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Article

Susceptibility of *Exopalaemon carinicauda* to the infection with Shrimp hemocyte iridescent virus

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Abstract: In this study, ridgetail white prawns *Exopalaemon carinicauda* were infected *per os* with debris of Shrimp hemocyte iridescent virus (SHIV)-infected *Penaeus vannamei* and via intramuscular injection (im) with raw extracts of SHIV. The infected *E. carinicauda* showed obvious clinical symptoms, including weakness, empty gut and stomach, pale hepatopancreas, and partial death with cumulative mortality of (50.0±26.5)% and (76.7±18.3)%, respectively. Results of TaqMan probe based real-time quantitative PCR showed that the moribund and survival individuals with clinical signs of infected *E. carinicauda* were SHIV-positive. Histological examination showed that there were dark eosinophilic inclusions, of which some were surrounded with or contained tiny basophilic staining, and pyknosis in cytoplasm of hemocytes in the hepatopancreatic sinus, hematopoietic cells, and cuticular epithelium, etc. Positive hybridization signals were observed in stomach, hematopoietic tissue, cuticular epithelium, and hepatopancreatic sinus of infected prawns from both *per os* and im groups, according to the results of *in situ* DIG-labeling-loop-mediated DNA Amplification (ISDL). Transmission electron microscopy of ultrathin sections showed that icosahedral SHIV particles existed in hepatopancreatic sinus and gills of the infected *E. carinicauda* of the *per os* group. The viral particles were also observed in the hepatopancreatic sinus, gills, pereiopods, muscles, and uropods of the infected *E. carinicauda* from the im group. The assembled virions mostly distributed outside of the assembling area near cellular membrane of infected cells, which were with envelope and about 150 nm in diagonal diameter. The results of molecular biological tests, histopathological examination, ISDL, and transmission electron microscopy confirmed that *E. carinicauda* is one of the susceptible hosts of SHIV. This study also reminded that *E. carinicauda* showed some degree of tolerance to the infection with SHIV *per os* challenge mimicking natural pathway.

Keywords: Shrimp hemocyte iridescent virus; *Exopalaemon carinicauda*; susceptibility; host; ISDL

1. Introduction

The iridescent virus family *Iridoviridae* contains large icosahedral double-stranded DNA viruses, which is divided into two subfamilies (i.e., *Alphairidovirinae* and *Betairidovirinae*) and composed of five known genera: *Lymphocystivirus*, *Megalocytivirus*, *Ranavirus*, *Chloriridovirus*, and *Iridovirus*. Of them, the genera *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* belong to *Alphairidovirinae*, and

Chloriridovirus and *Iridovirus* belong to *Betairidovirinae* [1-3]. Iridescent viruses of *Alphairidovirinae* lead a high mortality rate in significant fish and amphibians [3], While iridescent viruses *Betairidovirinae* infect insects and crustaceans. The first discovery of a possible iridescent virus in crustaceans was published in 1993 [4]. In the same year Lightner and Redman reported the observation of another suspected iridescent virus in Penaeid shrimp *Protrachypene precipua* [5]. Subsequently, Miao et al. reported that a suspected iridescent virus observed in lymphoid cell cultures on *Penaeus chinensis* in 1999 [6]. Years later, Tang et al. (2007) identified an iridovirus, i.e., Sergestid iridovirus (SIV), from diseased sergestid shrimp *Acetes erythraeus*, which is a likely causative agent, evidenced through *in situ* hybridization and PCR test [7]. Xu et al. (2016) reported *Cherax quadricarinatus* iridovirus (CQIV) isolated from diseased red claw crayfish *Cherax quadricarinatus* [8]. Qiu et al (2017) identified an iridescent virus named Shrimp hemocyte iridescent virus (SHIV), which isolated from farmed *Penaeus vannamei* in 2014 and also detected in *P. chinensis* and *Macrobrachium rosenbergii* [9].

The range of susceptible hosts is an important part of epidemiology and pathogen ecology. It is also highly concerned for international trade and control of the disease. The World Organisation for Animal Health (OIE) has adopted a chapter in "The Aquatic Animal Health Code" to provide criteria for determining which species are listed as susceptible in 2014 with emphasis that the route of transmission is consistent with natural pathways for the infection [10]. Ridgetail white prawn *Exopalaemon carinicauda* is one of major economic crustaceans in China, which could propagate throughout all the year round, naturally distributes in the coast of Yellow Sea and Bohai Sea of China [11,12]. *E. carinicauda* accounts for one third of the total yields of polyculture ponds in the eastern China [11]. It is also a species of wild prawn and commonly exists in the ponds farming *M. rosenbergii* or penaeid shrimp. To date, there is no report pointing out that *E. carinicauda* can be infected with iridescent viruses. Owing to the common existence in intensive prawn farming ponds, it is important for precaution of the spreading risk of SHIV, which is based on assessing the susceptibility of *E. carinicauda* to SHIV and evaluating SHIV potential transmission routes and possible reservoirs in the nature. In this study, *E. carinicauda* were challenged *per os* to mimic natural infection according to the OIE standards, while intramuscular injection was used as a positive control. A combination of TaqMan real-time PCR, histopathological observation, *in situ* DIG-labeling-loop-mediated DNA amplification (ISDL) and ultra-thin transmission electron microscopy were used to confirm the infection with SHIV in *E. carinicauda*.

2. Materials and Methods

2.1. Animals

Specific-pathogen-free (SPF) ridgetail white prawns *E. carinicauda*, (7.2±0.5) cm in body length, were reared and bred in the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences. Prawns *E. carinicauda* were cultivated in a 40 L plastic tanks containing 20 L seawater of 30 ppt in salinity with 90% daily exchange rate, at 27 °C water temperature, with continuous aeration, and fed three times per day with the formula feed for a week in our wet lab. For multiplication of SHIV, 30 healthy white leg shrimp *P. vannamei* of (10.2±0.8) cm in body length were purchased from a shrimp farm in Weifang of Shandong Province and then held in 40 L of 30 ppt salinity seawater with same management above mentioned.

