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# Detection of Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV, Decapod Penstylhamaparvovirus 1) in Commodity Red Claw Crayfish (*Cherax quadricarinatus*) Imported into South Korea

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**Abstract:** Freshwater crayfish, which are cultivated in aquaculture, are economically important for food and ornamental purposes. However, relatively few studies have focused on potentially pathogenic viruses in crayfish compared to in penaeid shrimp. Commodity red claw crayfish (*Cherax quadricarinatus*; 400 crayfish in 10 batches) and red swamp crayfish (*Procambarus clarkii*; 40 crayfish in 2 batches) imported into South Korea from Indonesia and China were screened by PCR to detect infectious hypodermal and hematopoietic necrosis virus (IHHNV or Decapod penstylhamaparvovirus 1). IHHNV was detected in tissue samples pooled from nine out of ten batches of red claw crayfish imported from Indonesia. Phylogenetic analysis of PCR amplicons from representative pools clustered the IHHNV strain with infectious-type II sequences commonly detected in Southeast Asian countries rather than with type III strains detected previously in whiteleg shrimp (*Penaeus vannamei*) cultured in South Korea. IHHNV DNA was detected most frequently in the muscle (eight batches, 66.7% samples), followed by in the hepatopancreas (five batches, 41.7% samples) and gills tissue (three batches, 25.0% samples). These data suggest that red claw crayfish could be a potential carrier of the virus and that quarantine procedures must be strengthened in South Korea to avoid importing infectious types of IHHNV in commodity crustaceans such as red claw crayfish.

**Keywords:** infectious hypodermal and hematopoietic necrosis virus; infectious type; red claw crayfish; type II; reservoir

## 1. Introduction

The red claw crayfish *Cherax quadricarinatus* is a large, highly productive, and rapidly growing freshwater decapod crustacean that can live in diverse environments [1]. Since the mid-1980s, red claw crayfish aquaculture has grown rapidly in tropical and sub-tropical regions including South Africa, Zimbabwe, Japan, America, China, and Chile [2]. Interest in both aquaculture and aquarium trade has resulted in the species being translocated worldwide [3]. Although it is farmed mostly in extensive pond systems, intensive rearing systems are becoming common [2,4]. Pathogens in crayfish hosts have not been widely

investigated, and such intensive production systems have increased the likelihood of disease impacts due to viruses such as white spot syndrome virus, *C. quadricarinatus* bacilliform virus, *Cherax giardiavirus*-like virus, spawner-isolated mortality virus, a putative gill parvovirus, reo-like virus, and *C. quadricarinatus* parvo-like virus [5–10].

Infectious hypodermal and hematopoietic necrosis virus (IHHNV, also named as *Decapod penstylhamaparvovirus* 1 for taxonomic consistency) is the smallest of the known crustacean viruses, with a non-enveloped icosahedral head and ssDNA genome of approximately 3.9 kb in length; the virus is a known shrimp pathogen causing cuticular deformities described as runt-deformity syndrome and had been listed as one of the notifiable crustacean pathogens by the World Organization for Animal Health (OIE) [11,12]. Until recently, a total of five genotypes (three infectious types including types I, II, and III and two non-infectious types including types A and B) of IHHNVs have been described [13]. Although the infectious type of IHHNV primarily infects penaeid shrimp [14], the virus can also infect the giant freshwater prawn *Macrobrachium rosenbergii*, grapsid crab *Hemigrapsus penicillatus*, and estuarine crab *Neohelice granulate* [14–16]. Natural and experimental infections of the red swamp crayfish *Procambarus clarkii* have also been reported [17–19], with an outbreak in wild *P. clarkii* at Weishan Lake, Shadong province in China resulting in nearly 100% mortality [17]. Additionally, disease transmission between shrimp and crayfish was confirmed by feeding IHHNV-infected *P. vannamei* tissue to *P. clarkii* [18]. Although some previous studies reported the presence of Cowdry type A inclusion bodies [20] and endogenous Brevidensovirus-like elements [21] in red claw crayfish, these studies did not provide clear evidence of IHHNV infection in the species.

In this study, we investigated the potential presence of IHHNV in commodity red claw crayfish and red swamp crayfish purchased from Korean fishery markets but originally imported from foreign countries (Indonesia and China) using conventional PCR and phylogenetic analysis.

## 2. Materials and Methods

### 2.1. Samples

Frozen *C. quadricarinatus* imported from Indonesia (400 crayfish in 10 batches, approximately 15–48 g) and *P. clarkii* imported from China (40 crayfish in 2 batches, approximately 52–63 g) were purchased from 9 retail fish markets in South Korea (Table 1). The samples were stored at −80 °C until processing.

