–10^7 copies of AbHV plasmid standards. Each of the amplification curves represents the mean of three replicates. (B) Standard curve from 10-fold serial dilutions (10^7–10^2 copies) of AbHV plasmids standard in three replicates measurements. CT: threshold cycle, Log C: logarithm of AbHV copy numbers; R^2: coefficient of determination.

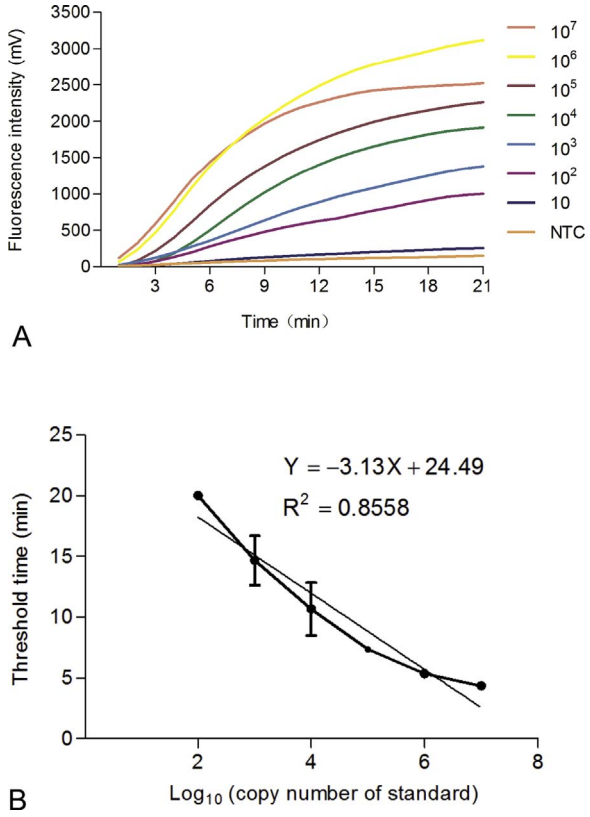

Fig. 3. Amplification curve and representative standard curve plots of RGNNV-qRPA assays. (A) Fluorescence amplification curves with 10–10^7 copies of RGNNV plasmid standards. Each of the amplification curves represents the mean of three replicates. (B) Standard curve from 10-fold serial dilutions (10^7–10^2 copies) of RGNNV plasmids standard in three replicates measurements. CT: threshold cycle, Log C: logarithm values of RGNNV copy numbers; R^2: coefficient of determination.

determined using a Qubit® 3.0 Fluorometer in order to calculate plasmid copy number. Plasmid stocks were stored at −20 °C.

2.8. Sensitivity of qPCR detection

Plasmids were serially diluted, as described above in the sensitivity of qRPA detection methods section. The AbHV-qPCR assay was performed following Corbeil et al. using the Premix Ex Taq™ kit (TAKARA, Dalian, China) (Corbeil et al., 2010). Each reaction included 12.5 μL Premix Ex Taq, 0.5 μL of each primer (10 μM), 1 μL Taq Man® Probe (10 μM), 8.5 μL ddH_2O, and 2 μL DNA template for a final reaction volume of 25 μL. Plasmid standards used ranged from 10 to 10^8. Reaction conditions were 95 °C for 30 s, followed by 95 °C for 5 s, and 60 °C for 30 s, with 30 cycles.

The RGNNV-qPCR assays used a fluorescent dye with the SYBR® Premix Ex Taq™II kit (Tli RNase H Plus) (Kuo et al., 2011). Each reaction contained 12.5 μL SYBR® Premix Ex, 1 μL of each primer (10 μM), 8.5 μL ddH_2O, 2 μL plasmid standards of RGNNV. The reaction conditions were as follows: 95 °C for 30 s, followed by 95 °C for 5 s, and 60 °C for 45 s, with 40 cycles.

2.9. Applicability comparison of qRPA and qPCR assays

To further verify the reliability of the AbHV-qRPA and RGNNV-qRPA detection methods, we selected 50 *H. diversicolor* and 40 *E. akaara* in this study. Each sample was quantitatively detected using both qRPA and qPCR, and the results of the two methods were compared.