Before challenge test, the prawns and the shrimp were sampled and detected for potential pathogens, including White spot syndrome virus (WSSV), Taura syndrome virus (TSV), Yellow head virus (YHV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), and acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (*Vp*$_{AHPND}$), using the nested PCR or TaqMan probe based quantitative real-time PCR (TaqMan qPCR) methods [13], Covert mortality nodavirus (CMNV) using TaqMan qPCR methods [14], and SHIV using nested PCR [15].

2.2. Preparation of Viral Inoculum

For preparation of material for challenge test, 30 healthy shrimp *P. vannamei* were infected by feeding with SHIV (strain 20141215) infected tissue, which was derived from diseased *P. vannamei*

collected in a farm in Zhejiang Province in Dec. 2014 [9]. After 5 days post-infection, 2.5 g cephalothoraxes (removing tergites) were taken from SHIV-infected shrimp and subsequently homogenized in 40 mL pre-cooled PPB-Tris (376.07 mM NaCl, 6.32 mM K_2SO4, 6.4 mM $MgSO_4$, 14.41 mM $CaCl_2$, and 50 mM Tris-HCl, pH 6.5–8.0) [16]. The suspension was centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet was resuspended in 25 mL PPB-Tris by homogenization and re-centrifuged. The supernatants from two steps were merged and filtered through a 500-mesh sieve and a 0.45 μm filter. SHIV dose in the suspension was quantified using TaqMan qPCR method [15]. For challenge test via intramuscular injection, an inoculum containing 10^4 copies/μL SHIV was prepared by diluting the suspension with PPB-His (replace 50 mM Tris-HCl with 50 mM Histidine in PPB-Tris) [16], and then the inoculum was dispensed in 100 μL aliquots before stored at −80 °C.

2.3. Challenge Tests

Challenge tests were performed with healthy *E. carinicauda* in 4 groups, including intramuscular injection (IM), *per os* (PO), control of intramuscular infection (CIM), and control of *per os* (CPO). Each group had 3 replicates with 10 prawn individuals per replicate. Each prawn in the IM group was injected 10 μL of viral inoculum (~10^5 copies). Prawns in the PO group were fed with total 3 g debris of SHIV-infected *P. vannamei* tissue (with a viral dose about 10^{10} copies/g). Prawns in the CIM group and the CPO group were injected with 10 μL sterile PPB-His and commercial formula feed, respectively. During the test period, the rearing conditions were kept as same as that prior to challenge.

Significance analysis of cumulative mortalities between each two groups was carried out using the t-test for homoscedasticity hypothesis of two group samples with the add-in tool of Data Analysis in Microsoft® Excel® 2016 MSO 64-bit.

For evaluation of the pathogenic model in the infection groups, mortality data were nonlinearly regressed following 3-parameter sigmoid equation: $M_{(t)} = \frac{a}{1+e^{-k(t-t_0)}}$, of which $M_{(t)}$ is the cumulative mortality at the time (day post infection); t is the time (day post infection); a is the maximum mortality; t_0 is the day post infection to the half of the maximum mortality; k is the instant incidence rate. The real pathogenic model shall deduct the average mortality of control groups.

2.4. Detection of SHIV Using TaqMan Probe Based Real-Time Quantitative PCR

Total DNA were extracted from the hepatopancreas of samples stored at -20 °C using a TIANamp Marine Animals DNA Kit (Tiangen, Beijing, China), respectively. TaqMan qPCR, using the total DNA samples as templates, was conducted as described previously [15] to detect and quantify SHIV. The forward and reverse primers were SHIV-F 5'-AGG AGA GGG AAA TAA CGG GAA AAC-3' and SHIV-R 5'-CGT CAG CAT TTGGTT CAT CCA TG-3', respectively. The TaqMan probe (5'-CTG CCC ATC TAA CAC CAT CTC CCG CCC-3') was labeled with 5'-6-carboxyfluorescein (FAM) and 3'-TAMRA. Each PCR mixture in 20 μL contained 10 μL 2× FastStart Essential DNA Probes Master (Roche, Indianapolis, IN, USA), 500 nM primer each, 200 nM probe,100 ng total DNA template. The program was initial denaturation at 95 °C for 10 min, following 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The amplification and date analysis were carried out in a CFX-96 Quantitative Fluorescence Instrument (BioRad, USA).

2.5. Histopathological Examination

The cephalothoraxes of *E. carinicauda*, sampled during the challenge tests, were fixed with Davison's AFA fixative (DAFA) for 24 h and processed for sectioning and hematoxylin and eosin (H&E) staining as described by Bell & Lightner [17]. The histological sections were analyzed and photographed under a light microscopy system (Eclipse 80i, Nikon, Japan).

2.6. Loop-mediated Isothermal Amplification (LAMP)

A set of specific primers, composed of FIP, BIP, LF, LB, F3, and B3, for LAMP detection of SHIV was designed targeting the gene of DNA-directed RNA polymerase II second largest subunit of SHIV

genomic sequence (GenBank accession No. MF599468), using PrimerExplorerV4 (http://primerexplorer.jp/elamp4.0.0/index.html). These primers were searched against the database in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to analyze the sequence similarities. The primers were synthesized by Sangon Biotech (Shanghai, China). Each LAMP mixture contained 1.6 μM each of inner primers FIP and BIP, 0.8 μM each of loop primers LF and LB, 0.2 μM each of outer primers F3 and B3, 1.4 mM of dNTP mix (TaKaRa, Dalian, China), 1.2 M betaine (Solarbio, Beijing, China), 25 μM calcein (Sigma, USA), 500 μM $MnCl_2$, 6 mM $MgCl_2$, 8 U Bst 2.0 DNA polymerase (New England Biolabs Inc., Beverly, USA), 1× supplied buffer and the specified amount of template DNA in a final volume of 25 μL. The procedure was 60 cycles for 60 °C, following 5 minutes at 85 °C on the CFX-96 Quantitative Fluorescence Instrument (BioRad, USA) using calcein fluorescent channel. Detection specificity of the LAMP primers were examined using 100 ng of total DNA extracted from uninfected prawns and shrimp infected with other pathogens, including WSSV, *Vp*$_{AHPND}$, IHHNV, and EHP.