**Table 1.** Crayfish species, country of origin, year collected, and PCR detection of IHHNV in DNA extracted from each of three different tissue types pooled from each batch of crayfish.

| Sample | Species | Origin | Year/Month | IHHNV Detection | | |
|---|---|---|---|---|---|---|
| | | | | Muscle | HP * | Gill |
| 20-002 | *Cherax quadricarinatus* | Indonesia | 2019/03 | + ** | + ** | - |
| 20-003 | *Procambarus clarkii* | China | 2018/10 | - | - | - |
| 20-004 | *Cherax quadricarinatus* | Indonesia | 2019/01 | + ** | + ** | + ** |
| 20-005 | *Cherax quadricarinatus* | Indonesia | 2019/04 | + *** | - | - |
| 20-006 | *Cherax quadricarinatus* | Indonesia | 2019/06 | - | - | - |
| 20-007 | *Cherax quadricarinatus* | Indonesia | 2019/02 | + ** | - | + ** |
| 20-008 | *Cherax quadricarinatus* | Indonesia | 2020/01 | + ** | + ** | + ** |
| 20-009 | *Cherax quadricarinatus* | Indonesia | 2019/04 | + ** | + ** | - |
| 20-010 | *Cherax quadricarinatus* | Indonesia | 2019/04 | + ** | - | - |
| 20-011 | *Cherax quadricarinatus* | Indonesia | 2019/02 | + *** | - | - |
| 20-012 | *Procambarus clarkii* | China | 2018/10 | - | - | - |
| 20-013 | *Cherax quadricarinatus* | Indonesia | 2018/12 | - | + *** | - |

* HP, hepatopancreas. ** Sequenced samples. *** Not sequenced due to low level of amplification.

### 2.2. DNA Extraction

Five crayfish were randomly selected from each batch, and their muscles (~6 mg from each crayfish for a total of 30 mg per batch), hepatopancreases (~6 mg from each crayfish for a total of 30 mg per batch), and gills (~6 mg from each crayfish for a total of 30 mg per batch) were collected. DNA was extracted from different tissues with DNeasy Blood & Tissue kits (69506, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each DNA sample was processed separately, after which the five samples for each organ were pooled, resulting in three samples (one for each organ type) for PCR analysis.

### 2.3. IHHNV PCR

According to the recommendation of the OIE [22], the viral DNA was amplified by IHHNV-389F/389R PCR primers which targeting the nonstructural protein-coding region of the IHHNV genome [23]. The reaction samples (25 µL) contained AccuPower® PCR PreMix (K-2016, Bioneer, Daejeon, South Korea), 1 µL of each DNA extract, and 1 µL (10 pmole) of each primer. The thermal cycling conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 5 min. The available PCR-positive amplicons were sequenced at Bioneer Inc. (Daejeon, Korea).

### 2.4. Sequence Comparison & Phylogenetic Analysis

The obtained nucleotide and its deduced amino acid sequences of presumptive IHHNV DNAs from the crayfish samples were compared with other available IHHNV sequences in the GenBank database using BLAST searches (available online: www.ncbi.nlm.nih.gov/BLAST (accessed on 1 March 2021)), which confirmed that the sequences resided within the IHHNV non-structural protein-coding region. The two IHHNV sequences from the hepatopancreas batches of *C. quadricarinatus* were selected and used for further phylogenetic analysis for these reasons; first, sample 20-002 showed multiple infections with white spot syndrome virus [24] and was selected for further analysis. Second, all the tested tissues (muscle, hepatopancreas, and gills) of sample 20-004 were positive for IHHNV detection and preferentially selected for further analysis. Trimmed sequences of IHHNV strains from different geographical origins and hosts were aligned using ClustalX (ver. 2.1) [25] and BioEdit Sequence Alignment Editor (ver. 7.1.0.3) [26]. A maximum-likelihood phylogenetic tree was constructed using the Jukes–Cantor model and 1000 bootstrap replicates in MEGA-X ver. 10.0 [27].

### 2.5. GenBank Accessions

The obtained partial sequences of the nonstructural protein-coding region in IHHNV detected from *C. quadricarinatus* hepatopancreas DNA of sample batches 20-002 and 20-004 were deposited in the GenBank database under accession numbers MT543324 and MT543323, respectively.

## 3. Results and Discussion

Although recent studies showed that *P. clarkii* can be infected by IHHNV both naturally and experimentally [17–19], the susceptibility of *C. quadricarinatus* to the viral infection remains unclear. Here, we detected IHHNV DNA in nine out of ten sample batches of *C. quadricarinatus* collected from Korean fish markets by PCR using 389F/389R primers (Table 1). Although IHHNV was not detected in the tissues of the two batches of *P. clarkii* imported from China, the sample number was small and may not be representative of the IHHNV infection status of this species farmed across different regions in China.

Among the different tissue types pooled from frozen *C. quadricarinatus* collected from fish markets, PCR detected IHHNV DNA in eight out of ten batches of muscle tissue, five out of ten batches of hepatopancreas tissue, and three out of ten batches of gill tissue. Vial DNA was not detected in one out of ten batches in any of the three tissue types (Table 1). The sequenced PCR-positive amplicons of presumptive IHHNV DNAs showed >99% sequence

identity within themselves. As a result of PCR analysis, a representative sample positively detected for IHHNV DNA in muscle tissue is shown in Figure 1. In penaeid shrimp, IHHNV primarily targets tissues of ectodermal and mesodermal origin, with the principal target organs including the gills, cuticular epithelium (or hypodermis), all connective tissues, hematopoietic tissues, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches, and its ganglia, but not enteric tissues such as the hepatopancreas, midgut, or its caeca [12,28,29]. IHHNV was detected in the hepatopancreas of *C. quadricarinatus* as described previously for IHHNV infection in *P. clarkii*, possibly because the virus replicated in either internal connective tissue cells or hemocytes circulating in the hemolymph, or because virus particles circulated freely in the hemolymph [18,19].