3. Results

3.1. Specificity of RPA detection

The specificity of the detection reactions for AbHV and RGNNV was determined by checking the cross-reactivity of the assay with different DNA viruses, bacteria, or genomic DNA. A positive signal was only detected for the AbHV-infected (positive) and RGNNV-infected (positive) samples, indicating that RPA detection methods have a good specificity (Fig. 1).

3.2. Sensitivity of qRPA detection

High concentrations of the two viruses (10^7–10^5 copies/μL) showed a strong amplification signal within 3–5 min (Figs. 2 and 3). The minimum detection limit is 100 copies for both viruses. The AbHV-qRPA reaction was completed in 30 min, and the RGNNV-qRPA reaction in only 20 min. In order to verify the repeatability of the test batches, we performed three independent tests. Each test yielded similar results. There was a strong linear correlation between the logarithmic concentration and the fluorescence threshold time (Ct) at different concentrations (R^2 of AbHV and RGNNV were 0.90 and 0.8558, respectively; Figs. 2 B and 3 B).

3.3. Sensitivity of qPCR detection

In this study, TaqMan® PCR and SYBR-Green real-time PCR were used to detect AbHV and RGNNV, respectively. The detection limits for AbHV and RGNNV were 1000 and 100 copies per reaction, respectively (Fig. 4A and B). Fig. 4C shows a strong linear relationship of TaqMan® PCR detection of AbHV plasmid standards between the different concentration range (10^8–10^3 copies per reaction) and the number of cycles (Ct) on the corresponding concentration range (R^2 = 0.99). The SYBR-Green technique used to detect RGNNV also shows a strong linear relationship between Ct and number of copies at values between 10^8–10^2 copies per reaction (Fig. 4D).

3.4. Comparison of qRPA and qPCR assays

In all, 50 *H. diversicolor* and 40 *E. akaara* were used in this study to test the correlation between the qRPA and qPCR assays. The detection percentage of samples infected with AbHV for both methods were 96% and 93%, respectively. The detection percentage of RGNNV was 97% and 95%, respectively. Additionally, these data show that there is a strong correlation between qRPA and qPCR detection (R^2 > 0.8) (Fig. 5).

4. Discussion

TaqMan® PCR is still the primary diagnostic method for detection of AbHV (Corbeil et al., 2010). This method can detect 30 copies of recombinant plasmids in one reaction. Previous studies have used this method to test 1673 samples from different abalone populations in Victoria and Tasmanias, showing that the sensitivity and specificity are 96.7% and 99.4%, respectively (Corbeil et al., 2010). However, the detection limit in present studies was 1000 copies, causing it to be below the level reported in the literature and also below the AbHV-qRPA assay presented here (100 copies). Furthermore, AbHV-qRPA has an advantage when considering the reaction time and temperature. The entire reaction takes place at 37 °C, without the need for a complicated heating and cooling system, and results can be obtained within 20 min. This can be compared to the TaqMan® PCR method which typically takes 1 h. Therefore, the qRPA detection method presented here can greatly improve the screening efficiency of AbHV and enable timely preventive measures.

