ORIGINAL ARTICLE

Detection of scale drop disease virus from non-destructive samples and ectoparasites of Asian sea bass, *Lates calcarifer*

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Abstract

Non-destructive sampling methods offer practical advantages to detection and monitoring of viral pathogens in economically important farmed fish and brood-stock. Here, we investigated whether blood, mucus and fin can be used as non-lethal sample sources for detection of *scale drop disease virus* (SDDV) in farmed Asian sea bass, *Lates calcarifer*. Detection of SDDV was performed in parallel from three non-destructive and seven destructive sample types, collected from both clinically sick fish and subclinical fish obtained from an affected farm. The results showed that SDDV was detectable in all 10 sample types with the percentage ranging from 20% to 100%. Blood was the best non-destructive sample source exhibited by the fact that it yielded 100% SDDV-positive tests from both sick ($n = 12$, 95% CI: 69.9–99.2) and clinically healthy fish ($n = 4$, 95% CI: 39.6%–97.4%) and is considered a "sterile" sample. This study also revealed concurrent infection of SDDV and two ectoparasites *Lernanthropus* sp. and *Diplectanum* sp., in all affected fish ($n = 8$, 95% CI: 46.7–99.3) during the disease outbreak. These ectoparasites also tested positive for SDDV by PCR, indicating that they were potential sample sources for PCR-based detection of SDDV and possibly other viruses infecting Asian sea bass.

KEYWORDS
carriers, detection, non-lethal samples, scale drop disease, SDDV

1 | INTRODUCTION

Scale drop disease virus (SDDV) is the causative agent of scale drop disease (SDD), a newly emerging disease of farmed Asian sea bass, *Lates calcarifer* in Singapore, Indonesia, Malaysia and Thailand (Gibson-Kueh et al., 2012; de Groof et al., 2015; Nurliyana et al., 2020; Senapin et al., 2019). SDDV is a double-stranded DNA virus, having an icosahedral shape (140–180 nm diameter), with a reported incomplete genome size of 124,244 bp (de Groof et al., 2015). The virus is currently classified as a novel *Megalocytivirus*, one of the five genera within the family *Iridoviridae*

(de Groof et al., 2015). The major capsid protein encoding gene of SDDV had a ~64%–65% nucleotide identity to other members in the same genus including infectious spleen and kidney necrosis virus (ISKNV), red seabream iridovirus (RSIV) and turbot reddish body iridovirus (TBIV) and 73.28% identity to a newly identified European chub iridovirus (de Groof et al., 2015; Halaly et al., 2019; Nurliyana et al., 2020; Senapin et al., 2019). Fish infected with SDDV reportedly exhibited loss of scale, darkened body, fin rot, tail erosion and sometimes accompanying signs of cloudy eyes and red belly. Mortality in natural disease outbreaks was reported up to 40%–50% in mainly subadult and adult fish, thus causing a

considerable economic loss to farmers (Gibson-Kueh et al., 2012; de Groof et al., 2015; Senapin et al., 2019). Several molecular diagnostic methods for SDDV are currently available including conventional single and semi-nested PCR (Charoenwai et al., 2019; Senapin et al., 2019), quantitative PCR (qPCR) (de Groof et al., 2015; Sriisan et al., 2020) and loop-mediated isothermal amplification (LAMP) (Dangtip et al., 2019). All aforementioned methods relied on destructive sampled fish tissues such as spleen, liver, kidney, brain, eyes and muscle. Lethal sampling approaches may not be desirable for a large sample size used in research and active surveillance programme or a smaller population size of valued broodstock and endangered animal species. Non-lethal sampling methods rather uphold animal welfare practice and have been considered for early screening of some viral infections in aquatic animals, especially for valued broodstock and ornamental fish. Previous studies reported detection of the virus in multiple organs (e.g. liver, kidney, spleen, gills, brain, eyes and fin), suggesting that SDDV caused systemic infection in Asian sea bass (Senapin et al., 2019; Charoenwai et al., 2019; Sriisan et al., 2020). Previously, Giray et al. (2005) reported blood as a reliable non-lethal sample type for the detection of infectious salmon anaemia virus (ISAV) in both moribund and asymptomatic salmon. Pectoral fin can be used for detecting infectious hematopoietic necrosis virus (IHNV) in rainbow trout (Dhar et al., 2008). Similarly, non-destructive sampling methods using blood and liver biopsy samples were used for detection of tilapia lake virus (TiLV) in subclinically infected tilapia, while mucus was used for virus detection in clinically sick fish by molecular diagnostic assays (Chiamkunakorn et al., 2019; Liamnimitr et al., 2018). Detection of viral haemorrhagic septicaemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV) from the leech parasites (*Myzobdella lugubris*, *Hemiclepsis marginata* and *Hirudo medicinalis*) indicated potential involvement of these ectoparasites in disease transmission (Faisal & Schulz, 2009; Salimi & Abdi, 2016). Other ectoparasites, *Argulus foliaceus* and *Piscicola geometra*, were reported as mechanical vectors of spring viraemia of carp virus (SVCV) in carp (Ahne, 1985). This suggests that common ectoparasites of Asian sea bass such as *Lernanthropus* sp. and *Diplectanum* sp. might be potential mechanical carriers of SDDV since they suck blood and mucus from the host. In this study, a comparison of destructive versus non-destructive sample sources for the PCR-based detection of SDDV in Asian sea bass was performed. Additionally, we determined two ectoparasites as mechanical carriers of the virus which are a potential source for non-lethal screening of SDDV by PCR in Asian sea bass.

