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A validated semi-nested PCR for rapid detection of scale drop disease virus (SDDV) in Asian sea bass (*Lates calcarifer*)

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ABSTRACT

Scale drop diseases virus (SDDV), a newly characterized virus of farmed Asian sea bass (*Lates calcarifer*), has been reported in several countries in Southeast Asia. However, no fully validated detection method is publicly available for disease diagnosis and surveillance. Here, we described a newly developed semi-nested PCR (snPCR) method for detection of the virus from field samples. The designed primers targeting a gene encoding *ATPase* generated amplicons of 738 bp and 412 bp in the first and second step PCR, respectively. The established protocol could detect down to 100 viral copies/μL template and was 100-fold more sensitive than single step PCR. A Specificity test against extracted DNA from ten bacterial pathogens, tissues from viral infected specimens and fish host revealed no cross amplification. The SDDV snPCR method could detect the virus from all clinical samples showing symptoms of scale drop disease (n = 25) and all samples from outbreaks of an unknown disease (n = 6) whereas all clinically healthy fish sea bass (n = 161) and grouper (n = 45) collected from different provinces tested negative. The newly established protocol might be useful for Asian sea bass farming countries to initiate disease diagnosis and surveillance.

1. Introduction

Asian sea bass (*Lates calcarifer*) is a highly economic marine fish that has been intensively farmed in Asia-Pacific region (e.g. Australia, Indonesia, Malaysia, Philippines, Thailand, Taiwan, and Vietnam) (FAO, 2012; de Groof et al., 2015). Several emerging diseases have been reported in recent years, however, scale drop disease (SDD) is considered as the top viral concern (de Groof et al., 2015). The disease was initially described as scale drop syndrome (SDS) with suspected viral aetiology (Gibson-Kueh et al., 2012). The causative virus was subsequently identified as a novel *Megalocytivirus*; termed scale drop disease virus (SDDV), a double-stranded DNA virus with icosahedral morphology with the near complete genome size of 124 Kb (de Groof et al., 2015).

The diseased fish were characterized by scale loss, fin and tail erosion, darkened bodies, gills pallor and sometimes exophthalmia. The disease affected both juvenile and adult fish, causing 40–50% mortality (de Groof et al., 2015; Senapin et al., 2019). Experimental infection using virus propagated in fish cell line induced ˜20-60% mortality and fulfilled Koch's postulates (de Groof et al., 2015). Up-to-date, presence of SDDV associated with mortality in famed Asian sea bass has been confirmed in Malaysia, Singapore, Indonesia (Gibson-Kueh et al., 2012; de Groof et al., 2015) and Thailand (Senapin et al., 2019). For disease diagnosis, published and patented single PCR and qPCR protocols (WO2014191445A1; de Groof et al., 2015) were developed for SDDV but not yet fully validated. The single PCR protocols employed primers binding to either *ATPase* (adenosine triphosphatase) or *MCP* (major capsid protein) genes of the SDDV (WO2014191445A1). Probe-based qPCR targeted to the putative DNA dependent RNA polymerase gene of the virus have also been developed (WO2014191445A1; de Groof et al., 2015). To our knowledge, many fish laboratories in Asia-Pacific are not able to access the relatively costly qPCR machine. This study, therefore, developed a validated conventional semi-nested PCR method that most laboratories in the region can access for purpose of SDDV disease diagnosis and surveillance.