RGNNV damages the health culture of grouper populations around

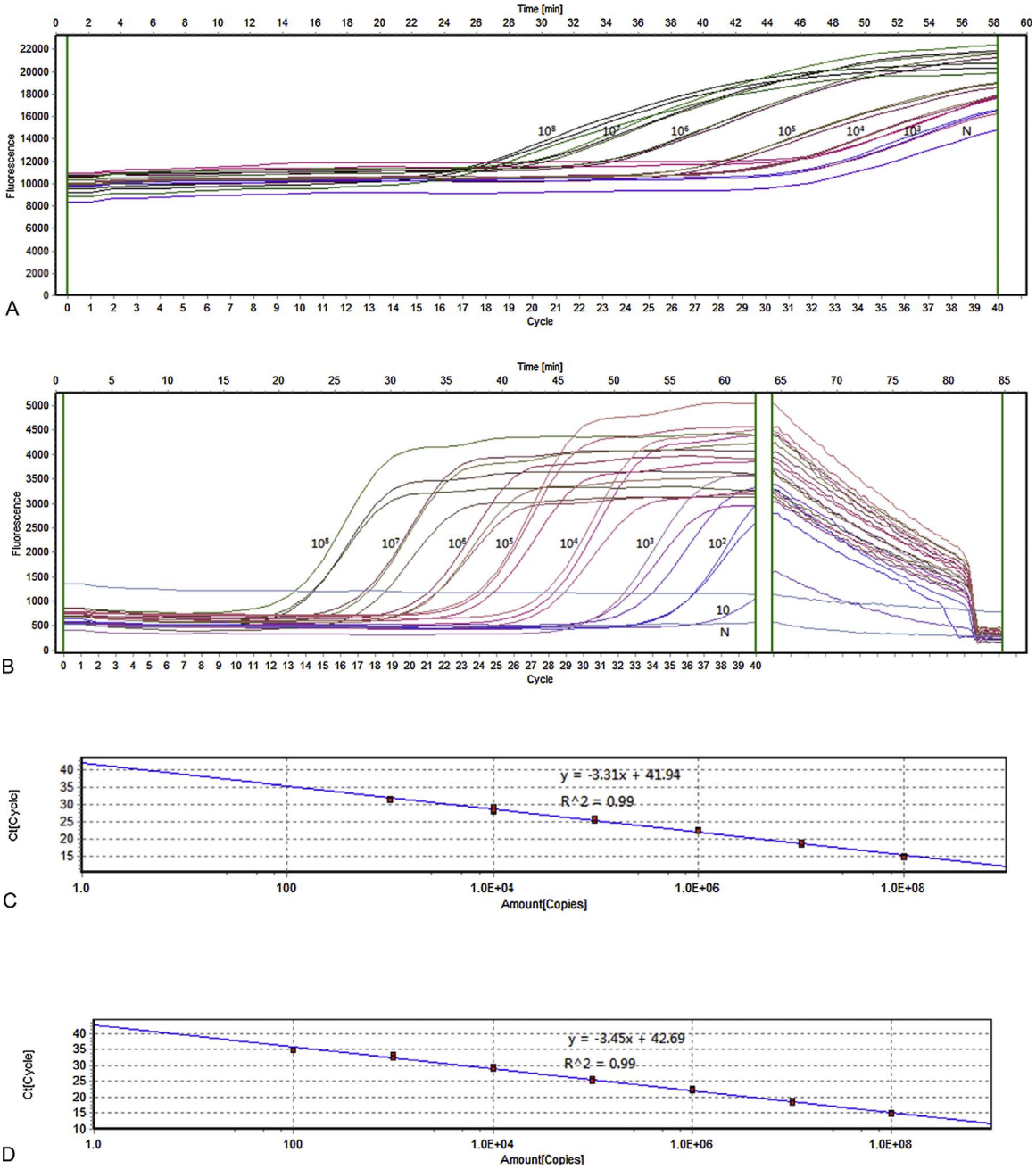

Fig. 4. Amplification curve and representative standard curve plots of AbHV-qPCR and RGNNV-qPCR assays. (A) Fluorescence amplification curve with 10^3–10^8 copies of AbHV plasmid standards (B) Fluorescence amplification curve with 10–10^8 copies of RGNNV plasmid standards; Standard curve from 10-fold serial dilutions from three replicate measurements (C) AbHV plasmid standards (10^8–10^3 copies) (D) RGNNV plasmid standards (10^8–10^2 copies).

longer historically, so there are more reported detection methods. Based on the presence or absence of nucleic acid amplification, they can be divided into two categories. For example, ELISA (Arimoto et al., 1992; Huang et al., 2001; Nunez-Ortiz et al., 2016), in situ hybridization (Comps et al., 1996), and molecular beacon (Su et al., 2015) belong to the detection category without nucleic acid amplification.