2 | MATERIALS AND METHODS

2.1 | Animal ethic

The use of animals in this study was approved by Burapha University Animal Ethics Committee (BUU-IACUC001/2563).

2.2 | Fish samples collection

In 2019, during SDD outbreak in an open cage-cultured farm (Farm 1) located in an Eastern province of Thailand, clinically sick fish (n = 15) and apparently healthy fish (n = 5) (60–280 g body weight) were collected within the same cage. Two groups of fish were captured using different nets and then kept separately to avoid cross-contamination. Additionally, five apparently healthy Asian sea bass from another farm (Farm 2) with no history of SDD located in the same province were also included in the present study. Two sample collection schemes for each fish were performed by non-destructive sampling (mucus, caudal fin clip and blood) followed by destructive sampling (liver, kidney, spleen, gills, brain, eye and muscle). Prior to non-destructive sample collection, individual fish were anesthetized using clove oil in 10 L of sea water (80 ppm) for 5 min. Approximately 0.2 ml blood was collected from the caudal vein of each fish using a 21G-needle connected to a 3 ml-syringe. Collected blood was carefully transferred into a microtube containing anticoagulant heparin and kept at 4°C. Mucus (~0.1 ml) was then collected from the fish body surface by scraping along the lateral line from cranial to caudal fin using a sterile microscope slide, while caudal fin (~0.1 g) was collected by cutting using a sterile scissor. The non-destructive samples were kept on ice and transported to the laboratory for further analysis. Prior to destructive sample collection, the fish were humanly killed using an overdose of clove oil (250 ppm). Each fish was aseptically necropsied, and different tissue types (liver, kidney, spleen, gills, brain, eye and muscle) were collected and preserved in 95% ethanol for PCR analysis.

2.3 | Gill parasite examination and preservation

After fish euthanasia, gill filaments from the individual fish (n = 20) collected from the disease affected Farm 1 were kept in 0.85% NaCl for parasite observation and count. *Lernanthropus* sp. was easily observed by naked eyes (Abdul Khalid & Shaharoum-Harrison, 2014; Chu et al., 2012), while examination for monogenean *Diplectanum* sp. was done under a light microscope (Figure S1). Parasites were classified into genus based on their morphology according to González-Lanza et al. (1991) and Bu et al. (1999). For SDDV detection, two pooled samples of parasites collected from each fish, *Lernanthropus* sp. (3–5 individuals per pool) and *Diplectanum* sp. (13–200 individuals per pool), were preserved in 95% ethanol until subsequent process.

