

# 1      **Development of a SYBR Green quantitative PCR assay for detection of** 2                      ***Lates calcarifer herpesvirus (LCHV) in farmed barramundi***

3              Watcharachai Meemetta<sup>1</sup>, Jose A. Domingos<sup>2</sup>, Ha Thanh Dong<sup>3\*</sup>, Saengchan Senapin<sup>1,4\*</sup>

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5              <sup>1</sup>Fish Health Platform, Center of Excellence for Shrimp Molecular Biology and  
6              Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok, Thailand,  
7              10400

8              <sup>2</sup>Tropical Futures Institute, James Cook University, Singapore, 387380

9              <sup>3</sup>Faculty of Science and Technology, Suan Sunandha Rajabhat University, Bangkok,  
10              Thailand, 10300

11              <sup>4</sup>National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science  
12              and Technology Development Agency (NSTDA), Pathum Thani, Thailand, 12120

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14      **Running head:** qPCR detection of LCHV

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16      \*Corresponding authors:

17      H.T. Dong (hathanh.do@ssru.ac.th)

18      S. Senapin (saengchan@biotec.or.th)

## 19 **Highlights**

- 20 • This study reported a new SYBR Green qPCR method for detection of LCHV
- 21 • The qPCR method had detection limit of 10 copies per  $\mu$ l plasmid DNA template when
- 22 spiked with genomic DNA from the host
- 23 • The aforementioned method is highly specific to LCHV
- 24 • Validation with clinical samples revealed that LCHV could be detected from multiple
- 25 organs with fin and brain the best organs for qPCR detection

## 26 ABSTRACT

27 *Lates calcarifer* herpes virus (LCHV) is a new virus of farmed barramundi in Southeast Asia.  
 28 However, a rapid detection method is yet to be available for LCHV. This study, therefore,  
 29 aimed to develop a rapid quantitative PCR (qPCR) detection method for LCHV and made it  
 30 timely available to public for disease diagnostics and surveillance in barramundi farming  
 31 countries. A newly designed primer set targeting a 93-bp fragment of the LCHV putative  
 32 major envelope protein encoding gene (*MEP*) was used for developing and optimizing a  
 33 SYBR Green based qPCR assay. The established protocol could detect as low as 10 viral  
 34 copies per µl of DNA template in a reaction containing spiked host DNA. No cross-  
 35 amplification with genomic DNA extracted from host as well as common aquatic pathogens  
 36 (12 bacteria and 3 viruses) were observed. Validation test of the method with clinical samples  
 37 revealed that the virus was detected in multiple organs of the clinically sick fish but not in the  
 38 healthy fish. We thus recommend that barramundi farming countries should promptly initiate  
 39 active surveillance for LCHV in order to understand their circulation for preventing possibly  
 40 negative impact to the industry.

41 **Keywords:** detection, *Lates calcarifer* herpes virus (LCHV), qPCR

## 42 INTRODUCTION

43 Barramundi (*Lates calcarifer*) or Asian sea bass is one of the economically important finfish  
 44 species in Asia-Pacific which has been farmed in a wide range of salinity in either open cage  
 45 systems or earthen ponds (Jerry et al., 2014). Barramundi, like other intensively farmed fish,  
 46 is susceptible to various infectious pathogens and often subject to serious outbreaks and  
 47 economic losses (Dong et al., 2017a, b; Jerry et al., 2014; Ransangan et al., 2010; Toranzo et  
 48 al., 2005). In recent years, three newly emerging viruses have been reported in farmed  
 49 barramundi in Asia-Pacific, including scale drop disease virus (SDDV) (Gibson-Kueh et al.,  
 50 2012; de Groof et al., 2015), *Lates calcarifer* herpes virus (LCHV) (Chang et al., 2017) and  
 51 *Lates calcarifer* birnavirus (LCBV) (Chen et al., 2019). Both SDDV and LCHV were  
 52 discovered from disease outbreaks where the fish showed clinical symptoms of “scale drop”  
 53 and laboratory infections with the cultivated virus from cell culture resulted in up to 60% and  
 54 77% cumulative mortality, respectively (de Groof et al., 2015; Chang et al., 2017). By  
 55 contrast, LCBV did not induce mortality in the controlled laboratory trial (Chen et al., 2019).