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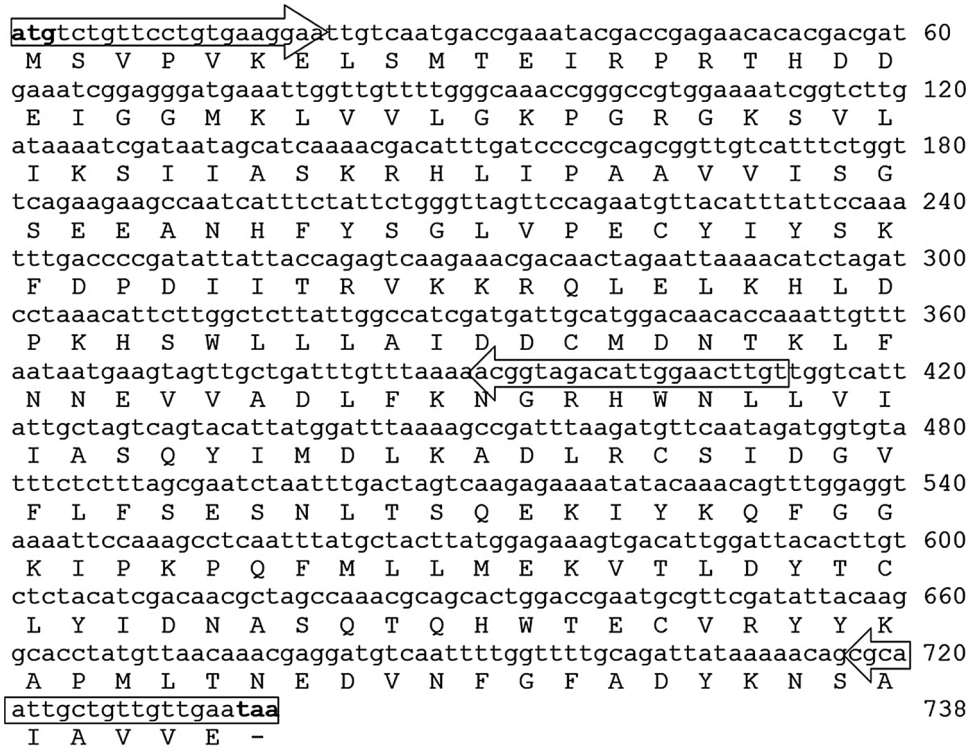

Fig. 1. Coding strand sequence of *ATPase* open reading frame (GenBank accession no. MH152407) of SDDV. Putative start and stop codons are indicated by bold texts. Arrow boxes mark positions of primers in semi-nested PCR assay described in this study.

2. Materials and methods

2.1. Primer design

Nucleotide sequences encoding *ATPase* genes of several members of *Megalocytivirus* were downloaded from GenBank e.g. infectious spleen and kidney necrosis virus, ISKNV (AB669097); red seabream iridovirus, RSIV (F264212); turbot reddish body iridovirus, TBIV (KX354229); Singaporean SDDV (KR139659) and Thai SDDV (MH152407). Multiple sequence alignments were performed using ClustalW of the MEGA-X software. Primer positions were chosen from the conserved regions and designed manually to bind to SDDV sequences. Specificity of the designed primers was initially examined using primer blast (www.ncbi.nlm.nih.gov/ tools/primer-blast/). Positions of the selected primers are indicated in Fig. 1. The forward primer RB-ATPase-F1/ 5′-ATG TCT GTT CCT GTG AAG GAA-3′ and reverse primer RB-ATPase-R1/ 5′-TTA TTC AAC AAC AGC AAT TGC G-3′ were designed for the first step PCR which aims to amplify a 738-bp complete SDDV *ATPase* sequence. For snPCR, RB-ATPase-F1 and inner reverse primer RB-ATPase-R2/ 5′-ACA AGT TCC AAT GTC TAC CGT-3′ were designed to target 412 bp internally to the first product.

2.2. Optimization of PCR conditions

Extracted DNA from liver specimens of SDDV-infected fish from our previous study (Senapin et al., 2019) were used to optimize PCR conditions using Biometra PCR Thermal Cyclers (Germany). Standard PCR mixtures contained 0.5 µL of each 10 µM primer (Bio Basic Inc, Canada), 2.5 µL of 10 X reaction buffer (RBC Bioscience, Taiwan), 0.5 µL of 10 mM dNTPs (Thermo Fisher Scientific), 0.25 µL of *Taq* DNA polymerase (RBC Bioscience, Taiwan, 5 units/µL), 2 µL of DNA template solution (100 ng/µL) and nuclease-free water in a final volume of 25 µL. Reaction without DNA template was used as a negative control. Gradient PCR were carried out for the first step (RB-ATPase-F1 and RB-ATPase-R1) and semi-nested (RB-ATPase-F1 and RB-ATPase-R2) amplification using annealing temperature ranging from 51.7 to 68 °C. The optimal annealing temperatures for the first and semi-nested PCR were 60 and 67 °C, respectively Thus, the final PCR conditions for the first step PCR were denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s with a final extension at 72 °C for 5 min.