The second category is based on nucleic acid amplification and includes RT-PCR (Gomez et al., 2004; Grotmol et al., 2000; Nishizawa et al., 1995), RT-qPCR (Dalla Valle et al., 2005; Hodneland et al., 2011; Kuo et al., 2011; Mazelet et al., 2011), and LAMP (Mekata et al., 2014; Sung and Lu, 2009; Wang et al., 2011), among others. The RT-qPCR assay introduced by Kuo et al. can detect as few as 10 copies of the plasmid (Kuo et al., 2011). The same method was attempted in this study, but we were only able to detect 100 copies of the plasmid as the same detection limit as the qRPA method. The RT-qPCR assay takes 40 min to complete, while qRPA yields results within 20 min. For high copy number plasmids, qRPA can obtain results within 10 min, causing it to have a large advantage over conventional PCR methods. Additionally, the detection rate of qRPA was slightly higher than that of qPCR with shorter time using both methods to analyze the same batch of grouper samples. LAMP is another isothermal amplification detection method. The sensitivity of RT-LAMP compared with single-step RT-PCR and nested RT-PCR was described by Sung and Lu (Sung and Lu, 2009); they found that the detection limit of RT-LAMP was approximately 1000-fold and 10-fold higher than that of one-step PCR and nested RT-PCR, respectively (Sung and Lu, 2009). However, LAMP is a qualitative or semi – quantitative detection method that cannot be accurately quantified. On the contrary, qRPA can perform quantification in the sample, as evidenced by a good correlation between quantification methods ($R^2 > 0.85$).

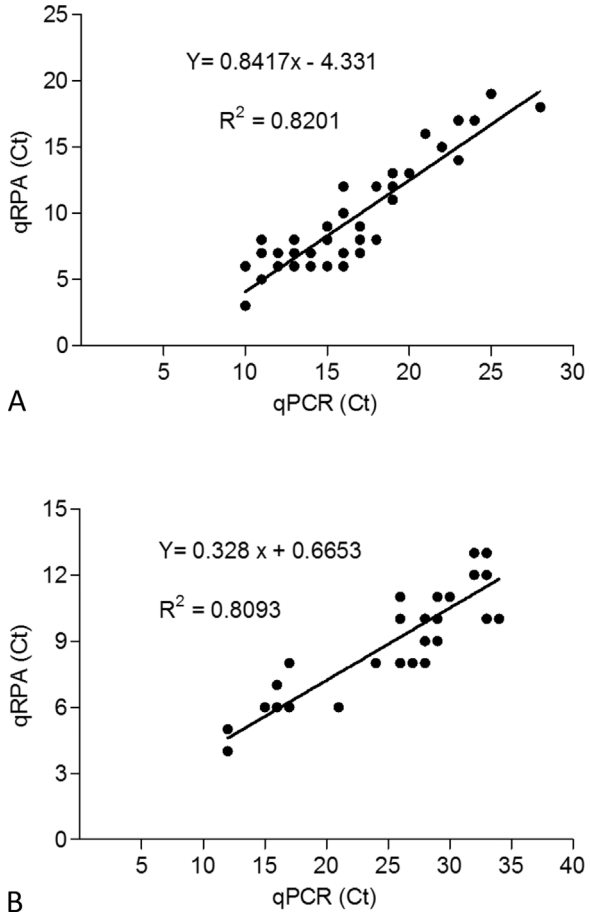

Fig. 5. Correlation of Ct values between qRPA and qPCR assays. Plot A: Correlation of Ct values between AbHV-qRPA and AbHV-qPCR assays; plot B: Correlation of Ct values between RGNNV-qRPA and RGNNV-qPCR assays.