56 LCHV discovered by Chang et al. (2017) is a novel member of the family *Alloherpesviridae*,  
 57 which is genetically most similar to *Ictalurid herpesvirus 1* (<60% nucleotide identity), a  
 58 pathogenic virus of channel catfish. LCHV is an enveloped virus with diameter of  
 59 approximately 100 nm, and genome size of ~130 kb while other members of  
 60 *Alloherpesviridae* are between 150-250 nm in diameter and 100-250 kb in genome size  
 61 (Hanson et al., 2011; Chang et al., 2017).

62 Both SDDV and LCHV infections cause similar scale drop disease-like gross signs which are  
 63 clinically indistinguishable. Therefore, molecular detection methods are required for diagnostic  
 64 and screening purposes. Several DNA-based detection methods for SDDV have been freely  
 65 available such as single PCR (Senapin et al., 2019), semi-nested PCR (Charoenwai et al.,

2019), loop-mediated isothermal amplification (LAMP) (Dangtip et al., 2019), probe-based qPCR (de Groof et al., 2015), and SYBR Green-based qPCR (Sriisan et al., 2020). The latter one is the most sensitive method with a detection limit of 2 copies of DNA template per reaction. In case of LCHV, following discovery of the virus, several primer sets for detection purpose were published in a patent (Chang et al., 2017), the use of these methods thus might be conditionally limited. According to requests from private sector, this study, therefore, developed a new, sensitive qPCR detection method for rapid diagnostics of LCHV and made it available to promote active surveillance for preventing wide-spread of this pathogen.

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## 75 MATERIALS AND METHODS

### 76 Fish samples and DNA extraction

77 In 2019, there were 3 batches of barramundi samples subjected to testing for LCHV in our  
78 laboratory. Batch 1 comprised of adult fish (n = 5) in which 4 of them exhibited scale drop  
79 clinical signs while one fish had healthy looking appearance. Eight different tissue types  
80 (liver, kidney, spleen, gills, fin, brain, eyes and muscle) from each fish were dissected and  
81 individually preserved in 95% ethanol. Batch 2 comprised of apparently healthy barramundi  
82 fry that were ethanol-preserved. Three whole fry were pooled and considered as one sample  
83 for the test (n = 5 pools). Batch 3 (n = 10) comprised of ethanol-preserved spleen samples  
84 collected from 5 apparently healthy juvenile fish and 5 clinically sick fish showing scale drop  
85 disease-like symptoms. Approximately 5 mg tissue was subjected to DNA extraction using  
86 conventional sodium dodecyl sulfate/proteinase K containing lysis solution followed by  
87 phenol/chloroform extraction and ethanol precipitation. The obtained DNA pellet was  
88 resuspended in sterile distilled water and quantified using spectrophotometry at OD 260 and  
89 280 nm.

## 90 **Primer design and PCR conditions**

91 LCHV primers were designed to target a 93 bp partial fragment of a putative major envelop  
 92 protein (*MEP*) gene of the virus. Forward primer LCHV-MEP93-qF: 5'-  
 93 GTACTTCATCGCCTACGGAGC-3' and reverse primer LCHV-MEP93-qR: 5'-  
 94 TACGTGTGCTTGAGGAGGTC-3' were synthesized from Bio Basic, Canada. Gradient  
 95 PCR was firstly conducted to find an optimal annealing temperature (*T<sub>a</sub>*) using *T<sub>a</sub>* ranging  
 96 from 58 to 65 °C. The reaction mixture of 20 µL contained 200 ng of DNA extracted from fin  
 97 of LCHV-infected fish, 1x iTaq Universal SYBR Green SuperMix (Bio-Rad Cat.no. 172-  
 98 5121) and 200 nM of each primer. The PCR amplification conditions were initial  
 99 denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 10 s and annealing at 58-  
 100 65 °C for 30 s (Bio-Rad CFX Connect Real-Time PCR) followed by melt peak analysis.  
 101 Finally, *T<sub>a</sub>* of 61°C was selected and the same thermocycling conditions were used  
 102 throughout the study.