2.7. In situ DIG-labeling-loop-mediated DNA Amplification (ISDL)

ISDL followed the method published by Jitrakorn et al. [18] with some modification to target SHIV. Paraffin sections were dewaxed and rehydrated according to the normal DIG-labeled *in situ* hybridization method [17-19]. Rehydrated slides were added with ddH_2O and denatured on a 100 °C heating block for 2 min, then subsequently placed in a wet box. Total 150 μL LAMP mixture, of which the components are as same as 2.6, above except that the dNTP mix was supplemented with 0.1 mM digoxigenin-11-dUTP (DIG-labeled dUTP) and without calcein and $MnCl_2$, were added dropwise to each slide. The mixture was incubated at 65 °C for 60 min, followed by 85 °C for 5 min. Subsequent steps were performed in accordance with a normal *in situ* hybridization [19]. Tissue sections of healthy prawns were used as the control.

2.8. Ultrathin Transmission Electron Microscopy

For transmission electron microscopy with ultrathin sections, samples were prepared as previous described [20,21]. Briefly, tissues of hepatopancreas, muscle, pereiopods, uropods, and gills of infected *E. carinicauda* were placed in TEM fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 160 mM NaCl and 4 mM $CaCl_2$ in 200 mM PBS, pH 7.2) and cut rapidly into ~ 1 mm^3 pieces with scalpels, fixed for 1 h at room temperature, and then post-fixed with OsO_4. Specimens were embedded in Spurr's plastic and dyed with uranyl acetate and lead citrate. Ultrathin sections were prepared on collodion-coated grids by the Equipment Center of the Medical College, Qingdao University (Qingdao, China). All grids were examined under a JEM-1200 electron microscope (JEOL, Japan) operating at 80–100 kV.

3. Results

3.1. Clinical Signs and Cumulative Mortality

The challenge test lasted for 15 days. After 3 days post infection (dpi), prawns in both intramuscular (IM) and *per os* (PO) groups were clinically infected, which displayed empty stomach and gut and pale hepatopancreas, the hematopoietic tissue under the base of rostrum showed slight white color (Figure 1, IM and PO); while prawns in the control groups (i.e., CPO and CIM) displayed normal gross signs, including feed-filled stomach and gut and light brown hepatopancreas, but the hematopoietic tissue was not visible (Figure 1, CPO).

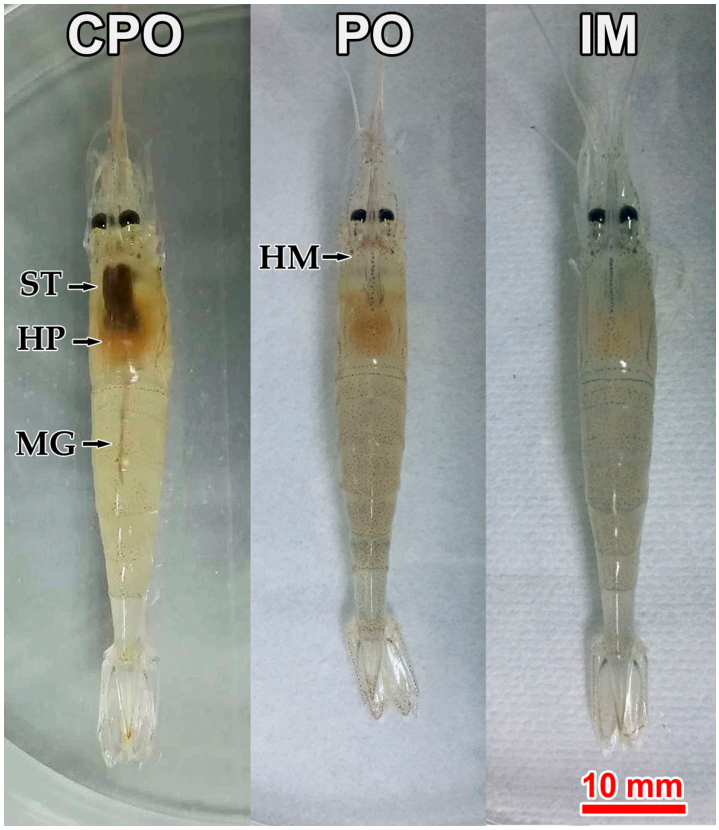

Figure 1. Gross signs of prawns *Exopalaemon carinicauda* in different groups of the challenge test. **CPO**: Prawn in *per os* control group; **PO**: Prawn in *per os* group; **IM**: Prawn in intermuscular injection group. Solid arrows indicate stomach (ST), hepatopancreas (HP), midgut (MG), and hematopoietic tissue (HM). Red bar = 10 mm.

Prawns in IM and PO groups suffered from rapid increasing of mortalities during the period between 2 dpi and 5 dpi. The average cumulative mortalities stabilized at (76.7±18.3)% and (50.0±26.5)% in the IM group after 5 dpi and PO group after 10 dpi, respectively, which were both significantly higher ($P < 0.01$) than that of CIM and CPO groups. Additionally, the cumulative mortality of the IM group was significantly higher ($P < 0.05$) than that of the PO group at 3 dpi and 4 dpi (Figure 2). Significance analysis of the overall data of four groups showed very significant differences among IM, PO, and control groups ($P<0.01$).

Based on the nonlinear regression following the 3-parameter sigmoid equation, the pathogenic model of infection with SHIV on *E. carinicauda* via im and *per os* were $M_{IM(t)} = \frac{70.77\pm0.21}{1+e^{-(4.76\pm2.49)[t-(2.64\pm0.21)]}}$ and $M_{PO(t)} = \frac{42.52\pm1.22}{1+e^{-(0.82\pm0.51)[t-(3.83\pm0.87)]}}$, respectively. The functions indicated the infection of *E. carinicauda* with SHIV via im caused (70.77±0.21)% accumulative mortality, while the infection *per os* caused (42.52±1.22)% accumulative mortality. The former reached the half level of accumulative mortality in (2.64±0.21) dpi with an instant incidence rate at (4.76±2.49)/day; and the latter did in (3.83±0.87) dpi with an instant rate at (0.82±0.51)/day.