2.4 | DNA extraction

The conventional phenol–chloroform DNA extraction method was applied for seven destructive sample types (liver, kidney, spleen, gills brain, eye and muscle), caudal fin and parasites (entire body), while

TABLE 1 Comparative detection results of SDDV in destructive and non-destructive samples from clinically sick and healthy fish collected from 2 farms

	Destructive sampling							Non-destructive sampling		
	Liver	Kidney	Spleen	Gill	Brain	Eye	Muscle	Blood	Mucus	Fin
Farm 1 (affected farm)										
Clinically sick fish										
Infection prevalence (95% CI)	86.7 (58.4–97.7)	100 (74.7–99.4)	93.3 (66.0–99.6)	100 (74.7–99.4)	93.3 (66.0–99.6)	73.3 (44.8–91.1)	93.3 (66.0–99.6)	100 (69.9–99.2)	100 (56.1–98.7)	73.3 (44.8–91.1)
n	15	15	15	15	15	15	15	12	7	12
Subclinical fish										
Infection prevalence (95% CI)	60 (17.1–92.7)	60 (17.1–92.7)	60 (17.1–92.7)	60 (17.1–92.7)	60 (17.1–92.7)	20 (1.1–70.1)	100 (46.3–98.1)	100 (39.6–97.6)	100 (39.6–97.6)	80 (29.9–98.9)
n	5	5	5	5	5	5	5	4	4	5
Farm 2 (non-affected farm)										
Healthy fish										
Infection prevalence (95% CI)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)	0 (1.9–53.7)
n	5	5	5	5	5	5	5	5	5	5

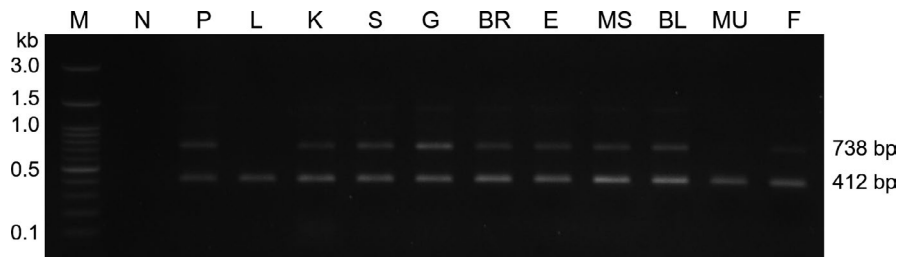Abbreviation: SDDV, scale drop disease virus.

FIGURE 1 Representative detection of SDDV from an individual sick fish from Farm 1. M, 3 kb DNA ladder; N, no template negative control; P, positive control using DNA extracted from SDDV-infected fish as template, liver (L), kidney (K), spleen (S), gill (G), brain (BR), eye (E), muscle (MS), blood (BL), mucus (MU) and fin (F). SDDV, scale drop disease virus

a commercial kit (High Pure Viral Roche Kit) was used for blood and mucus samples. DNA concentration and purity were measured using a spectrophotometer at 260 and 280 nm, and the concentration was then adjusted to 100 ng/μl for PCR tests.

2.5 | Detection of SDDV by PCR

Semi-nested PCR (Charoenwai et al., 2019) was employed for SDDV detection in both destructive and non-destructive sample types as well as the parasites. The method has a detection limit of 100 copies per μL template and highly specific for SDDV. PCR mixture and thermocycling condition were performed as previously described by Charoenwai et al. (2019). DNA extracted from a confirmed SDDV-infected fish (Senapin et al., 2019) was used as a positive control, while distilled water without DNA template was used as a negative control. Expected PCR products for the first and second rounds of PCR were 738 bp and 412 bp, respectively. The presence of two bands indicated heavy infection (++), while an appearance of only 412 bp band was considered light infection (+). PCR products were analysed by 1.2% agarose gel, stained with SERVA Green (Serving Scientists, German) and photographed under UV light.

2.6 | Application of blood sample for SDDV detection

Blood sample was identified as the best non-destructive sample type for PCR detection of SDDV. To validate that blood sample can be used for non-destructive detection of SDDV by PCR in field samples, apparently healthy Asian sea bass (n = 20) were collected from the same previously affected Farm 1, 13 months after the disease outbreak. The blood samples were non-lethally collected from the caudal vein of each fish in the same manner as mentioned above and subjected to DNA extraction and SDDV diagnosis by semi-nested PCR (Charoenwai et al., 2019). All fish were alive after sample collection. In order to confirm that the positive sample(s) was truly positive, the positive sample(s) was then assayed with the SYBR Green quantitative polymerase chain reaction (qPCR) method according to Sriisan et al. (2020).

2.7 | Statistical analysis

Per cent prevalence of SDDV infection was analysed using the EpiCalc 2000 program at 95% confidence interval.