The semi-nested PCR mixtures contained 0.4 µL of each 10 µM primer RB-ATPase-F1 and RB-ATPase-R2 (Bio Basic Inc, Canada), 2.0 µL of 10 X supplied reaction buffer, 0.4 µL of 10 mM dNTPs (Thermo Fisher Scientific), 0.2 µL of *Taq* DNA polymerase (RBC Bioscience, Taiwan, 5 units/µL), 5 µL of the first PCR product and nuclease-free water in a final volume of 20 µL. The snPCR conditions were denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 67 °C for 30 s, 72 °C for 30 s with a final extension at 72 °C for 5 min. Amplified products were analyzed by 1% agarose gel electrophoresis, stained with RedSafe™ (ABC Scientific) and visualized under UV light.

2.3. Specificity test

Specificity of the semi-nested PCR protocol was examined against genomic DNA of 10 common bacterial pathogens of fish and extracted DNA from fish infected with either infectious spleen and kidney necrosis virus (ISKNV) or nervous necrosis virus (NNV) (Table 1). Extracted DNA from SDDV-infected fish was used as a positive control while extracted DNA from apparently healthy Asian sea bass was included as a negative control. PCR mixtures and conditions were carried out as mentioned above.

2.4. Sensitivity test

A complete DNA sequence encoding *ATPase* gene (738 bp) of the Thai SDDV strain were cloned into pGEM®-T easy cloning vector (Promega) as previously described (Senapin et al., 2019). Recombinant plasmid containing inserted *ATPase* gene was then used for sensitivity assay. Ten-fold serial dilutions of plasmid copies from 10^8 to 1 copy/µL

Table 1
Details of samples used for specificity test.

Pathogens/samples	Code	Source
Streptococcus iniae	VN2396	Laboratory strain
Vibrio harveyi	SDMN-Y6	Dong et al., 2017b
Vibrio parahaemolyticus	XN89	Phiwsaiya et al., 2017
Vibrio tubiashi	SDMN-G4	Dong et al., 2017b
Vibrio vulnificus	N/A	Laboratory strain
Vibrio alginolyticus	N/A	Laboratory strain
Vibrio cholera	NK8	Dong et al., 2015
Pleisiomas shigelloides	NK10	Dong et al., 2015
Tenacibaculum litopenaei	SDMN-T4	Dong et al., 2017b
Nocardia seriolae	VN2391	Laboratory strain
ISKNV infected tissue	N/A	Dong et al., 2017a
NNV infected tissue	N/A	Laboratory sample

were used as DNA template for PCR test. Additionally, fish DNA extracted from a clinically healthy Asian sea bass of 100 ng/reaction was spiked into the first step PCR reactions to mimic the test samples. Semi-nested PCR was then carried out as mentioned above. The last dilution that showed positive result was considered as detection limit of the test. The sensitivity tests using plasmids with and without spiked fish DNA was performed in duplicates.

2.5. Effect of DNA purification methods

In this study, effect of DNA purification methods on SDDV detection was also investigated. Two DNA column purification kits, namely A and B, and a conventional phenol/chloroform extraction were used. Starting DNA materials were the serial dilutions of control plasmid plus spiked fish DNA as described above. After purification steps, the obtained DNA was subjected to the SDDV snPCR assays.

2.6. Detection of SDDV in fish samples

Archived samples (n = 237) of Asian sea bass (*L. calcarifer*) and grouper (*Epinephelus* sp.) were previously collected from Eastern and Southern provinces of Thailand in the period of 2016–2018 including Chanthaburi, Chachoengsao, Chonburi, Trang, Krabi, Phang Nga, Phuket, Satun, Songkhla, and Pattani (Table 2). Tissue samples (pool of liver and kidney) were preserved in 95% ethanol. DNA was extracted from the mixed kidney and liver using conventional phenol/chloroform extraction or commercial kit (genomic DNA isolation kit, PureDireX). DNA concentration was quantified by measuring absorbance at 260 nm. The purity of the samples was also checked by measuring the ratio of OD_{260} nm and OD_{280} nm using spectrophotometer with acceptable values between 1.7 and 1.8 and adjusted to 100 ng/μL for PCR amplification. PCR assays were conducted as described above and amplified products were analyzed by agarose gel electrophoresis.