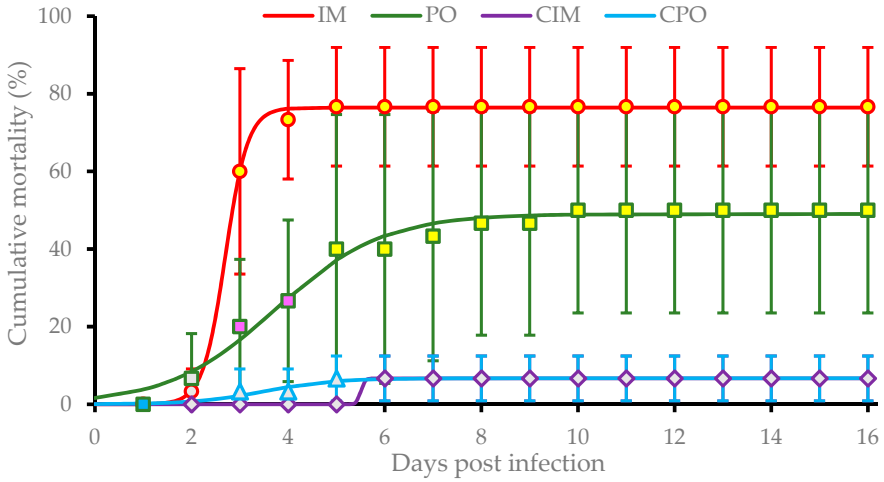

Figure 2. Cumulative mortalities of *Exopalaemon carinicauda* in the challenge test. IM group, prawns were challenged with filtrated viral suspension via intermuscular injection; PO group, prawns were fed with tissues of SHIV-infected *P. vannamei*; CIM group, prawns were injected with sterile PPB-His buffer; CPO group, prawns were fed with commercial feed. Cumulative mortalities are shown as means of data from 3 replicates for each experimental group (each replicate contained 10 individuals). The mean points with same color indicate no significant difference ($P>0.05$), and the mean points with different colors indicate a significant difference ($P<0.05$). Overall analysis indicated there are very significant differences among groups IM, PO, and the two controls ($P<0.01$). The curves were drawn based on the nonlinear regression following the 3-parameter sigmoid equation.

3.2. TaqMan qPCR Detection

Moribund prawns in IM and PO groups were sampled for the experimental duration, and all the survival prawns in each group were collected at the end of the challenge experiment. All the prawn samples were detected for potential pathogens, using the TaqMan qPCR method. The SHIV-positive rates were 80.0% (24/30) and 46.7% (14/30) in the IM group and the PO group, respectively. The results showed that tests of SHIV were negative for the survival prawns in IM and PO groups. No SHIV positive was detected in CIM and CPO groups. The tests of *Vp*$_{AHPND}$, IHHNV and WSSV were negative for all of prawn samples. The logarithmic SHIV loads (in copies/ng-DNA) of IM and PO group were 5.65±2.31, 3.08±0.60 in prawns, respectively; while CIM and CPO groups were negative. The viral loads in challenged prawns in the IM group were significantly higher ($P < 0.01$) than that in the PO group (Figure 3). The loads of SHIV in 21 moribund and died prawns in the IM group were detected to reach $(4.20\pm3.88)\times10^{6}$ copies/ng-DNA, while that in 3 non-clinical prawns were detected at only $(5.47\pm4.16)\times10^{-1}$ copies/ng-DNA. The loads of SHIV in 7 died prawns in the PO group during 5 – 7 dpi averaged at $(3.63\pm1.44)\times10^{4}$ copies/ng-DNA, which were much higher than that in 7 survival prawns in the same group at $(3.57\pm3.42)\times10^{2}$ copies/ng-DNA (Figure S1). These results showed that prawns in IM and PO groups were successfully infected by SHIV.

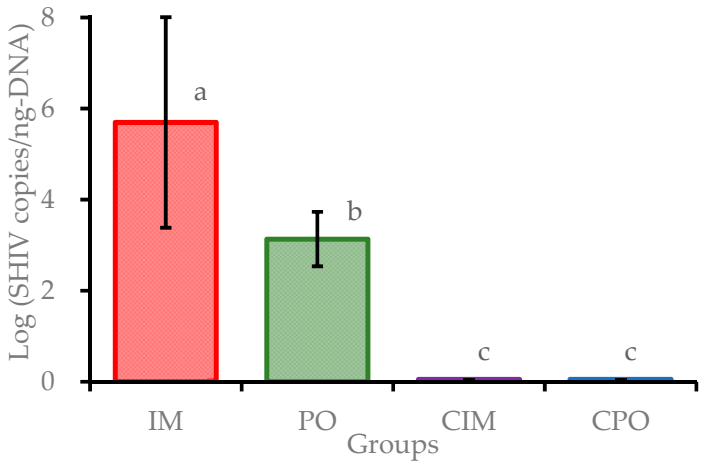

Figure 3. *SHIV loads in prawns* Exopalaemon carinicauda *from the challenge test. Different letters above the bars indicate significant difference* ($P < 0.01$). *CIM and CPO were negative.*

3.3. Histopathology

Histopathological examination of moribund prawns showed that nuclear pyknosis and acidophilic inclusions could be observed in the hemocytes of hepatopancreatic sinus (Figure 4a), hemotoblasts of hematopoietic tissues (Figure 4c), and cuticular epithelium (Figure 4e); while the tissues of the control prawns showed normal (Figure 4b, 4d, and 4f). Typical cytoplasmic and dark eosinophilic inclusions, of which some were surrounded with or contained tiny basophilic staining, appeared beside the nuclei in hematopoietic tissues, hemocytes, and epithelium. The inclusions were more easily found in the infected hematopoietic tissues (Figure 4b).