A previous study on RPA suggests that the small volume of the reaction solution can promote the dynamic reaction of DNA template in the pool (Lillis et al., 2016). Therefore, we tried to reduce the reaction volume in this study. However, the RPA buffer is so viscous that it is difficult to obtain an admixture of the reaction solution. This may explain why the qRPA repeatability is not as accurate as qPCR. Before preparing the RPA reaction solution, we first configured the upstream and downstream primers, probe, and buffer. Next, we thawed the freeze-dried powder (in TwistAmp kit), and then add MgAc to the fully mixed solution to initiate the reaction. This process is more cumbersome than PCR and LAMP, resulting in increased susceptibility to environmental contamination. Although RPA has the advantage of rapid reaction and low reaction temperature, it still requires more effort to reduce reagent costs and simplify reaction steps in order to play a better role in field detection. Twist Amp® DNA amplification kits and Twist Amp® exo Probe are very expensive; the cost of each reaction of 25 μL is approximately 25 Yuan. In contrast, the price of qPCR assays is comparatively lower than that of qRPA assays; each reaction costs approximately 5 Yuan. If SYBR@Green dye is used, the cost is reduced further.

Overall, we present a method of AbHV-qRPA and RGNNV-qRPA that facilitates the simple, rapid, and specific detection of pathogens in samples from animal populations in aquaculture. Our method improves the detection efficiency of viral infection and will play a positive role in the widespread monitoring of diseases in aquaculture.

Acknowledgments

We express our thanks to Mr. Xu Li-Wen, Dr. Guo Zhixun and Dr. Su Youlu (South China Sea Fisheries Research Institute, Guangzhou, China) for providing E. akaara, WSSV positive shrimp and Aeromonas hydrophila genomic DNA, respectively. This work was supported by the "Central Public-interest Scientific Institution Basal Research Fund, CAFS" (2016RC-LX05), the "Earmarked Fund for Modern Agro-industry Technology Research System" (CARS-49), the "Technology Extension Foundation of Marine Fishery in Guangdong Province" (A201601B13) and the "Guangdong Special Support Program" (00-201620641).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.09.024.

References

Abd El Wahed, A., Patel, P., Heidenreich, D., Hufert, F.T., Weidmann, M., 2013. Reverse transcription recombinase polymerase amplification assay for the detection of middle East respiratory syndrome coronavirus. PLoS Curr. 5.

Arimoto, M., Mushiake, K., Mizuta, Y., Nakai, T., Muroga, K., Furusawa, T., 1992. Detection of striped jack nervous necrosis (SJNNV) by enzyme-linked immunosorbent assay (ELLASA). Fish Pathol. 27, 191–195.

Babu, B., Washburn, B.K., Miller, S.H., Poduch, K., Sarigul, T., Knox, G.W., Ochoa-Corona, F.M., Paret, M.L., 2017. A rapid assay for detection of Rose rosette virus using reverse transcription-recombinase polymerase amplification using multiple gene targets. J. Virol. Methods 240, 78–84.

Boyle, D.S., McNerney, R., Teng Low, H., Leader, B.T., Perez-Osorio, A.C., Meyer, J.C., O'Sullivan, D.M., Brooks, D.G., Piepenburg, O., Forrest, M.S., 2014. Rapid detection of Mycobacterium tuberculosis by recombinase polymerase amplification. PLoS One 9.

Chi, S.C., Wu, Y.C., Cheng, T.M., 2005. Persistent infection of betanodavirus in a novel cell line derived from the brain tissue of barramundi Lates calcarifer. Dis. Aquat. Org. 65, 91–98.

Comps, M., Trindade, M., Delsert, C., 1996. Investigation of fish encephalitis viruses (FEV) expression in marine fishes using DIG-labelled probes. Aquaculture 143, 113–121.

Corbeil, S., Colling, A., Williams, L.M., Wong, F.Y.K., Savin, K., Warner, S., Murdoch, B., Cogan, N.O.I., Sawbridge, T.I., Fegan, M., Mohammad, I., Sunarto, A., Handlinger, J., Pyecroft, S., Douglas, M., Chang, P.H., Crane, M.S., 2010. Development and validation of a TaqMan (R) PCR assay for the Australian abalone herpes-like virus. Dis. Aquat. Org. 92, 1–10.

Corbeil, S., McColl, K.A., Williams, L.M., Mohammad, I., Hyatt, A.D., Crameri, S.G., Fegan, M., Crane, M.S., 2012a. Abalone viral ganglioneuritis: establishment and use of an experimental immersion challenge system for the study of abalone herpes virus infections in Australian abalone. Virus Res. 165, 207–213.