3 | RESULTS

3.1 | Comparative SDDV detection results from destructive and non-destructive sample types

Polymerase chain reaction detection results for both destructive and non-destructive sample types are shown in Table 1. The 10 tested tissue types of clinically diseased and healthy fish yielded different percentages of SDDV-positive test results. From Farm 1 (affected farm), diseased fish showed loss of scale, dark body, fin and tail erosion, exophthalmos and mortality reaching approximately 40%–50%. Among 15 clinically sick fish showing typical signs of "scale drop," 15/15 (95% CI: 74.4–99.4) kidney and gills tested positive for SDDV, while 11/15 to 14/15 (73.3%–93.3%) of other tested destructive sample types yielded positive tests. The kidney and gills are the best destructive sample types for detection of SDDV from clinically sick fish (n = 15, 95% CI: 74.4–99.4). Three non-destructive sample types showed SDDV positive in 100% (n = 12, 95% CI: 69.9–99.2), 100% (n = 7, 95% CI: 56.1–98.7) and 73.3% (n = 15, 95% CI: 44.8–91.1) samples from blood, mucus and fin, respectively (Table 1). It should be noted that, due to technical issues, we were unable to collect some blood and mucus samples as planned (i.e. rapid clotting of blood after death, loss of mucus on some diseased fish making volume of samples to not be sufficient for DNA extraction) resulting in different number of each sample type. Figure 1 demonstrates semi-nested PCR detection results of SDDV from sick fish number 13. With respect to samples derived from the clinically healthy fish collected from the same Farm 1, 100% (n = 5, 95% CI: 46.3–98.1) muscle samples were SDDV-positive, while the other lethal sample types gave 20% (n = 5, 95% CI: 1.1–70.1) to 60% (n = 5, 95% CI: 17.1–92.7) positive results (Table 1). Detection results for non-destructive sample types were 4/4 for blood and mucus while 4/5 for fin samples (Table 1). All destructive and non-destructive sample types collected from five clinically healthy fish from Farm 2 were negative for SDDV (Table 1). Taken together, the results indicated

that three non-destructive sample types (blood, mucus and caudal fin) are feasible for non-lethal detection of SDDV in both clinically sick and subclinical Asian sea bass. Among three types of non-destructive samples, blood was the best giving 100% ($n = 16$, 95% CI: 75.9–99.4) positive test results.

3.2 | Detection of SDDV from non-lethal blood samples

Among 20 blood samples collected from another batch of apparently healthy Asian sea bass using non-lethal means, 5% samples ($n = 20$, 95% CI: 0.3–26.9) tested positive for SDDV. The positive sample resulted in a single band of 412 bp, indicating low viral loads or light infection (figure not shown). The positive sample was also confimed by SDDV qPCR assay (122 copies/200 ng DNA).

3.3 | Concurrent infections of SDDV and two ectoparasites in Asian sea bass

Gill parasite examination revealed that all 20 SDDV-infected sea bass from an outbreak in Farm 1 were also infested with two

TABLE 2 Comparative parasitic infestation and SDDV detection results from the parasites

	Lernanthropus sp.	*Diplectanum* sp.
Farm 1 (affected farm)		
Clinically sick fish		
Parasite infection prevalence (95% CI)	100 (74.7–99.4) ($n = 15$)	100 (74.7–99.4) ($n = 15$)
Number of parasites per fish (mean ± *SD*)	5 ± 4.3	154 ± 165.6
SDDV infection prevalence (95% CI)	87.5 (46.7–99.3) ($n = 8$)	100 (74.7–99.4) ($n = 15$)
Subclinical fish		
Parasite infection prevalence (95% CI)	100 (46.3–98.1) ($n = 5$)	100 (48.3–98.1) ($n = 5$)
Number of parasites per fish (mean ± *SD*)	4 ± 3.9	89 ± 17.1
SDDV infection prevalence (95% CI)	40 (7.3–82.9) ($n = 5$)	60 (17.0–92.7) ($n = 5$)
Farm 2 (non-affected farm)		
Healthy fish		
Parasite infection prevalence (95% CI)	20 (1.1–70.1) ($n = 5$)	100 (46.3–98.1) ($n = 5$)
Number of parasites per fish (mean ± *SD*)	2 ± 1.4	110 ± 32.4
SDDV infection prevalence (95% CI)	0 (4.9–80.2) ($n = 2$)	0 (1.9–53.7) ($n = 5$)

Abbreviation: SDDV, scale drop disease virus.

