Partial Sequence of a Novel Virus Isolated from *Pelodiscus sinensis* Hemorrhagic Disease

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Key Words

Pelodiscus sinensis · Hemorrhagic disease · Random PCR · Partial sequence

Abstract

Outbreaks of hemorrhagic syndrome-like disease with high mortality rates have frequently occurred in *Pelodiscus sinensis* farms. The purpose of this study was to investigate the pathogen through challenge infection assays and partial sequencing of the genome of the pathogen. A 453-bp amplicon was obtained by random PCR using the nucleic acid extracted from the tissue homogenate filtrate and showed 32% identity at the amino acid level with the replicase polyprotein of the porcine reproductive and respiratory syndrome virus by Blastx. Multiple alignments indicated the putative protein sequence has some similarities to the replicase polyprotein of Arteriviridae, and the phylogenetic tree showed it was closely related to equine arteritis virus. This sequence was found in the lung of the diseased *P. sinensis* by in situ hybridization. Dot blot hybridization and quantitative RT-PCR showed that the lung had the highest content of virus. The peak replication of *P. sinensis* hemorrhagic syndrome virus (TSHSV) in the lung occurred 4 days after infection. The ribonucleic nature of the viral genome was con-

firmed by RNase A or DNase I treatments. We named the virus TSHSV in this study as *P. sinensis* is also known as *Trionyx sinensis*. These results provide a fundamental basis for further understanding the biology and the molecular mechanisms of TSHSV.

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Introduction

Pelodiscus sinensis, also known as *Trionyx sinensis* or the Chinese soft shell turtle, is a member of Chelonii in the taxonomy and is mainly distributed in China, Japan and Southeast Asia. Because of its high economic value, *P. sinensis* has become an important freshwater aquaculture species in China. Similar to other aquaculture species, disease remains one of the major limiting factors affecting the sustainable development of aquaculture of this species [1]. Compared to fish, relatively few studies have been performed on *P. sinensis*. The majority of the studies reported previously were mainly focused on diagnosis of diseases based on clinical features and emergency treatment of the disease, while the etiology of the disease was not known.

It has been reported that irido-like virus, herpes-like virus, reo-like virus, rhabdo-like virus and 30- to 39-nm

spherical viruses were isolated from *P. sinensis* in China. Tan and Xiang [2] reported that herpes virus can cause significant surface ulceration, abdominal bleeding and bleeding red plaques. Ye [3] reported that the etiology of parotitis (popular name of *P. sinensis* infection) might be a virus, the conclusion of which was mainly based on the fact that antibacterial drugs cannot cure the disease. Zhang et al. [4] first isolated a 30-nm spherical virus from *P. sinensis* and named it *T. sinensis* virus (TSV). Subsequent studies showed that TSV can cause lesions in carp epithelial tumor cells, eel gonadal cells and grass carp ovary cells [5, 6]. However, information on the nucleic acid sequence and taxonomy of TSV is currently not available. Chen et al. [7] discovered a spherical virus with a diameter of 35–39 nm in the lung, stomach, throat mucosa and ventral cortex of *P. sinensis* suffering from hemorrhagic disease. This virus was primarily present in small blood vessels and capillary endothelial cells of the target organs. Chen et al. [8, 9] isolated an iridovirus with a diameter of 120–160 nm from *P. sinensis* with ‘red neck’ disease. This is the first fully sequenced and taxonomically confirmed *P. sinensis* virus. Subsequent studies investigated the immunology and molecular characteristics of this iridovirus [10–14], but outbreaks and deaths caused by iridovirus in aquaculture have not been reported to date.

Currently, diagnosis of *P. sinensis* viral infection is mainly based on the description of the symptoms, which is easily confused with bacterial infection or noninfectious diseases. Commonly used methods for rapid identification of the virus are mainly through detection of specific proteins or nucleic acids, thus obtaining nucleic acid information is particularly important to establish rapid detection methods of viruses. Isolation and amplification of genetic materials from unknown viruses in the highly complex host cell nucleic acids is key to identifying unknown and emerging viruses [15], which is also the bottleneck in acquiring unknown and emerging viral genome sequences.