## 103 **Sensitivity, qPCR efficiency, and specificity assays**

104 Positive control plasmid namely pMEP93 was constructed for use in diagnostic sensitivity  
 105 test. This was done by cloning the 93-bp *MEP* amplified fragment obtained above into pGEM  
 106 T-easy vector (Promega) and transforming into *Escherichia coli* XL-1 blue. After colony  
 107 PCR verification of potential correct clones, one recombinant clone was sent for DNA  
 108 sequencing at Macrogen (South Korea). Copy number of pMEP93 was calculated based on  
 109 plasmid size and concentration at <https://cels.uri.edu/gsc/cndna.html> and the pMEP93 was 10  
 110 fold-serially diluted from 10<sup>7</sup> to 1 copies/µl. Plasmid dilutions (2 µl) were then used as  
 111 template in qPCR conditions described above. To mimic a real test, each reaction also  
 112 contained spiked 100 ng DNA extracted from a healthy barramundi. Control reaction without  
 113 pMEP93 was used as a negative control. Analytical sensitivity experiment was conducted in

3 replicates within the same run. Standard curve was then automatically generated from quantification cycle (C<sub>q</sub>) values being plotted versus log<sub>10</sub> pMEP93 quantity. Formula for copy number calculation, coefficient of correlation (R<sup>2</sup>), and amplification efficiency (E) values were also provided by the Bio-Rad Maestro Software.

The optimized qPCR protocol was subsequently used to test for specificity against extracted genomic DNA from i) clinically healthy fish, ii) from 12 common aquatic bacterial species, and iii) from fish samples infected with either infectious spleen and kidney necrosis virus (ISKNV), nervous necrosis virus (NNV), or scale drop disease virus (SDDV). Sample sources and preparation were previously described (Charoenwai et al., 2019; Sriisan et al., 2020). DNA extracted from fin of LCHV-infected fish was used as positive control. No template control was used as negative reaction. Specificity test was performed in 2 replicates by 2 qPCR runs.

## **LCHV detection in field samples**

The newly developed qPCR was used to detect and quantify LCHV loads in the barramundi DNA samples prepared from 3 fish batches. 200 ng DNA template was used in each qPCR reaction. The obtained C<sub>q</sub> was used to calculate viral copy numbers in the samples using the equation, copy number = 10<sup>(C<sub>q</sub> - Intercept)/Slope</sup> i.e. 10<sup>(C<sub>q</sub> - 41.34)/-3.539</sup> derived from the stand curve described above. Comparative evaluation of the viral loads in different fish tissue types was performed using samples from batch 1.

## **RESULTS**

### **SYBR Green based LCHV qPCR**

The LCHV qPCR protocol developed in this study had a detection limit of 10 copies/ $\mu$ l template i.e. 20 copies/reaction. Mean Cq  $\pm$  SD values of the detection limit were  $37.91 \pm 0.33$  (**Fig. 1a**). In other words, samples with  $Cq \leq 37.91 \pm 0.33$  were considered as LCHV positive tests. The amplified products yielded uniform melting temperatures ( $T_m$ ) at  $84.0^\circ\text{C}$  (**Fig. 1b**), indicating that the primers and the condition assayed were specific. The 93-bp amplicon had a relatively high  $T_m$  due to its 58% GC content of the sequence. Note that there was 1 nucleotide difference (**Supplemental Fig. 1**) between the target sequence in this study and that from the previous data (Chang et al., 2017). Based on the standard curve shown in **Fig. 1c**, the performance of the newly developed qPCR was high determined by its amplification efficiency (E) of 91.7% with  $R^2$  of 0.995. When evaluated the protocol specificity, the LCHV qPCR was demonstrated to be highly specific because it only detected LCHV infected sample but not DNA extracted from 3 other viruses, 12 bacteria, or clinically healthy fish tested. Data from one of the two replicates is shown in **Fig. 2**.