3. Results

3.1. Specificity of SDDV snPCR

Investigation of primer blast to GenBank using primer sequences of RB-ATPase-F1 and RB-ATPase-R1 or RB-ATPase-F1 and RB-ATPase-R2 indicated specificity of the designed primers to only SDDV (as of March 2019). Subsequently, snPCR assay against genomic DNA of 10 common fish bacterial pathogens from five genera (*Vibrio*, *Streptococcus*, *Pleisiomonas*, *Tenacibaculum,* and *Nocardia*), 2 viral (ISKNV and NNV) infected tissues, and host DNA revealed no cross amplification (Fig. 2). This indicated that the established SDDV snPCR is highly specific for SDDV.

3.2. Sensitivity of SDDV snPCR

The sensitivity test of snPCR against 10-fold serial dilutions of recombinant plasmid containing SDDV *ATPase* gene in the presence of spiked fish DNA revealed that the first step PCR could detect a 738 bp-target product from the template as low as 10^4 copies/μL (Fig. 3). When subjected to semi-nested PCR, the detection sensitivity was 10^2 copies/μL template (Fig. 3). Note that two expected amplicons (738 and 412 bp) were generated in dilutions from 10^8 to 10^4 copies/μL template while only single internal amplicon (412 bp) was detected in the dilutions of 10^3 and 10^2 copies/μL template. A band of ~1.4 kb possibly derived from cross-hybridized products could be observed from high concentrated template e.g. 10^8 to 10^4 copies (Fig. 3). The same detection limit of 10^2 copies/μL template was also obtained when using the serial plasmid controls without spiked fish DNA (figures not shown). The results were consistent in two replicates and the representative sensitivity test result with the presence of spiked DNA is shown in Fig. 3. When compared the effect of DNA purification kits, the results shown in Fig. 4 demonstrated that the detection sensitivity varied depending on the method used. The 3 extraction approaches yielded a 10- to 1,000-fold reduction in PCR sensitivity (Fig. 4). The best result was obtained from using kit A, although the detection limit was lower to 10^3 copies/μL (Fig. 4).

3.3. Detection of SDDV from fish samples

The established snPCR method was employed to investigate the presence of SDDV in the samples collected from 3 Eastern provinces and

Table 2
Details of fish samples used for SDDV snPCR test and the test results.

Region	Province	Year	Fish health status	+ve/number of tested samples	
				Asian sea bass	Grouper
Eastern Thailand	Chanthaburi	2016	Sick fish with scale drop symptoms	18/18	–
		2016	Unknown diseased fish	6/6	–
		2017	Sick fish with scale drop symptoms	5/5	–
		2017	Clinically healthy fish	0/12	
		2018	Sick fish with scale drop symptoms	2/2	–
	Chachoengsao	2017	Clinically healthy fish	0/80	–
	Chonburi	2017	Clinically healthy fish	0/10	–
Southern Thailand	Satun	2017	Clinically healthy fish	0/10	0/10
	Trang	2017	Clinically healthy fish	0/10	0/10
	Krabi	2017	Clinically healthy fish	0/10	0/10
	Phangnga	2017	Clinically healthy fish	0/5	0/5
	Phuket	2017	Clinically healthy fish	0/10	0/10
	Pattani	2017	Clinically healthy fish	0/1	–
	Songkhla	2017	Clinically healthy fish	0/13	–
Total				31/192	0/45