With the development of molecular biology technologies, there are several methods that can acquire nucleic acid information without any known sequences available. For example, the random primer PCR method allows the amplification of nucleic acids and subsequent sequencing. BLAST analysis can allow the identification of both RNA and DNA from sequences obtained from viruses in the tissue samples. Using this method, Allander et al. [16], identified a human parvovirus in the specimens of respiratory tract infections. Stang et al. [17], unexpectedly detected HSV-1 virus in mouthwash of patients with chronic fatigue syndrome. Fouchier et al. [18], discovered a new type of coronavirus HCoV-NL. Allander et al. [19] and

Gaynor et al. [20] also identified a new polyomavirus with this random primer PCR method.

In this study, we analyzed the etiology of *P. sinensis* deaths, which occurred at farms in Zhejiang Province, China. It was tentatively determined as a viral infection by challenge assay. Subsequent random primer PCR yielded an amplicon whose sequence was determined, and several experiments were used to verify the origin of the sequence by RT-PCR, in situ hybridization and dot blot assays. The dynamics of viral replication and distribution of virus in different organs were analyzed by detecting this specific nucleic acid sequence.

Materials and Methods

Sample Collection and Preliminary Analysis of the Etiology

Sick *P. sinensis* with a disease onset time in early September 2013 were collected from the outside pond of turtle farms in Zhejiang Province. The average weight of the dying *P. sinensis* was approximately 1,000 g. Serious hemorrhage occurred in the internal organs of the sick *P. sinensis*, including liver, intestine, kidney, lung, gonads and spleen. Bacterial pathogens and viral pathogens were analyzed for this outbreak, which had a high mortality rate. The potential bacterial etiology was explored by trying isolating bacteria from liver, spleen, kidney, lung and blood using soybean casein agar (Tryptone Soya Agar). The possible viral etiology was explored by artificial infection of healthy turtles with 200 µl of the sick *P. sinensis* tissue homogenates after centrifugation (12,000 rpm/min) and filtration (0.22 µm). All challenge assays were performed under the condition of water temperature of 25–28°. All animal experiments were conducted according to the Institutional Animal Care Committee guidelines for the care and use of animals in research.

Amplification of Virus Nucleic Acid Using Random Primers

Liver, spleen, lung, kidney and intestine were isolated from sick *P. sinensis* and homogenized on ice. The homogenate was centrifuged and the supernatant was filtrated through a 0.22-µm filter. The filtrate was centrifuged at 40,000 g for 3 h, and the pellet was resuspended in TN buffer as virus crude extract. The host's nucleic acids were removed by RNase A and DNase I treatment. Briefly, virus crude extract was treated with DNase I and RNase A (Takara, Dalian, China) at 37° for 3 h, and inactivated by incubating in 75° for 10 min. Virus nucleic acids were extracted from 200-µl nuclease-digested samples using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany). The random primer for reverse transcription was FR26V-N 5'-GCCG GAGCTCTGCAGATATCNNNNNN, and the random amplification primer was FR20RV 5'-GCCGGAGCTCTGCAGATATC-3' [9]. The PCR template was prepared prior to amplification. The viral nucleic acids were divided into two groups, RNA and DNA samples, for subsequent experiments. RNA samples were reverse transcribed using an Invitrogen reverse transcription kit (SuperScript III Reverse Transcriptase) and FR26V-N as the primer. After reverse transcription, the product was denatured at 94° for 3 min, rapidly cooled and placed on ice for 2 min, and 3'-

5' exo-Klenow DNA polymerase (NEB) was added to synthesize the second cDNA strand. The reaction was performed at 37° for 1 h and inactivated at 75° for 10 min. Subsequently, the reverse-transcribed products were used as templates for PCR amplification. For preparation as DNA samples, tissue extraction was added to the reaction mixture, denatured at 94° for 3 min and cooled on ice for 2 min. Subsequently, 3'-5' exo-Klenow DNA polymerase was added and incubated at 37° for 1 h. Double-stranded DNA filling reaction contained 10× Klenow buffer 3 µl, dNTP (2.5 mM) 4 µl, FR26RV-N primer 2 µl, DNA template 3 µg, 3'-5' exo-Klenow DNA polymerase 1 µl and H₂O to make up a total volume of 20 µl. The reaction product was used as a subsequent amplification template.