#### LCHV detection in fish samples

Tissue tropism of LCHV was revealed using the sample batch 1. Among all 8 tissues (liver, kidney, spleen, gills, fin, brain, eyes and muscle) tested from 4 diseased barramundi, the qPCR assay detected LCHV DNA at variable loads in 3-7 tissue types of each fish but not in the kidney samples (**Table 1**). There was only 1 in 4 liver sample which tested positive for LCHV with low viral loads (32 copies/200 ng DNA). DNA extracted from the fin, gills and muscle had averagely higher LCHV loads (24-597 copies/200 ng DNA) when compared to that of the brain, eyes, spleen and liver (13.7-184 copies/200 ng DNA). LCHV was not detected in any of the 8 tissue types of a clinically healthy fish from the same batch (**Table 1**).



The established qPCR was also applied to diagnose field samples from batches 2 and 3. DNA from all five pools of clinically healthy fry from batch 2 tested negative for by LCHV (**Table 2**). In batch 3, LCHV was detected from 5 clinically sick fish with viral loads ranging from 18.7 to 115.9 copies per 200 ng DNA (Cq 36.84-34.04) and undetectable in 5 clinically healthy fish (**Table 2**).

## DISCUSSION

LCHV and SDDV infections reportedly cause similar gross sign of “scale drop” in infected barramundi (de Groof et al., 2015; Chang et al., 2017). Despite the fact that both SDDV and LCHV have been recently discovered, the “scale drop” syndrome has been recognized in Southeast Asia since 1992 (Gibson-Kueh et al., 2012; de Groof et al., 2015). Therefore, it has raised a concern that both of these pathogens may have been long undiagnosed in farmed barramundi due to unavailability of respective diagnostic tools at that time. Nevertheless, currently several molecular detection methods for SDDV are available to support disease investigation. However, following discovery of LCHV as an emerging virus in Singapore in 2017 (Chang et al., 2017), there was no continuous research up-to-date. Although several sets of primers were described in the original patent document by Chang et al. (2017), their detection limits and test specificity remain uninvestigated. The validated qPCR method developed in this study might serve as a useful diagnostic tool for rapid screening of the suspected cases as well as active surveillance and early monitoring of the pathogen for the barramundi aquaculture industry.

Detection of LCHV in multiple organs of the clinically sick fish suggests that the virus caused systemic infection, similar to that of SDDV (Senapin et al., 2019; Charoenwai et al., 2019; Sriisan et al., 2020). Interestingly, the liver and kidney tissues which are normally used for PCR diagnostics of fish viruses appeared to be unsuitable for LCHV detection while the

fin seemed to be the best targeted tissue due to its highest viral loads, followed by gills, muscle, spleen and brain. There was a limitation of fish numbers in this study, further comparative analysis should be done with larger sample numbers in order to gain a better understanding of virus tissue tropism as well as viral loads in the fish at different stages of infection. Nevertheless, this knowledge might be useful for establishment of cost-effective and non-destructive sampling strategies of fin and/or gills of farmed fish for periodical monitoring of the LCHV.

The present study focused primarily on the development and validation of a sensitive qPCR detection method for LCHV. Apart from LCHV, several pathogens have been reported to cause similar clinical signs of “scale drop” including SDDV (Gibson-Kueh et al., 2012; de Groof et al., 2015; Senapin et al., 2019), a pathogenic strain of *Vibrio harveyi*, and *Tenacibaculum maritimum* (Dong et al., 2017a; Gibson-Kueh et al., 2012). Relatively low viral loads present in the clinically sick fish with scale drop disease-like symptoms suggests that LCHV might be an opportunistic pathogen rather than the true causative agent of the diseased fish investigated in this study. However, identification of other pathogens in field samples was not done in this study. We thus recommend that investigation of the at least four aforementioned agents should be considered for the fish showing scale drop symptoms in order to weigh involvement of each pathogen in field outbreaks.