ectoparasites (Table 2), indicating triple infections. The crustacean parasite was morphologically identical to *Lernanthropus* sp., while the monogenean parasite was morphologically similar to that of *Diplectanum* sp. (Figure 2). *Lernanthropus* sp. were visible to the naked eyes with length of ~ 5–6 mm. Under microscopy, unique features of *Lernanthropus* sp. were their thoracic legs, the shape of the dorsal plate and the female two egg strings (Figure 2a) (Abdul Khalid & Shaharoum-Harrison, 2014; Chu et al., 2012). The examined *Diplectanum* sp. had three pairs of head organs, two pairs of pigmented eyespots, haptor with two pairs of hamuli and two squamodiscs (one on ventral and another on the dorsal surface of haptor) (Figure 2b) (Bu et al., 1999; González-Lanza et al., 1991; Wu et al., 2005). Prevalence of the two parasites was 100% in all 20 fish examined from Farm 1 (Table 2). The mean intensity of infections in clinically sick fish and subclinical fish was 5 ± 4.3 and 4 ± 3.9, respectively, for *Lernanthropus* sp. and 154 ± 165.6 and 89 ± 17.1, respectively, for *Diplectanum* sp. The mean intensity of infections in apparently healthy fish from non-affected farm (Farm 2) was 2 ± 1.4 for *Lernanthropus* sp. and 110 ± 32.4 for *Diplectanum* sp. (Table 2).

3.4 | Detection of SDDV from ectoparasite

Semi-nested PCR detection of SDDV in the crustacean parasite *Lernanthropus* sp. revealed that 7/8 (95% CI: 46.7–99.3) pools from clinically sick fish and 2/5 (95% CI: 7.3–82.9) pools from subclinical fish tested positive for SDDV (Table 2). For gill monogenean *Diplectanum* sp., 15/15 (95% CI: 74.7–99.4) and 3/5 (95% CI: 17.0–92.7) pools were tested positive, respectively (Table 2). Majorities of positive samples from clinically sick fish resulted in two bands (738 bp and 412 bp), indicating high viral loads while only a single band of 412 bp that was seen from samples collected from subclinical fish indicated low viral loads (Figure 3). The parasites from Farm 2 tested negative for SDDV (Table 2).

4 | DISCUSSION

The present study demonstrated that non-destructive sample sources, for example blood, mucus and caudal fin, are feasible for detection of SDDV in farmed Asian sea bass. The use of these sample types might be useful and cost-effective for early screening of SDDV in relatively expensive broodstock or periodical monitoring of the pathogen in grow-out farms. Nonetheless, blood sample might be a better non-destructive sample source for SDDV detection than the mucus and fin, as it may reduce the risk of contamination from external environment.