Single-primer PCR was performed using FR20RV as a primer and double-stranded cDNA or filled DNA as the template. The PCR reaction program included 94° for 2 min and 40 cycles of 94° for 1 min, 65° for 100 s and 72° for 2 min, followed by 72° for 10 min. After agarose gel electrophoresis, the product purified using the PCR purification kit product was digested by EcoR V at 37° for 3 h. The product with an expected size of 500–1,500 bp was purified and ligated to pSIMPLE 18 EcoRV/BAP Vector (Takara). The ligation product was transformed into *Escherichia coli* TOP10 competent cells. Positive clones with >500-bp insert were sequenced. Sequences were analyzed by Blastn and Blastx. The phylogenetic tree was constructed by neighbor-joining bootstrapping using MEGA 4.

Detection of Viral Nucleic Acid by Dot Blot and in situ Hybridization

Based on the sequences, we designed specific primers F1 5'-AAGGCACATCTGACCCATTT-3' and R1 5'-AAGAGCCGA GACCAGCGAAG-3' to amplify the target sequence with an expected size of 308 bp. PCR reaction conditions were 95° for 3 min, followed by 40 cycles of 95° for 30 s, 58° for 30 s and 72° for 30 s, and a final extension at 72° for 10 min. The pair of primers described above was used to detect the presence of viral nucleic acid in the tissues of the infected animals with noninfected tissue as control. All primers were synthesized by Invitrogen.

Hybridization probes were prepared using the PCR DIG Probe Synthesis Kit (Roche). Total RNA was extracted from the lung, and cDNA was synthesized using MMLV reverse transcriptase (Promega). Subsequently, probes with an expected size of 308 bp were synthesized using F1 and R1 as primers and cDNA as the template. The probe was used for follow-up of dot blot hybridization and in situ hybridization, which would be used as further evidence to prove the origin of the section of nucleotide sequence.

Dot blot hybridization was performed according to the following steps. Total RNA was extracted from the liver, spleen, lung, kidney, intestine and heart. After adding RNA inhibitor, total RNA (3 µg) was spotted on a nylon membrane. UV cross-linking (1,200 lux for 2 min) was performed after drying. Prehybridization was performed at 45° for 20 min. A DIG-labeled probe was denatured by boiling for 5 min and added to preheated DIG Easy Hyb at 45° overnight. Following overnight incubation, 2× SSC, 0.1% SDS was used to wash twice at 15–25° with 5 min for each wash. Subsequently, 0.5× SSC, 0.1% SDS was used to wash at 65–68° twice with 15 min for each wash. NBT substrates were added for visualization of the membrane.

In situ hybridization was carried out according to the following protocol:

- (1) After fresh lung tissue was imbedded by freezing microtome, frozen sections (5-µm thickness) were prepared and fixed with 4% PFA at room temperature in a humid chamber for 10 min
- (2) The sections were treated with acetic anhydride (pH 8.0) for acetylation; prehybridization was performed at 65° for 1 h by covering the sections with hybridization solution
- (3) A digoxin-labeled probe was then added and incubated at 65° for 24 h
- (4) After washing with blocking solution at room temperature for 30 min, digoxin antibody was added and incubated at 4° overnight
- (5) FITC-labeled antidigoxin secondary antibody was added at room temperature
- (6) Finally, confocal microscopy was used for visualization

Quantitative PCR Detection of Viral Distribution in Tissues and Replication in vivo