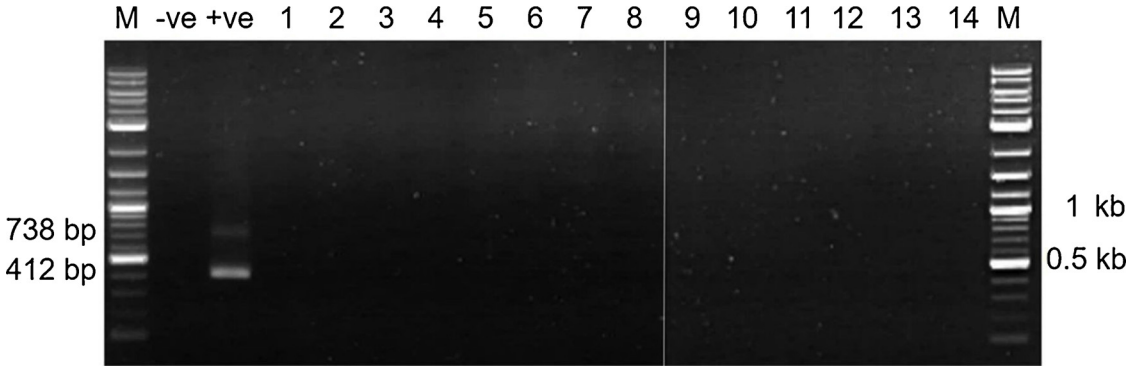

Fig. 2. Agarose gel showing the specificity test result of SDDV snPCR. Lanes 1–12 were *Streptococcus iniae*, *Vibrio harveyi*, *V. parahaemolyticus*, *V. vulnificus*, VNN-infected tissue, ISKNV-infected tissue, *V. tubiashi*, *Tenacibaculum litopenaei*, *Nocardia seriolae*, *V. alginolyticus*, *Pleisiomas shigelloides* and *V. cholera*, respectively. Lanes 13–14 were DNA extracted from healthy Asian sea bass. M, 2 log DNA marker (New England Biolabs); -ve, no template control, +ve, positive control.

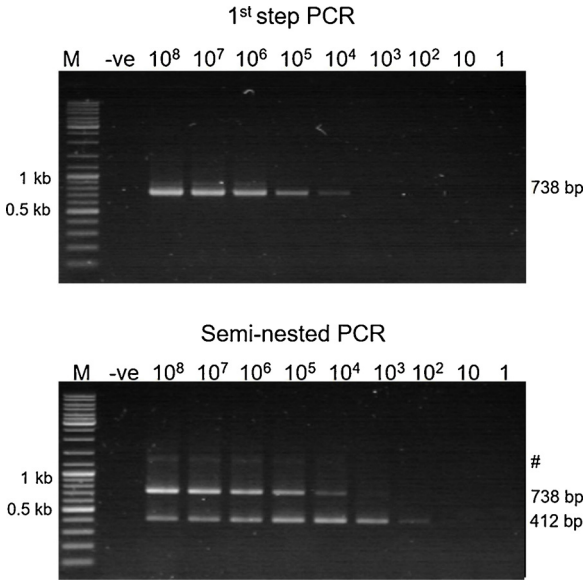

Fig. 3. Sensitivity tests of the first step PCR and snPCR using serial dilutions of plasmid containing SDDV *ATPase* gene. Number of copies were from 10^8 to 1 copy/μL. The first reactions contained spiked fish DNA extracted from a clinical healthy Asia sea bass. A representative test result from duplicate experiment is shown. M, 2 log DNA marker; -ve, no template control; #, cross hybridized PCR products.

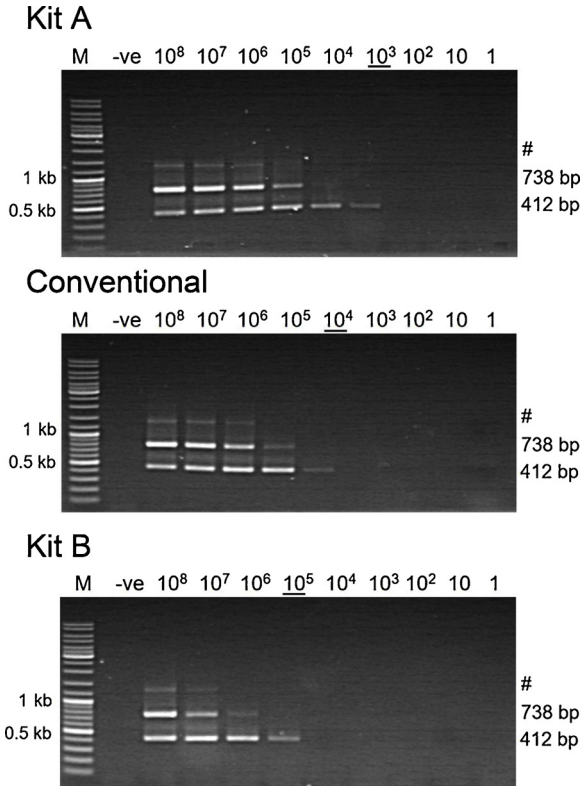

Fig. 4. Effect of DNA purification methods on SDDV snPCR detection. DNA templates with known concentration were prepared from serial dilutions of the plasmid control and spiked fish DNA. Two commercial column purification kits and a conventional phenol/chloroform DNA extraction protocol were used for comparison. After purification procedure, the obtained DNA from each method was subjected to SDDV snPCR detection. Underlines indicate the detection sensitivity. M, 2 log DNA marker; -ve, no template control; #, cross hybridized PCR products.