## Acknowledgements

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## Conflict of interest

206 The authors declare no conflict of interest.

## References

- Chang SF, Ng KS, Grisez L, De Groof A, Vogels W, Van Der Hoek L, Deijs M. 2017. Novel fish pathogenic virus. International patent no. WO 2018/029301 A1. USA: World Intellectual Property Organization.
- Charoenwai, O., Meemetta W., Sonthi, M., Dong, H.T., Senapin, S., 2019. A validated semi-nested PCR for rapid detection of scale drop disease virus (SDDV) in Asian sea bass (*Lates calcarifer*). J Virol Methods. 268, 37-41.
- Chen, J., Toh, X., Ong, J., Wang, Y., Teo, X. H., Lee, B., Wong, P. S., Khor, D., Chong, S. M., Chee, D., Wee, A., Wang, Y., Ng, M. K., Tan, B. H., Huangfu, T. (2019). Detection and characterization of a novel marine birnavirus isolated from Asian seabass in Singapore. Virology Journal, 16(1), 71.
- Dangtip, S., Kampeera, J., Suvannakad, R., Khumwan, P., Jaroenram, W., Sonthi, M., Senapin, S., Kiatpathomchai, W., 2019. Colorimetric detection of scale drop disease virus in Asian sea bass using loop-mediated isothermal amplification with xylenol orange. Aquaculture. 510, 386-391.
- 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., Taengphu, S., Sangsuriya, P., Charoensapsri, W., Phiwsaiya, K., Sornwatana, T., Khunrae, K., Rattanarojpong, T., Senapin, S. (2017a). 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., Jitrakorn, S., Kayansamruaj, P., Pirarat, N., Rodkhum, C., Rattanarojpong, T., Senapin, S., Saksmerprome, V. (2017b). Infectious spleen and kidney necrosis

disease (ISKND) outbreaks in farmed barramundi (*Lates calcarifer*) in Vietnam. Fish Shellfish Immunol., 68, 65-73.

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.

Hanson, L., Dishon, A., & Kotler, M. (2011). *Herpesviruses* that infect fish. Viruses, 3(11), 2160-2191.

Jerry, D.R. (2014) Biology and Culture of Asian Seabass *Lates calcarifer*. CRC Press, Boca Raton, FL, USA.

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<sup>Vp</sup> pirB<sup>Vp+</sup> mutant kills shrimp but produces neither Pir<sup>Vp</sup> toxins nor acute hepatopancreatic necrosis disease lesions. Appl. Environ. Microbiol. 83(16): e00680-00617.

Ransangan, J., & Manin, B. O. (2010). Mass mortality of hatchery-produced larvae of Asian seabass, *Lates calcarifer* (Bloch), associated with viral nervous necrosis in Sabah, Malaysia. Vet Microbiol. 145, 153-157.

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.

Sriisan, S., Boonchird, C., Thitamadee, S., Sonthi, M., Dong, H.T., Senapin, S. (2020) A sensitive and specific SYBR Green-based qPCR assay for detecting scale drop disease virus (SDDV) in Asian sea bass. Dis Aquat Organ. <https://doi.org/10.3354/dao03484>.

- 256 Toranzo, A. E., Magariños, B., & Romalde, J. L. (2005). A review of the main bacterial fish  
257 diseases in mariculture systems. *Aquaculture*, 246, 37-61.

## 258 Tables and Figures

259 **Table 1** LCHV loads in 8 different tissues from 5 barramundi samples in batch 1

Sample	Fish clinical status	Cq and LCHV load*/200 ng template)							
		Liver	Kidney	Spleen	Gills	Fin	Brain	Eyes	Muscle
1	“Scale drop”	ND	ND	33.33 [184]	35.91 [34.3]	34.35 [94.7]	34.4 [82.4]	ND	ND
2	“Scale drop”	ND	ND	36.05 [31.3]	32.39 [339]	34.26 [100]	ND	35.07 [59.3]	34.43 [89.9]
3	“Scale drop”	ND	ND	ND	ND	33.65 [149]	37.32 [13.7]	ND	36.46 [24.0]
4	“Scale drop”	36.02 [32]	ND	35.55 [43]	32.92 [240]	31.52 [597]	36.20 [28.4]	34.81 [70.2]	32.12 [404]
5	Healthy	ND	ND	ND	ND	ND	ND	ND	ND