The viral distribution in tissues was detected based on the sequence in this study. A 308-bp specific fragment was amplified by RT-PCR and cloned into T vector (Promega), and transformed into *E. coli* DH5α. The plasmid was extracted and the Ct value for the plasmid template (0.5 pg) was used as a standard for quantitative PCR. Specific primers used for fluorescence quantitative RT-PCR were F2 5'-GGCATGGTAACCACCGGATTTCAAG-3' and R2 5'-AGAACAAGAGCCGAGACCAGCGAAG-3'. The size of the product was 197 bp. Total RNA was extracted from liver, spleen, lung, kidney, intestine and heart after 4 days of infection. A total of 2 µg of RNA was used as a template and cDNA was reverse transcribed using MMLV reverse transcription reagent (Promega). Synthesized cDNA was diluted at 1:5, which was used as a PCR template. Quantitative PCR reaction was performed with SYBGreen reaction mixture (Bio-Rad) at 95° for 10 min, followed by 40 cycles of 95° for 30 s, 60° for 15 s and 72° for 30 s. The melting curve was obtained by determining the fluorescence intensity from 60° to 95° at a rate of 0.5°/5 s. The Ct value of each sample was compared with those obtained using a 0.5-pg plasmid as a template and the relative viral load was calculated.

To investigate the replication in vivo, the tissue filtrate from the sick *P. sinensis* was used to challenge healthy *P. sinensis* by intramuscular injection. Subsequently, total RNA was extracted from the lung every day for a total for 5 days. Viral load in the lung tissue was determined by quantitative RT-PCR as described above.

Determination of the Nature of Viral Nucleic Acid

Total RNA (2 µg) from lung tissues were treated with RNase A (10 mg/ml) or DNase I (5 U/µl) by incubating at 37° for 30 min, followed by inactivation at 65° for 15 min. Subsequently, F1 and R1 primers were used for RT-PCR amplification of virus-specific nucleic acid sequences.

Results

*Preliminary Analysis of Etiology for the Sick *P. sinensis**

The diseased *P. sinensis* had a weak neck. The back and plastron had a normal appearance without body enlarge-

Table 1. Challenge assay using filtered tissue homogenates from the naturally infected *P. sinensis* individuals

Groups	Challenge route	Turtles after 7-day challenge		Survival rate, %
		n	death, n	
Tissue homogenate	0.2-ml muscle injection	8	8	0
Filtered tissue homogenate	0.2-ml muscle injection	8	8	0
PBS control	0.2-ml muscle injection	5	0	100

ment. After dissecting, parotid gland inflammation was observed. Liver, spleen, kidney, lung, intestine and gonads had different levels of congestion. Other symptoms included hepatomegaly, fragility, piebald, severe congestion and intestinal bleeding without perforation. No bacterial pathogen was isolated from liver, spleen, kidney, lung and blood. Challenge of the healthy *P. sinensis* with tissue homogenates (liver, spleen, kidney, lung) from the diseased *P. sinensis* or the filtered tissue homogenates (0.22- μ m filter) resulted in the onset of death on the 4th day of infection; all *P. sinensis* died by day 7 (table 1). Autopsy of the challenged *P. sinensis* revealed characteristic symptoms in liver, spleen, kidney, lung, intestine and gonads, which were similar to the original case. These results indicated that the pathogen for the infection can be filtered through a 0.22- μ m filter further, suggesting that the pathogen was a virus. We tentatively named the pathogen as acute *Trionyx sinensis* hemorrhagic syndrome virus (TSHSV).

Sequencing Analysis of Viral Nucleic Acid

Nucleic acid was isolated after nuclease treatment of tissue homogenate filtrate to reduce the background interference. Given the fact that the nature of the nucleic acid was unknown, we synthesized either cDNA or double-stranded DNA followed by PCR amplification using random primers. The intensity of the band (at the size of >500 bp) amplified when the nucleic acid was processed as RNA was stronger than that when the nucleic acid was processed as DNA. PCR products in the range of 500–1,500 bp were purified and cloned. A total of 35 clones were picked for sequencing, and only one clone contained a sequence (453 bp) that was homologous to viral genome. The sequence (GenBank accession No. KR187104) Blast/Blastx analysis showed that the 453-bp nucleotide sequence and its deduced protein had 32% identity with the partial sequence of replicase polyprotein from porcine reproductive and respiratory syndrome virus (PRRSV). The sequence was compared with the replicase polyproteins of several vi-