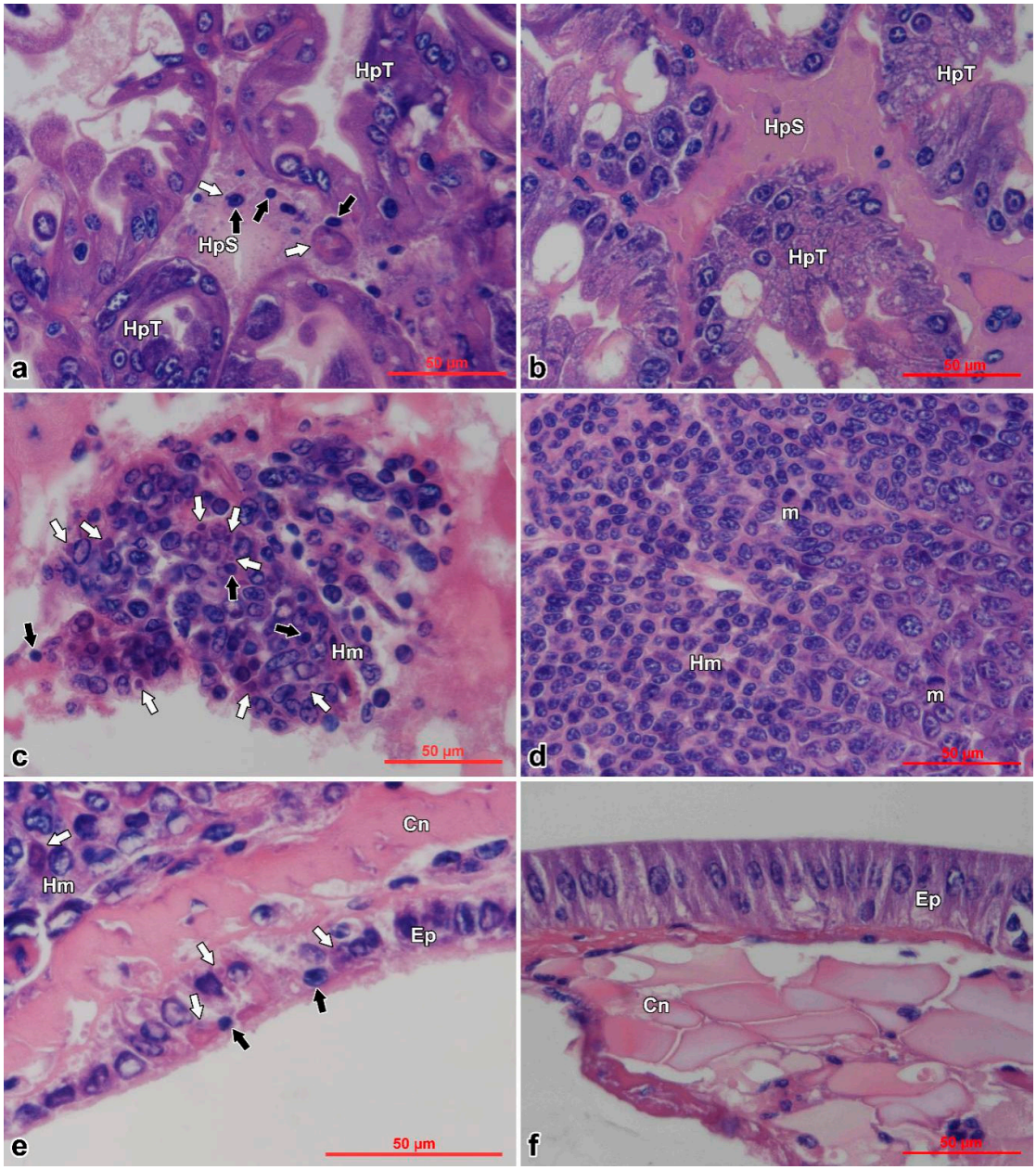

Figure 4. Histopathological examination of *E. carinicauda* tissues infected with SHIV and controls. Black arrows show the karyopyknosis and white arrows show the eosinophilic inclusions. Hepatopancreas (**a and b**); hematopoietic tissues (**c** and **d**), and cuticular epithelium (**e** and **f**) on which the cuticle were removed before dehydration. The left column (**a**, **c**, and **e**) are the tissues of the infected prawn; the right column (**b**, **d**, and **f**) are the tissues of the control prawn. HpT: hepatopancreatic tubule; HpS: hepatopancreatic sinus; Hm: haematopoietic tissue; Ep: epithelium; Cn: connective tissue; m: mitotic phase. Bar = 50 µm

3.4. Primer Design and LAMP Reaction

Primers for LAMP were designed targeting the gene of DNA-directed RNA polymerase II second largest subunit locating between 69047—69263 of SHIV genome (GenBank access no. MF599468), of which the sequence of was completely complementary to that of CQIV 77416—77200 (Figure 5). In addition, results of LAMP primer specificity assay showed that the reactions were only positive for SHIV-infected shrimp, while the reactions were negative for WSSV, *Vp*$_{AHPND}$, IHHNV or

EHP infected shrimp. It indicated that the LAMP primers in this study were specific to SHIV and should be available for ISDL.

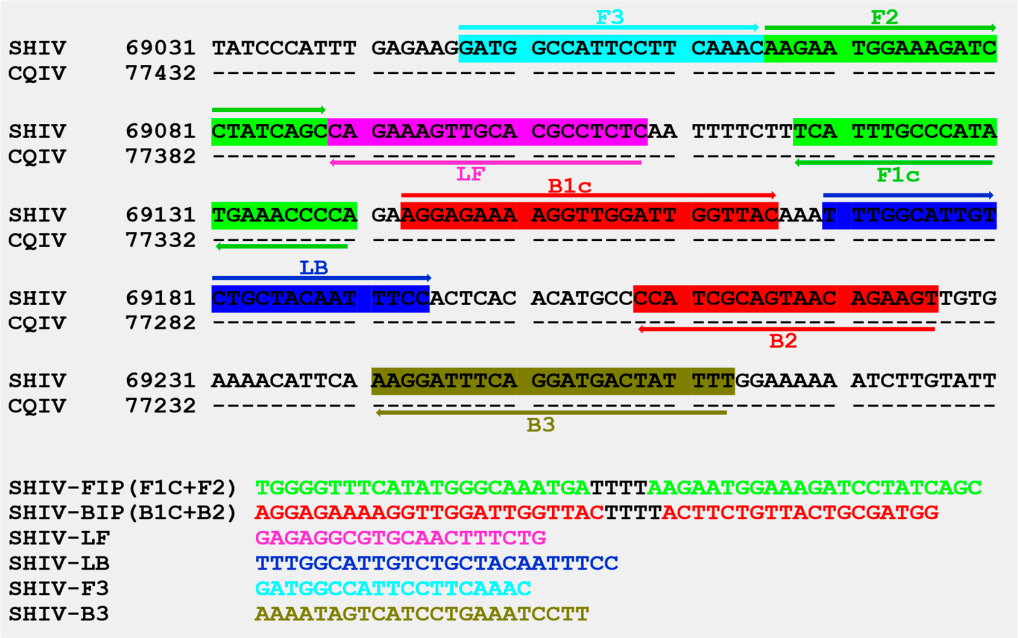

Figure 5. Information of primer design and primers used for LAMP, based on the reference sequence of SHIV. Sequences of SHIV (MF599468) and CQIV (MF197913) were obtained from GenBank.