Corbeil, S., Williams, L.M., Bergfeld, J., Crane, M.S.J., 2012b. Abalone herpes virus stability in sea water and susceptibility to chemical disinfectants. Aquaculture 326, 20–26.

Dalla Valle, L., Toffolo, V., Lamprecht, M., Maltese, C., Bovo, G., Belvedere, P., Colombo, L., 2005. Development of a sensitive and quantitative diagnostic assay for fish nervous necrosis virus based on two-target real-time PCR. Vet. Microbiol. 110, 167–179.

Doan, Q.K., Vandeputte, M., Chatain, B., Morin, T., Allal, F., 2017. Viral encephalopathy and retinopathy in aquaculture: a review. J. Fish Dis. 40, 717–742.

Gomez, D.K., Sato, J., Mushiake, K., Isshiki, T., Okinaka, Y., Nakai, T., 2004. PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. J. Fish Dis. 27, 603–608.

Gomez, D.K., Baeck, G.W., Kim, J.H., Choresca Jr., C.H., Park, S.C., 2008. Molecular detection of betanodavirus in wild marine fish populations in Korea. J. Vet. Diagn. Invest. 20, 38–44.

Grotmol, S., Nerland, A.H., Biering, E., Totland, G.K., Nishizawa, T., 2000. Characterization of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut Hippoglossus hippoglossus and design of an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. Dis. Aquat. Org. 39, 79–88.

Hill-Cawthorne, G.A., Hudson, L.O., El Ghany, M.F., Piepenburg, O., Nair, M., Dodgson, A., Forrest, M.S., Clark, T.G., Pain, A., 2014. Recombinations in staphylococcal cassette chromosome mec elements compromise the molecular detection of methicillin resistance in Staphylococcus aureus. PLoS One 9.

Hodneland, K., Garcia, R., Balbuena, J.A., Zarza, C., Fouz, B., 2011. Real-time RT-PCR detection of betanodavirus in naturally and experimentally infected fish from Spain. J. Fish Dis. 34, 189–202.

Huang, B., Tan, C., Chang, S.F., Munday, B., Mathew, J.A., Ngoh, G.H., Kwang, J., 2001. Detection of nodavirus in barramundi, Lates calcarifer(Bloch), using recombinant coat protein-based ELISA and RT-PCR. J. Fish Dis. 24, 135–141.

Kersting, S., Rausch, V., Bier, F.F., von Nickisch-Rosenegk, M., 2014a. Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. Mikrochim. Acta 181, 1715–1723.

Kersting, S., Rausch, V., Bier, F.F., von Nickisch-Rosenegk, M., 2014b. Rapid detection of Plasmodium falciparum with isothermal recombinase polymerase amplification and lateral flow analysis. Malar. J. 13, 99.

Kuo, H.C., Wang, T.Y., Chen, P.P., Chen, Y.M., Chuang, H.C., Chen, T.Y., 2011. Real-time