ruses from Arteriviridae, including PRRSV (GI: 12240325), equine arteritis virus (GI: 46397774), simian hemorrhagic fever virus (GI: 62510949) and lactate dehydrogenase-elevating virus (GI: 62510951). The putative protein from the partial sequence of TSHSV has a small domain that has sequence similarities to replicase polyprotein of Arteriviridae virus (fig. 1a). In terms of the deduced sequence, the MEGA 4 phylogenetic tree showed that TSHSV is closely related to equine arteritis virus (fig. 1b). To further verify whether the nucleic acids were derived from the virus genome, RT-PCR was performed using as templates the total RNA isolated from liver, lung and kidney of the naturally sick animals with typical hemorrhagic symptoms and artificially infected *P. sinensis*. The results showed that the expected fragments were amplified from the tissues of all of the infected *P. sinensis*, either naturally or artificially, while no PCR product was amplified from the tissue of healthy *P. sinensis* (data not shown). These primary results suggested that the nucleic acid was from an unknown virus genome.

Detection of Virus in the Tissues

Dot blot hybridization detected nucleic acid in all organs of the infected *P. sinensis*. In contrast, the healthy *P. sinensis* were negative. These results indicated that virus was present in all of the detected organs, with the highest level in the lungs (fig. 2a). Quantitative RT-PCR also indicated that the lungs contained the highest level of virus (fig. 2b).

In situ hybridization using a digoxin-labeled probe can detect nucleic acid in lung tissues, indicating that this nucleic acid was present in the lungs (fig. 3a). Meanwhile, quantitative RT-PCR was applied to determine the virus replication in the lungs after different times of infection. The results showed that virus started to replicate 1 day after infection and that replication was significantly increased at 2 days after infection and reached a peak at day 4 after the infection. Virus replication was significantly decreased at day 5 after infection (fig. 3b). The infected

Fig. 1. Bioinformatics analysis of the partial nucleotide sequence from TSHSV. **a** Multiple alignments between the putative protein based on the partial nucleotide sequence of TSHSV and the replicase polyprotein of Arteriviridae virus. Colored background: blank, invariant residues; red, green and pink residues conserved in 80, 60 or 40%, respectively, of the sequences. The National Center for Biological Information sequenceID:PRRSV (GI:12240325), equine arteritis virus (EAV) (GI: 46397774), simian hemorrhagic fever virus (SHFV; GI: 62510949) and lactate dehydrogenase-elevating virus (LDEV; GI: 62510951). **b** Phylogenetic tree built by the neighbor-joining bootstrapping using MEGA 4.

P. sinensis had breathing difficulties and started to die at day 5 after infection, while the clinical symptoms at days 3 and 4 were not obvious.

Determination of Viral Nucleic Acid Nature

The PCR product could be amplified using the DNase I-treated sample, while the RNase A-treated sample did not amplify a specific PCR product, suggesting that the viral nucleic acid was RNA (data not shown).

Discussion

Generally, the evidence of viral infection includes (1) negative bacterial isolation, (2) reproducible symptoms after artificial infection with filtered tissue homogenate from the sick animals, (3) observable virus particles by electron microscopy and (4) inefficiency of antimicrobial treatment. Our artificial infection assay using filtered tissue homogenate reproduced the symptoms of the naturally infected *P. sinensis*, indicating that the disease was caused by highly pathogenic lethal viral pathogens. Virus-like particles with sizes of 60–80 nm were also ob-

Fig. 2. Distribution of the nucleotide sequence in tissues of sick *P. sinensis*. **a** Dot blot hybridization for the infected tissue and control tissue. Equal amounts of total RNA from various tissues were hybridized with DIG-labeled probe. Lungs contained the highest amount of viral nucleic acid. **b** Quantitative RT-PCR indicated that the lungs contained the highest level of virus.

Fig. 3. Verification of the nucleotide sequence and dynamic of viral replication in the lungs after infection. **a** Confocal microscopic observation of viral nucleic acid by situ hybridization in lung tissues: FITC fluorescence using DIG antibody (A), under the same vision, DAPI staining of lung tissue nuclei (B), and merged image of A and B showing the location of specific fragments of viral nucleic acid (C). **b** Dynamic of viral replication in the lungs after infection. The results indicated that peak viral replication was achieved at day 4 after infection.