3.5. ISDL

A LAMP system was applied to *in situ* hybridization, used to examine the histological sections. ISDL results showed that blue-violet hybridization signals were detected in the hepatopancreatic sinus, stomach epithelium, cuticular epithelium, and hematopoietic tissues (Figure 6a, 6c, 6e, and 6g) of prawns in the IM and PO groups, but no signal was detected in the control groups (Figure 6b, 6d, 6f, and 6h). The results of ISDL provided further evidences that prawns in IM and PO groups were infected by SHIV.

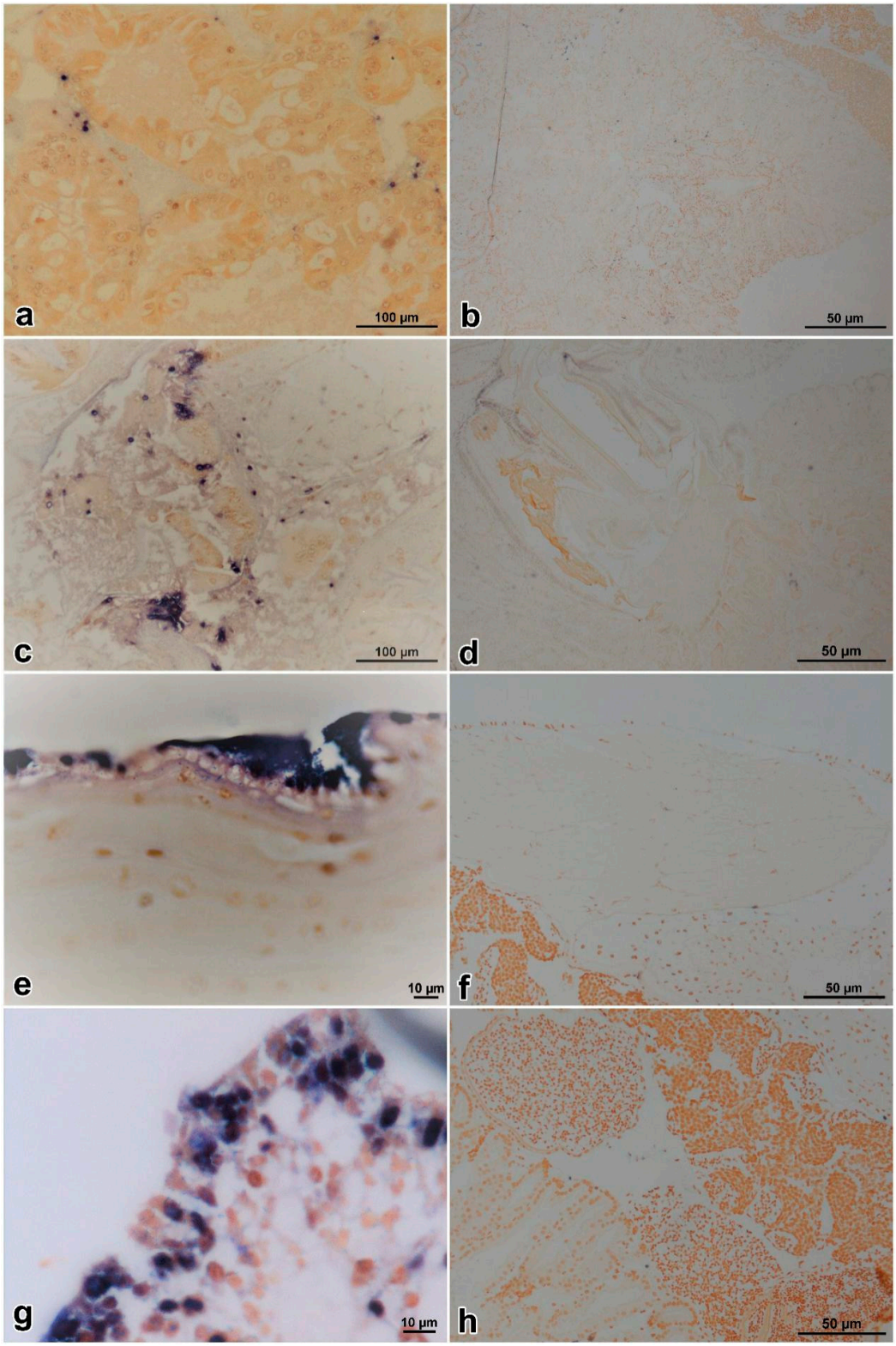

Figure 6. ISDL micrographs of diseased and healthy *E. carinicauda*. Hepatopancreas, stomach, cuticular epithelium, and hematopoietic tissue from a health prawn of PO group, respectively (**a**, **c**, **e**, and **g**); hepatopancreas, stomach, cuticular epithelium, and hematopoietic tissue from a diseased prawn of CPO group, respectively (**b**, **d**, **f**, and **h**).

3.6. TEM

Icosahedral virions and cytoplasmic inclusions were observed in hepatopancreatic sinus, pereiopods, uropods, muscle and gills of *E. carinicauda* in the IM group (Figure 7A–7E, and 7H). Assembled virions mostly distributed outside of the assembling area near cellular membrane of infected cells (Figure 7A, E). Similarly, virions were observed in the hepatopancreatic sinus and gills of prawns in the PO group (Figure 7F, G). Virions were hexagonal (i.e., icosahedral) and about 150 nm in diagonal diameter which matched with characteristics of SHIV.