260 \*Cq values are the above number while the LCHV loads are shown in [ ]. Grey highlights

261 samples with LCHV load more than 90 copies. ND, not detected

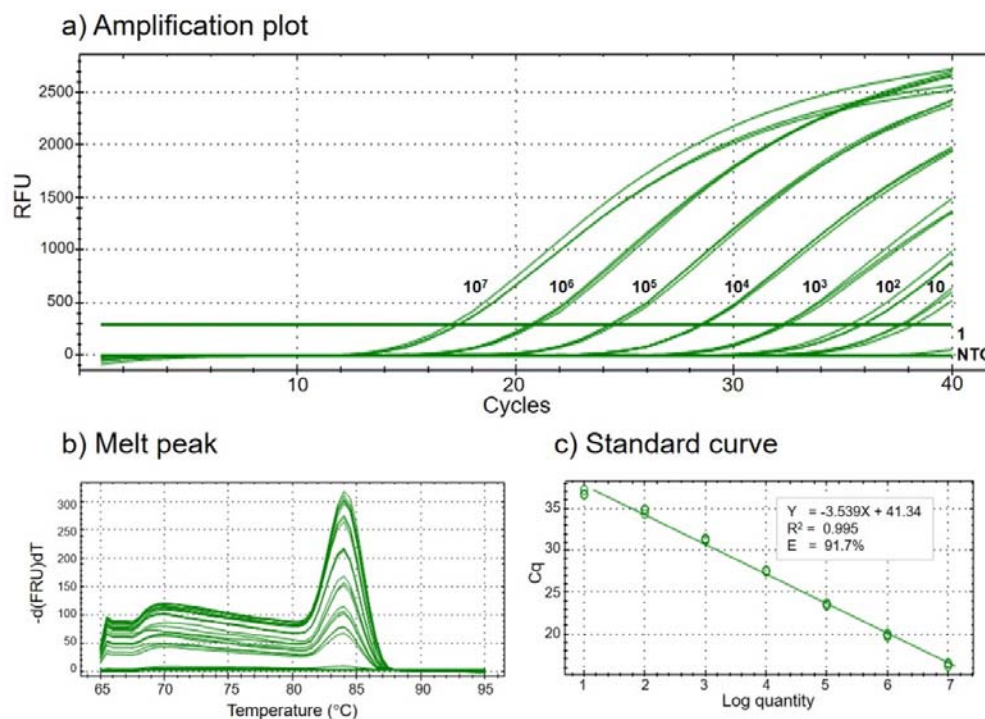
262

263 **Table 2** LCHV detection test results of samples from batches 2 and 3

Batch no.	Sample no.	Fish clinical status	Tested tissue	Cq	LCHV loads/200 ng DNA template
2 (fry)	Pool 1	Healthy	whole body	ND	Negative test
	Pool 2	Healthy	whole body	ND	Negative test
	Pool 3	Healthy	whole body	ND	Negative test
	Pool 4	Healthy	whole body	ND	Negative test
	Pool 5	Healthy	whole body	ND	Negative test
3 (juvenile)	Fish no. 1	Healthy	spleen	ND	Negative test
	Fish no. 2	Healthy	spleen	ND	Negative test
	Fish no. 3	Healthy	spleen	ND	Negative test
	Fish no. 4	Healthy	spleen	ND	Negative test
	Fish no. 5	Healthy	spleen	ND	Negative test
	Fish no. 6	“Scale drop”	spleen	35.39	48.2
	Fish no. 7	“Scale drop”	spleen	34.04	115.9
	Fish no. 8	“Scale drop”	spleen	35.48	45.4
	Fish no. 9	“Scale drop”	spleen	36.23	27.9
	Fish no. 10	“Scale drop”	spleen	36.84	18.7