7 Southern provinces of Thailand (Table 2). In total, 192 Asian sea bass and 45 grouper samples were tested. The result showed that SDDV was detected from only Asian sea bass samples from Chanthaburi whereas Asian sea bass samples from the remaining 9 provinces and groupers from Southern Thailand tested negative for SDDV (Table 2). All 31 clinically sick sea bass showing clinical signs of scale drop disease collected from 2016 to 2018 tested positive for SDDV. Representative results of positive samples are shown in Fig. 5. According to the sensitivity test above, the presence of 2 bands (738 and 412 bp) or 1 band (412 bp) could be indicative of SDDV being present in the infected fish at high- and low-level viremia, respectively. Additionally, there were 6 samples from an unknown disease outbreak in 2016 that tested positive for SDDV (Table 2). All clinically healthy sea bass (n = 161) and groupers (n = 45) tested negative for SDDV (Table 2).

4. Discussion

Since SDDV was discovered, single PCR and qPCR methods have been initially developed for disease diagnosis (WO2014191445A1; de Groof et al., 2015). Upon detection using the established qPCR, samples of scale drop syndrome fish collected from Singapore during 2010 to 2011 and Indonesia in 2012 tested positive for the virus (de Groof et al., 2015). This indicated that SDDV has been affecting farmed Asian sea bass in the region for a relatively long period. Therefore, appropriate planning and implementation are required to prevent any potential risk of transboundary spread of SDDV. A validated PCR method is also important for sea bass farming countries as it would assist relevant authorities and scientists to initiate diagnosis and active surveillance. The present study provides the first validated and relatively sensitive snPCR method for detection of SDDV from fish samples. Since SDDV snPCR method does not require expensive qPCR machine, it might be

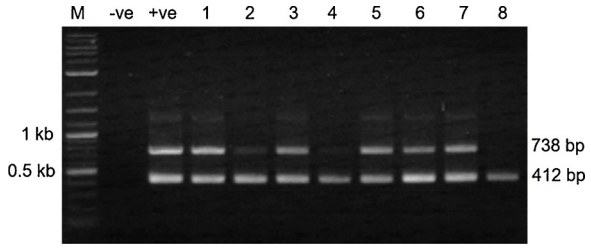

Fig. 5. Representative detection results of field samples using SDDV snPCR. Lanes 1–8 represented for clinically sick sea bass showing clinical signs of scale drop disease. M, 2 log DNA marker; -ve, no template control; +ve, positive control.

suitable for the majorities of laboratories in Southeast Asia.

The current SDDV snPCR method has a detection limit of 100 copies/μL, less sensitive than the previous SDDV qPCR by half (50 copies/μL) (de Groof et al., 2015) and 100 times more sensitive than single step PCR (10,000 copies/μL) in this study. These detection limits were expressed as the concentration of an already extracted DNA. The evidence from our study, and many others (for example, Chakraborty et al., 2005; Lucentini et al., 2006), suggested that efficiency of DNA extraction and purification methods especially considered from recovery yield, DNA integrity & purity, and inhibitor removal capability had significant impact to PCR detection sensitivity. Thus, selecting suitable DNA extraction/purification kits or approaches for certain biological sources is one of the major considerations for molecular diagnostic application and many downstream experiments.

In comparison to previously published nested or semi-nested PCR protocols for piscine viruses, SDDV snPCR was approximately 10 times less sensitive (Bergmann et al., 2010; Dong et al., 2017c). This may be due to high annealing temperatures used in this protocol, especially in the second step PCR (67 °C) to increase specificity. However, tested results of the field samples proved that the method is capable of being used for diagnosis purposes. The cross-hybridized products of around 1.4 kb could be observed in the sample with high viral loads. This phenomenon has also been observed in previous studies (Bergmann et al., 2010; Dong et al., 2017a, c) but did not interfere with interpretation of the test results.