quantitative PCR assay for monitoring of nervous necrosis virus infection in grouper aquaculture. J. Clin. Microbiol. 49, 1090–1096.
Lillis, L., Siverson, J., Lee, A., Cantera, J., Parker, M., Piepenburg, O., Lehman, D.A., Boyle, D.S., 2016. Factors influencing Recombinase polymerase amplification (RPA) assay outcomes at point of care. Mol. Cell. Probes 30, 74–78.
Ma, H., Wen, W., Su, Y., Feng, J., Xu, L., Peng, C., Guo, Z., 2015. Epidemiological characterization of VNNV in hatchery-reared and wild marine fish on Hainan Island, China, and experimental infection of golden pompano (Trachinotus ovatus) juveniles. Arch. Virol. 160, 2979–2989.
Mazelet, L., Dietrich, J., Rolland, J.L., 2011. New RT-qPCR assay for viral nervous necrosis virus detection in sea bass, Dicentrarchus labrax (L.): application and limits for hatcheries sanitary control. Fish Shellfish Immunol. 30, 27–32.
Mekata, T., Satoh, J., Inada, M., Dinesh, S., Harsha, P., Itami, T., Sudhakaran, R., 2015. Development of simple, rapid and sensitive detection assay for grouper nervous necrosis virus using real-time loop-mediated isothermal amplification. J. Fish Dis. 38, 873–879.
Mekuria, T.A., Zhang, S., Eastwell, K.C., 2014. Rapid and sensitive detection of Little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification. J. Virol. Methods 205, 24–30.
Miles, T.D., Martin, F.N., Coffey, M.D., 2015. Development of rapid isothermal amplification assays for detection of Phytophthora spp. in plant tissue. Phytopathology 105, 265–278.
Murinda, S.E., Ibekwe, A.M., Zulkaffly, S., Cruz, A., Park, S., Razak, N., Paudzai, F.M., Ab Samad, L., Baquir, K., Muthaiyah, K., Santiago, B., Rusli, A., Balkcom, S., 2014. Real-time isothermal detection of Shiga toxin-producing Escherichia coli using recombinase polymerase amplification. Foodborne Pathog. Dis. 11, 529–536.
Nguyen, H.D., Nakai, T., Muroga, K., 1996. Progression of striped jack nervous necrosis virus (SJNNV) infection in naturally and experimentally infected striped jack Pseudocaranx dentex larvae. Dis. Aquat. Org. 24, 99–105.
Nishizawa, T., Mori, K., Furuhashi, M., Nakai, T., Furusawa, I., Muroga, K., 1995. Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fis. J. Gen. Virol. 76, 1563–1569.
Nunez-Ortiz, N., Stocchi, V., Toffan, A., Pascoli, F., Sood, N., Buonocore, F., Picchietti, S., Papeschi, C., Taddei, A.R., Thompson, K.D., Scapigliati, G., 2016. Quantitative immunoenzymatic detection of viral encephalopathy and retinopathy virus (betanodavirus) in sea bass Dicentrarchus labrax. J. Fish Dis. 39, 821–831.
Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. DNA detection using recombination proteins. PLoS Biol. 4, 1115–1121.
Su, Y.-C., Wang, C.-H., Chang, W.-H., Chen, T.-Y., Lee, G.-B., 2015. Rapid and amplification-free detection of fish pathogens by utilizing a molecular beacon-based microfluidic system. Biosens. Bioelectron. 63, 196–203.
Sung, C.H., Lu, J.K., 2009. Reverse transcription loop-mediated isothermal amplification for rapid and sensitive detection of nervous necrosis virus in groupers. J. Virol. Methods 159, 206–210.
Toubanaki, D.K., Margaroni, M., Karagouni, E., 2015a. Development of a novel allele-specific PCR method for rapid assessment of nervous necrosis virus genotypes. Curr. Microbiol. 71, 529–539.
Toubanaki, D.K., Margaroni, M., Karagouni, E., 2015b. Nanoparticle-based lateral flow biosensor for visual detection of fish nervous necrosis virus amplification products. Mol. Cell. Probes 29, 158–166.
Wang, C.H., Lien, K.Y., Wang, T.Y., Chen, T.Y., Lee, G.B., 2011. An integrated microfluidic loop-mediated-isothermal-amplification system for rapid sample pre-treatment and detection of viruses. Biosens. Bioelectron. 26, 2045–2052.
Xia, X., Yu, Y., Weidmann, M., Pan, Y., Yan, S., Wang, Y., 2014. Rapid detection of shrimp white spot syndrome virus by real time, isothermal recombinase polymerase amplification assay. PLoS One 9.
Yang, Y., Qin, X., Wang, G., Jin, J., Shang, Y., Zhang, Z., 2016a. Development of an isothermoal amplification-based assay for rapid visual detection of an Orf virus. Virol. J. 13, 46.
Yang, Y., Qin, X., Zhang, W., Li, Y., Zhang, Z., 2016b. Rapid and specific detection of porcine parvovirus by isothermal recombinase polymerase amplification assays. Mol. Cell. Probes 30, 300–305.