264 ND, not detected





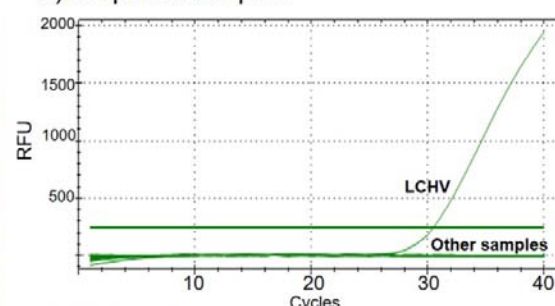
**Fig. 1** Performance and sensitivity of LCHV SBYR Green-based qPCR. (a) Amplification plots of positive control plasmid pMEP93 serial dilutions from  $10^7$  to 1 copies containing 100 ng spiked fish DNA in each reaction. Three technical replicates were done for each dilution. (b) Melt peak analysis of the products obtained in (a). (c) Standard curve derived by plotting Cq values versus  $\log_{10}$  pMEP93 concentrations. Formula for copy number calculation,  $R^2$  and E values are shown in the box.

# a) List of samples used in specificity test

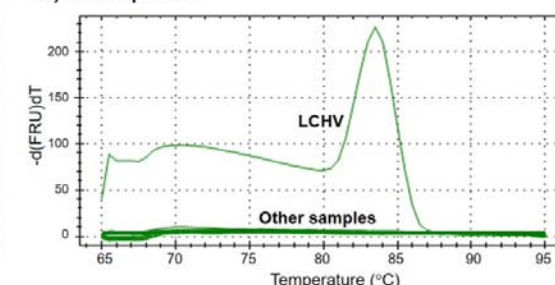
Pathogens/samples	Code	Source	Ave. Cq
<i>Streptococcus iniae</i>	VN2396	Laboratory strain	ND
<i>Vibrio harveyi</i>	SDMN-Y6	Dong et al. 2017a	ND
<i>Vibrio parahaemolyticus</i>	XN89	Phiwsaiya et al. 2017	ND
<i>Vibrio tubiashi</i>	SDMN-G4	Dong et al. 2017a	ND
<i>Vibrio vulnificus</i>	-	Laboratory strain	ND
<i>Vibrio alginolyticus</i>	-	Laboratory strain	ND
<i>Vibrio cholera</i>	NK8	Dong et al. 2015	ND
<i>Pleisomonas shigelloides</i>	NK10	Dong et al. 2015	ND
<i>Tenacibaculum litopenaei</i>	SDMN-T4	Dong et al. 2017a	ND
<i>Nocardia seriolae</i>	VN2391	Laboratory strain	ND
<i>Aeromonas hydrophila</i>	-	Laboratory strain	ND
<i>Aeromonas dhakensis</i>	-	Laboratory strain	ND
ISKNV infected tissue	-	Dong et al. 2017b	ND
NNV infected tissue	-	Laboratory sample	ND
SDDV infected tissue	-	Senapin et al. 2019	ND
LCHV infected tissue	-	This study	30.35
Healthy fish	-	Laboratory sample	ND
No template control	-	-	ND

ND, not detected

# b) Amplification plot



# c) Melt peak



**Fig. 2** Specificity test of LCHV SBYR Green-based qPCR. (a) DNA samples extracted from bacterial isolates and viral infected fish as well as control reactions (DNA from healthy fish and no template control) were used in the specificity assay. Average Cq values from technical replicates are shown. ND, not detected. (b) Amplification plots and (c) melt peak analysis of products from samples shown in table (a).

gtacttcatcgccctacggagcgctgggtcaccctgtacatcataaccaccatgggcctcacg  
Y F I A Y G A L V T L Y I I T T M G L T  
gacgtgacccctggacccctcctcaagcacacgta  
D V T L D L L K H T

**Supplemental Fig. 1** Nucleotide sequence of the LCHV qPCR target. Putative translated amino acid sequence is shown in capital alphabets. qPCR primers (double underlined) were designed to generate a 93-bp fragment of LCHV *MEP* gene. Compared to previously documented sequence (Chang et al. 2017), there is one silent mutation (gray highlighted) found in this study.