Detection of SDDV in multiple types of samples (seven destructive and three non-destructive) implied that SDDV infection is systemic during a clinical disease outbreak as previously suggested (Sriisan et al., 2020). Moreover, the presence of the virus in blood and mucus of all tested samples suggested that infected fish (both clinically sick and subclinical) from a disease outbreak can shed the
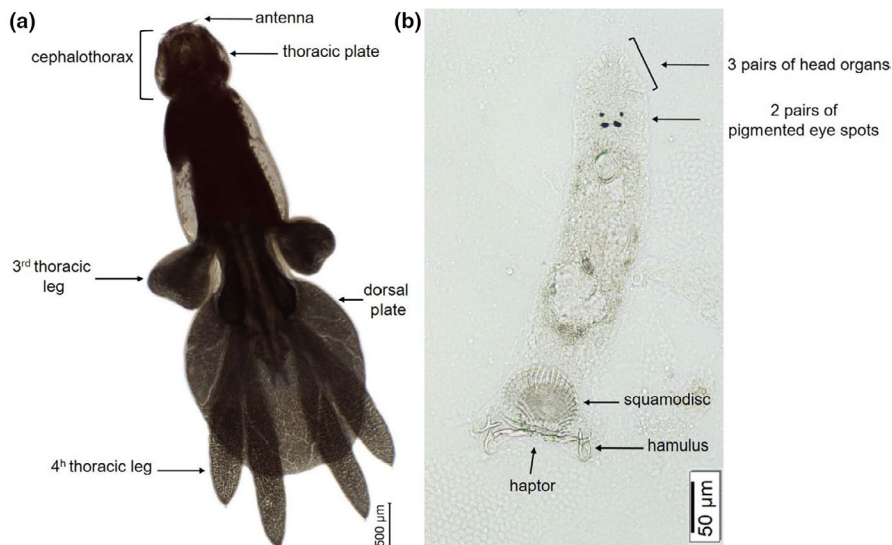
FIGURE 2 Characteristics of ectoparasites observed under light microscopy. (a) Ventral view of female *Lernanthropus* sp. without the egg strings revealing its characteristic thoracic legs and dorsal plate. (b) Ventral view of *Diplectanum* sp. showing body elongate, two pairs of eye spots, two squamodiscs and haptor with two pairs of hamuli
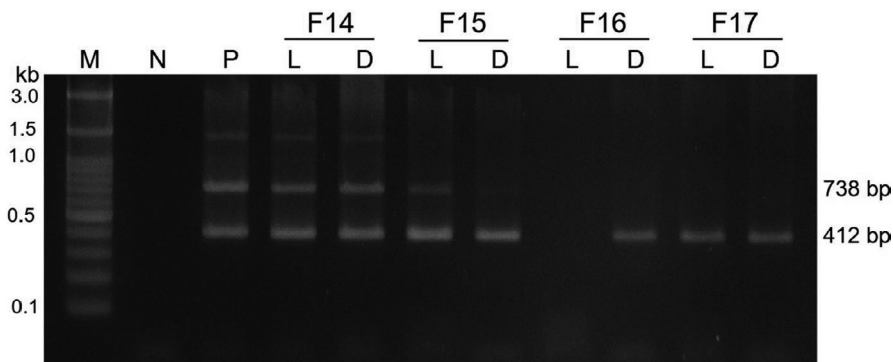

FIGURE 3 Representative SDDV detection results of ectoparasite samples using semi-nested PCR. M, 100 bp DNA ladder; N, no template negative control; P, positive control using DNA extracted from SDDV-infected fish as template. Parasites (L, *Lernanthropus* sp. and D, *Diplectanum* sp.) were collected from fish numbers 14–17 (F14–F17) from Farm 1.

virus to both systemic and mucosal systems similar to that of other viruses in finfish such as TiLV in tilapia (Chiamkunakorn et al., 2019; Liamnimitr et al., 2018) and ISAV in salmon (Aamelfot et al., 2015; Giray et al., 2005). The active viruses in the mucus might likely be able to be released into the cultured water and horizontally transmitted to other fish in the same population through direct contact or ingestion. If this is the case, assessment of viral loads in cultured water might be useful for SDDV detection and for early forecasts of the disease outbreaks (Kawato et al., 2016; Løvdal & Enger, 2002).

Even though *Lernanthropus* sp. and *Diplectanum* sp. are common parasites of Asian sea bass (Chu et al., 2012; Khrukhayan et al., 2016; Sonthi et al., 2016), concurrent infections of SDDV and two parasites *Lernanthropus* sp. and *Diplectanum* sp. were firstly revealed in this study. Detection of SDDV from parasites with relatively high viral load (indicated by presence of band products from semi-nested PCR) suggests that these two parasites are potentially mechanical carriers of the virus. Similarly, previous studies reported detection of VHSV and IPNV from the leech parasites (Faisal & Schulz, 2009; Salimi & Abdi, 2016). Nevertheless, the current study might suggest a novel approach of using parasites as one type of non-destructive sample for indirect monitoring of SDDV in Asian sea bass. The use of *Lernanthropus* sp. for such purpose might be more practical given the fact that it is a relatively larger parasite. Further studies should investigate on

prevalence of coinfections and the role ectoparasites play in fish viruses' transmission.

In conclusion, the present study explored different types of destructive and non-destructive samples for PCR diagnosis of SDDV in Asian sea bass. The results revealed that blood and mucus were considered the best sample sources for this purpose. Concurrent infestation of SDDV and two gill parasites were firstly detected. Interestingly, both parasites were potentially mechanical carriers of SDDV which can be considered as potential sources of samples for indirect diagnosis of SDDV and possibly other viruses infecting Asian sea bass.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Charoenwai O, Senapin S, Dong HT, Sonthi M. Detection of scale drop disease virus from non-destructive samples and ectoparasites of Asian sea bass, *Lates calcarifer*. J Fish Dis. 2020;00:1–7. https://doi.org/10.1111/jfd.13290