Conflict of interest

The authors declare no conflict of interest.

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References

Bergmann, S.M., Riechardt, M., Fichtner, D., Lee, P., Kempter, J., 2012. Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus. J. Virol. Methods 163, 229–233.

Chakraborty, A., Sakai, M., Iwatsuki, Y., 2005. Museum fish specimens and molecular taxonomy: a comparative study on DNA extraction protocols and preservation techniques. J. Appl. Ichthyol. 22, 160–166.

de Groof, A., Guelen, L., Deijs, M., Van Der Wal, Y., Miyata, M., Ng, K.S., Van Grinsven, L., Simmelink, B., Biermann, Y., Grisez, L., Van Lent, J., De Ronde, A., Chang, S.F., Schrier, C., Van Der Hoek, L., 2015. A novel virus causes scale drop disease in *Lates calcarifer*. PLoS Pathog. 11, e1005074.

Dong, H.T., Nguyen, V.V., Le, D.H., Sangsuriya, P., Jitrakorn, S., Saksmerprome, V., Senapin, S., Rodkhum, C., 2015. Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. Aquaculture 448 (1), 427–435.

Dong, H.T., Jitrakorn, S., Kayansamruaj, P., Pirarat, N., Rodkhum, C., Rattanarojpong, T., Senapin, S., Saksmerprome, V., 2017a. Infectious spleen and kidney necrosis disease (ISKND) outbreaks in farmed barramundi (*Lates calcarifer*) in Vietnam. Fish Shellfish Immunol. 68, 65–73.

Dong, H.T., Taengphu, S., Sangsuriya, P., Charoensapsri, W., Phiwsaiya, K., Sornwatana, T., Khunrae, P., Rattanarojpong, T., Senapin, S., 2017b. Recovery of *Vibrio harveyi* from scale drop and muscle necrosis disease in farmed barramundi, *Lates calcarifer* in Vietnam. Aquaculture 473, 89–96.

Dong, H.T., Siriroo, S., Meemetta, W., Santimanawong, W., Gangnonngiw, W., Pirarat, N., Khunrae, K., Rattanarojpong, T., Vanichviriyakit, R., Senapin, S., 2017c. Emergence of tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection. Aquaculture 476, 111–118.

FAO, 2012. Regional Overview of Fisheries and Aquaculture in Asia and the Pacific 2012. RAP Publication 2012/26: Available at. http://www.fao.org/docrep/017/i3185e/i3185e3100.htm.

Gibson-Kueh, S., Chee, D., Chen, J., Wang, Y.H., Tay, S., Leong, L.N., Ng, M.L., Jones, J.B., Nicholls, P.K., Ferguson, H.W., 2012. The pathology of' scale drop syndrome' in Asian seabass, *Lates calcarifer* Bloch, a first description. J. Fish Dis. 35, 19–27.

Lucentini, L., Caporali, S., Palomba, A., Lancioni, H., Panara, F., 2006. A comparison of conservative DNA extraction methods from fins and scales of freshwater fish: a useful tool for conservation genetics. Conserv. Genet. 7, 1009–1012.

Phiwsaiya, K., Charoensapsri, W., Taengphu, S., Dong, H.T., Sangsuriya, P., Nguyen, G.T.T., Pham, H.Q., Amparyup, P., Sritunyalucksana, K., Taengchaiyaphum, S., Chaivisuthangkura, P., Longyant, S., Sithigorngul, P., Senapin, S., 2017. A natural *Vibrio parahaemolyticus* $\Delta pirA^{Vp}$ $pirB^{Vp+}$ mutant kills shrimp but produces neither Pir^{Vp} toxins nor acute hepatopancreatic necrosis disease lesions. Appl. Environ. Microbiol. 83, e00680–17.

Senapin, S., Dong, H.T., Meemetta, W., Gangnonngiw, W., Sangsuriya, P., Vanichviriyakit, R., Sonthi, M., Nuangsaeng, B., 2019. Mortality from scale drop disease in farmed *Lates calcarifer* in Southeast Asia. J. Fish Dis. 42, 119–127.

WO2014191445A1, 2014. Scale Drop Disease (SDD) Causative Virus and Derivatives Thereof. Available at. https://patents.google.com/patent/WO2014191445A1/en.