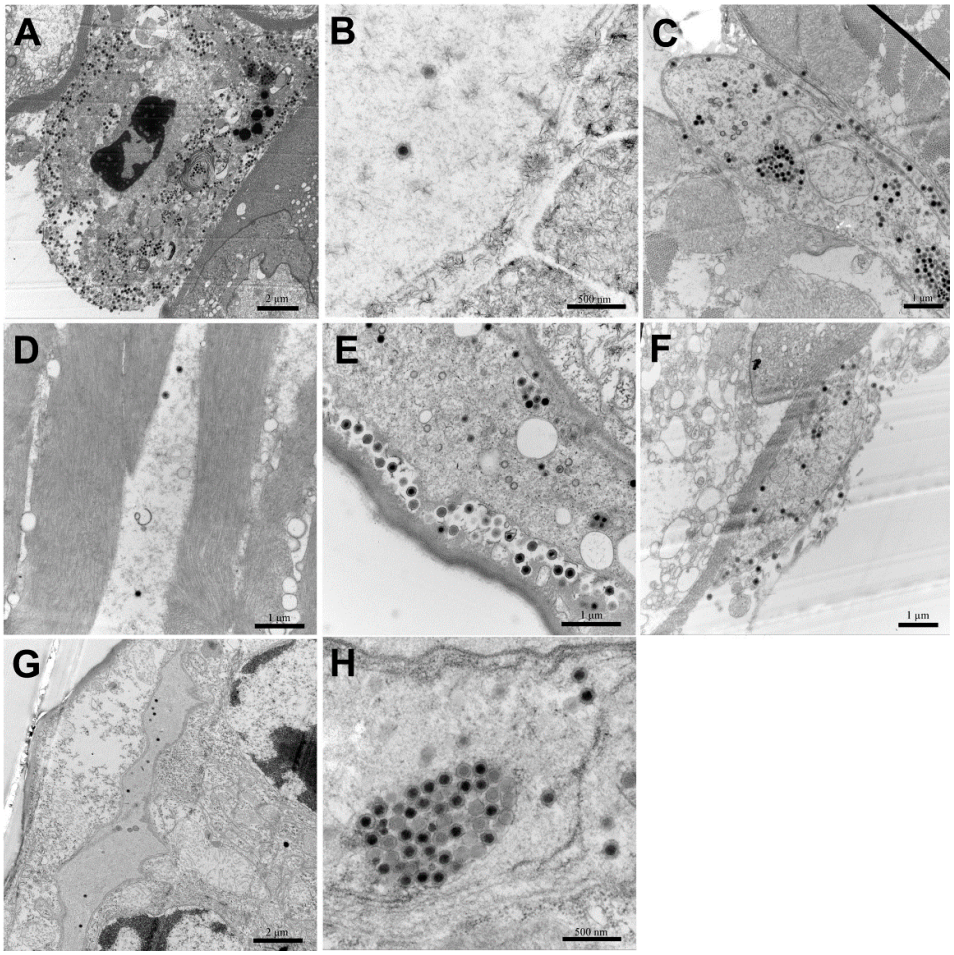

Figure 7. Transmission electron microscopy (TEM) of diseased *E. carinicauda*. **A-E:** hepatopancreatic sinus, pereiopods, uropods, muscle and gills, respectively, of a prawn in the IM group; **F** and **G**: hepatopancreatic sinus and gills, respectively, of a prawn in the PO group; **H**: cytoplasmic inclusions with a cluster of viral particles.

4. Discussion

Understanding the susceptibility of host range for a virus is important information for both study on host and virus. On the host aspect, knowing an infectious pathogen of a host species will provide important information for prevention of the relevant disease; on the virus aspect, the information of a susceptible host range can be used for identification of virus species, finding of a specific viral replication host or cell line, and understanding of viral ecology. Susceptible host range also is important for international trade to prevent from the transboundary spreading of a specific disease. For the purpose, OIE has issued criteria to determine susceptibility for a specific disease in 2014, which require evidences from either natural infection or experimental challenge mimicking natural infection [10]. As a newly found virus, the susceptible host range of SHIV, including its homologous strain named as CQIV sharing 99% genome similarity, remains large unknown space to be investigated. For the natural infection pathway, Positive detections of SHIV by PCR or qPCR have

been reported in different natural samples of crustacean, including *P. vannamei* [9], *P. chinensis* [9], *M. rosenbergii* [9,22], *C. quadricarinatus* [8], *Procambarus clarkii* [22,23], *P. japonicus* [22], *M. nipponense* [22,23], *M. superbum* [23], and Cladocera [23]. Severe disease with high mortality has been reported and infection with SHIV/CQIV has been demonstrated in *C. quadricarinatus*, *P. vannamei*, and *M. rosenbergii* [8,9,23], these species are the confirmed susceptible species for SHIV/CQIV which concord to the criteria of OIE standards for natural infection. Due to lack of the evidence for infection, usually presented by histopathology, TEM, or *in situ* hybridization, only detected positives by PCR or qPCR for SHIV cannot confirm the susceptibility of *P. chinensis*, *P. japonicus*, and *M. superbum* [22,23]. However, they may remain as suspicious susceptibility as there are confirmed susceptible species in the same genus. Cladocera has been detected as positive by qPCR, but the ISDL result showed negative. It has been considered as a non-susceptible species [23]. For the experimental infection pathway, The experimental challenges of *P. vannamei*, *Pr. clarkii*, Chinese mitten crab *Eriocheir sinensis*, and wild crab *Pachygrapsus crassipes* with CQIV have been reported [8,24]. Unfortunately, all of these experimental infections used only intramuscular injection, the results were not enough to support the confirmation of susceptibility for these species according to the criteria of OIE standards requiring the challenge pathway mimicking natural infection. Our present study is the first report to confirm susceptibility for the newly found virus by experimental pathway mimicking the natural infection fully following the criteria of OIE standards.

E. carinicauda is one of the important economic local crustacean distributing in Yellow Sea and Bohai Sea, which has been cultivated broadly in Jiangsu, Zhejiang, Shandong, and Liaoning Provinces of China [11,12]. It has been reported that *E. carinicauda* is a natural host of some viral pathogens, such as WSSV [25] and CMNV [26]. *E. carinicauda* was also considered as a potential model animal for researching shrimp viral pathogens [27].