Fig. 4. Identification of viral particles by electronic microscopy (arrow). Tissue homogenates were filtered using a 0.22- μ m filter, and density gradient centrifugation was performed. Viral particles with a size of 60–80 nm were observed by electronic microscopy.

served in the ultrafiltered tissue homogenate (fig. 4). Because suitable cell lines for the *in vitro* passage of the virus are not available, a higher purity of the virus was not obtained. Furthermore, electronic microscopy of the ultra-thin tissue section did not find direct evidence of virus infection.

TSHSV observed in this study was similar to TSV reported by Zhang et al. [4]. Both TSHSV and TSV are highly pathogenic viruses. However, the clinical symptoms caused by TSV are different from those caused by TSHSV. TSV infection causes a bleeding spot on the plastron, while TSHSV only causes weak neck, but does not have

obvious gross damage. TSV causes hepatomegaly and splenomegaly, while the size of the spleen is normal in TSHSV infection. Because nucleic acid information for TSV is not available, it is difficult to accurately determine the virus. In addition, artificial infection assay showed that TSHSV causes 100% mortality in both adult and young *P. sinensis*. These results suggest that TSHSV is highly pathogenic and can easily cause large deadly outbreaks.

Due to the lack of viral nucleic acid information, it is difficult to conduct rapid screening and accurate identification of the viruses in *P. sinensis*. Furthermore, commercial *P. sinensis* passage cells are not available, which makes it difficult for artificial propagation of unknown viruses and immunological identification of virus-specific protein antigens. Therefore, obtaining unknown virus nucleic acid sequences and establishing specific molecule-detection methods have become an important strategy to accurately detect this viral infection in *P. sinensis*. For the new unknown viruses, it is not appropriate to use conventional detection methods that are based on specific immunological antigens or known nucleic acid sequences. In contrast, the rapidity to acquire nucleic acid sequence of unknown viruses is key for virus characterization. Unknown virus nucleic acid sequences can be obtained by many approaches, e.g. representational difference analysis [21], suppression subtractive hybridization [22], sequence-independent single-primer amplification and its derivative method [17, 23], random PCR [24], and the recently developed high-throughput sequencing method [25]. Each method has its own advantages and disadvantages. In this study, a random PCR was used to identify unknown virus-related genes. At the 5' end of the primer, a universal sequence containing restriction sites was added, which can facilitate following cloning and is theoretically more efficient than sequence-independent single-primer amplification. Using this method, we successfully obtained a partial sequence of an unknown virus from clinical specimens, which provided a solid foundation for future study.

Clinical samples generally contain low levels of virus and therefore it is difficult to identify target virus sequences from highly complex clinical specimens. It is an important prerequisite to effectively reduce the interference of nucleic acids from host cells and other microorganisms, and increase the abundance of viral nucleic acid. In this study, host and bacterial cells were first removed by filtration. Considering that complete virus particles are resistant to nuclease, we used DNase I and RNase A

to treat the samples to reduce the background nucleic acid interference before extraction of viral nucleic acid. The fact that we only obtained one clone (out of 35 clones) containing viral sequences suggested that background interference is still the main obstacle to obtaining an unknown virus nucleic acid sequence directly from clinical specimens.

Although we only obtained a partial sequence of an unknown virus, the results are significant for follow-up studies. The sequence analysis implied TSHSV has a similar domain of replicase polyprotein of Arteriviridae, which is related to virus-coded proteinase [26, 27]. RT-PCR and fluorescent quantitative RT-PCR based on the sequences we obtained are highly specific and can be used for molecular detection of this pathogen. In situ hybridization and dot blot hybridization further confirmed that the nucleic acid sequences were present only in the tissue of infected animals, but not in the healthy animals. Quantitative PCR proved that there were high levels of virus in the lung tissue, while the liver and small intestine had low level of virus. Meanwhile, RNase A and DNase I treatment experiments further verified that the nucleic acid of the virus was RNA. The determination of dynamics of virus replication in the lungs contributes to the accurate diagnosis of the corresponding symptoms. Because this virus has a rapid disease onset and causes 100% lethality, effective vaccines are critical to control the disease. The sequence similarity between TSHSV and PRRSV [28–30] suggest ways for future studies on pathogenic features and vaccine development.

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