In order to investigate the susceptibility of *E. carinicauda* to SHIV, *E. carinicauda* were experimentally challenged with SHIV *per os* and intramuscular injection, following the OIE standards. After three days post challenge, infected prawns from both PO and IM groups displayed same clinical symptoms, including debility, empty guts and stomach, pale hepatopancreas, slight whitish hematopoietic tissue, and mortalities. Much higher doses of SHIV were detected in the moribund and died prawns in the intermuscular injection group than those in the *per os* group. The significant difference in viral dose may due to that injection of SHIV caused extensive and synchronous infection in different target cells. The clinical signs observed in *E. carinicauda* were similar to that of *P. vannamei* affected by SHIV in the study of Qiu et al. [9] and also similar to that of lethargy, anorexia, and mortality affected by CQIV [8]. According to the results of our challenge test, in the duration of 20 days, cumulative mortalities of *E. carinicauda* were (76.7±18.3)% and (50.0±26.5)% in groups of im and *per os*, respectively. Based on the pathogenic model resulted from nonlinear regression, the bioassay revealed that *E. carinicauda* showed some tolerant to the infection with SHIV *per os* challenge mimicking natural infection, which caused only (60.1±6.3)%-fold mortality within (1.45±0.35)-fold time spending at (17.2±14.0)%-fold slow instant speed comparing with the direct im challenge. The SHIV positive survival prawns in *per os* challenged group possessed viral loads at $(3.57\pm3.42)\times10^2$ copies/ng-DNA, which were about 100 folds lower than that detected in died prawns via im challenge. Previous studies showed that mortalities of experimental *P. vannamei*, *C. quadricarinatus* and *Pr. clarkii* challenge with SHIV or CQIV reached 100% [8,9]. These results indicated that *E. carinicauda* were likely resistant to SHIV to some degrees, which may provide a object for future study on mechanism and breeding of SHIV resistance. As *E. carinicauda* is a broadly distributing local species, the partial resistance of the ridgetail white prawn to SHIV may also provide a possible reservoir of the virus after the virus transports to the species in the area of the Yellow Sea, the Bohai Sea, and the East China Sea.

Positive tests of TaqMan qPCR and ISDL provided further evidences to the infection of *E. carinicauda* by SHIV. According to the results of ISDL, positive detection signals were detected in the hepatopancreatic sinus, hematopoietic tissue and cuticular epithelium of infected *E. carinicauda*. Previous studies showed that cuticular epithelium is a major target tissue of WSSV infection [25,28]. However, cuticular epithelium of *P. vannamei* is not a susceptible tissue to SHIV infection. Our results

showed difference in the tissue tropism between *E. carinicauda* and *P. vannamei* to SHIV infection. ISDL [18] is a highly sensitive and time-saving method, compared to normal *in situ* hybridization (ISH). In addition, the histopathological examination of diseased *E. carinicauda* were consistent or similar to that previously reported in *P. vannamei*, *C. quadricarinatus*, and *Pr. clarkii* [8,9]. Moreover, in this study, massive SHIV virions with dense cores, characterized by icosahedral and ~150 nm in diameter, consistent with the report of Qiu et al. [9], were observed in hemocytes of hepatopancreatic sinus and gills of *E. carinicauda* from IM and PO groups. The virions primarily existed in cytoplasm of hemocytes and branchial cells. Additionally, SHIV virions were also observed in pereiopods, muscles and uropods of *E. carinicauda* from the IM group. These results expanded the knowledge of tissue tropism of SHIV. Different infection paths of SHIV, including intramuscular injection and *per os* infection, contributed to significant differences on mortality and viral loads in *E. carinicauda* between the IM group and the PO group.

Taken together, the results of clinical observation, molecular biological diagnosis, histological examination, and TEM observation supported that *E. carinicauda* is a newly demonstrated susceptible species to SHIV, following the criteria for listing species as susceptible to infection with a specific pathogen developed by the OIE [10]. Currently, there was no naturally diseased SHIV case of *E. carinicauda* reported in farm or in sea area. As *E. carinicauda* has local economic importance, Biosecurity strategies [29,30] should be taken into account on the risk that SHIV can causes disease in *E. carinicauda* and the risk that the species becomes a possible reservoir of SHIV. It also should not be ignored that *E. carinicauda* showed some degree of tolerance to infection with SHIV, especially *per os* challenge mimicking natural pathway.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Positive data of SHIV qPCR detection within 40 cycles in IM and PO groups.

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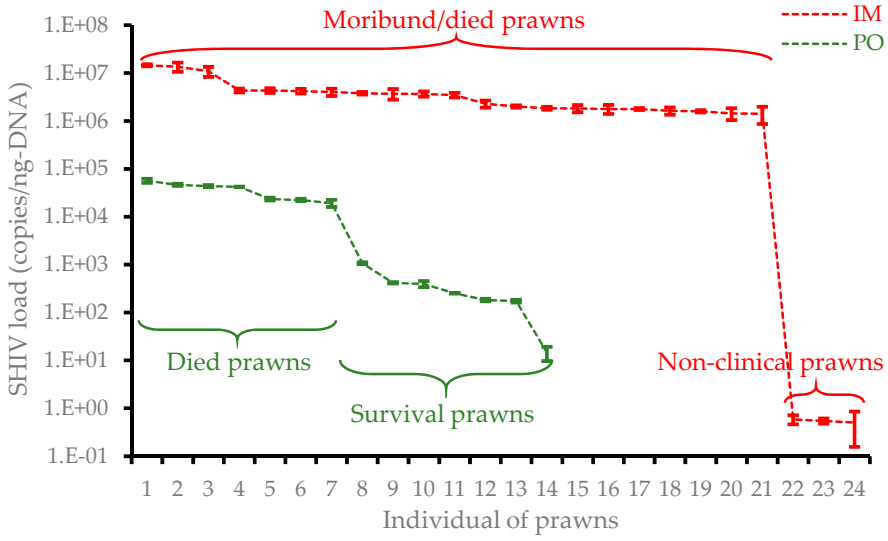

Figure S1. Positive data of SHIV qPCR detection within 40 cycles in IM and PO groups.