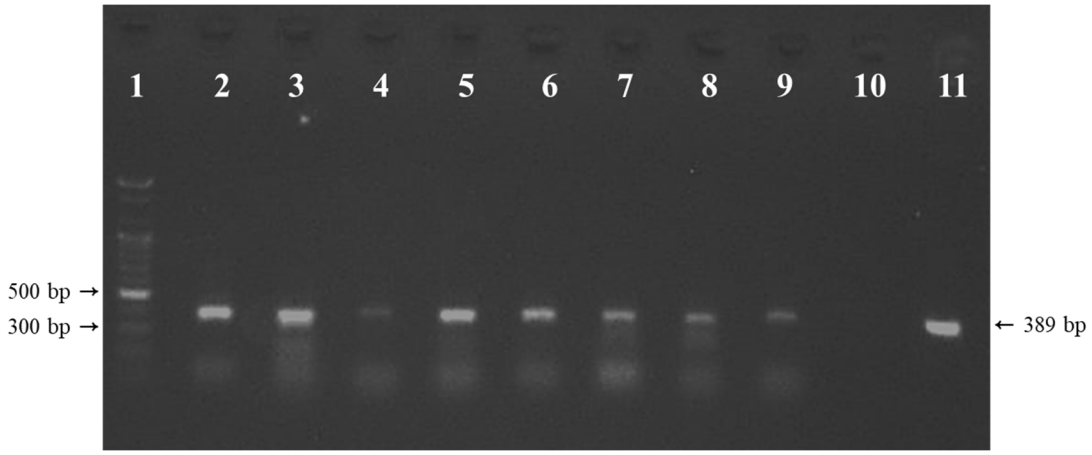


**Figure 1.** Agarose gel showing the 389-bp DNA amplified by IHHNV 389F/389R PCR primers using DNA extracts of muscle tissue pooled from imported red claw crayfish. Lane 1, 100-bp DNA maker (D-1030, Bioneer, Daejeon, South Korea); Lane 2, sample 20-002; Lane 3, sample 20-004; Lane 4, sample 20-005; Lane 5, sample 20-007; Lane 6, 20-008; Lane 7, sample 20-009; Lane 8, sample 20-010; Lane 9, sample 20-011; Lane 10, negative control; Lane 11, positive control.

To date, two distinct types of infectious IHHNV emerging in global shrimp culture farms have been identified as type II (primarily from Southeast Asia) and type III (primarily from the Americas and East Asia) [30,31]. Non-infectious types of endogenous IHHNV-related sequences have been found integrated in the genome of *P. monodon* from Africa and Australia [23], and a similar phenomenon was reported in *C. quadricarinatus* from Australia. Brevidensovirus-like elements showing high DNA homology but relatively low identities in its deduced amino acids with IHHNV were inserted into the crayfish genomes; however, their exact function remains unclear [21]. Moreover, the 389F/389R primer set used can amplify non-infectious endogenous types of IHHNV [32]. Therefore, we conducted further sequence comparisons and phylogenetic analyses using IHHNV DNA obtained from *C. quadricarinatus* to discriminate infectious and non-infectious endogenous types and also determine the type of the infectious virus.

The IHHNV sequences obtained in this study exhibited high similarities (>96% and >98% identities each) with non-structural protein 1 and non-structural protein 2 of the reference IHHNV strain (Decapod penstylhamaparvovirus 1, NC_039043.1) detected in *P. stylirostris* from the Gulf of California in 1998 [33], as well as with other IHHNV strains detected in shrimp and mollusks from diverse geographical locations. Moreover, in contrast to a previous study [21], the deduced amino acids of the PCR amplicons obtained showed high similarity (>96% and >98% identities, each) with non-structural protein 1 and non-structural protein 2 of the reference IHHNV strain (NC_039043.1). Phylogenetic analyses of representative 389F/389R PCR amplicons (~380 bp) from 2 batches of *C. quadricarinatus* (samples 20-002 (MT543324) and 20-004 (MT543323)) from Indonesia clustered closely together with type II strains mainly reported from Southeast Asian countries (Figure 2). Considering the evidence that (i) not all the *C. quadricarinatus* samples tested in this

study were positive in the PCR analysis, (ii) the nucleotide sequences of the positive PCR amplicons exhibited high similarities (>96%) to the nonstructural protein-coding region in IHHNV, and (iii) the sequences were phylogenetically clustered with the IHHNV strain with infectious-type II, the IHHNV DNA obtained from *C. quadricarinatus* in the current study was the infectious type rather than non-infectious endogenous IHHNV-related sequences. As only IHHNV type III strains were previously detected in South Korea [29,30], the IHHNV-positive imported commodity *C. quadricarinatus* is a potential reservoir that can inadvertently release this type into wild and farmed shrimp and crayfish in South Korea.

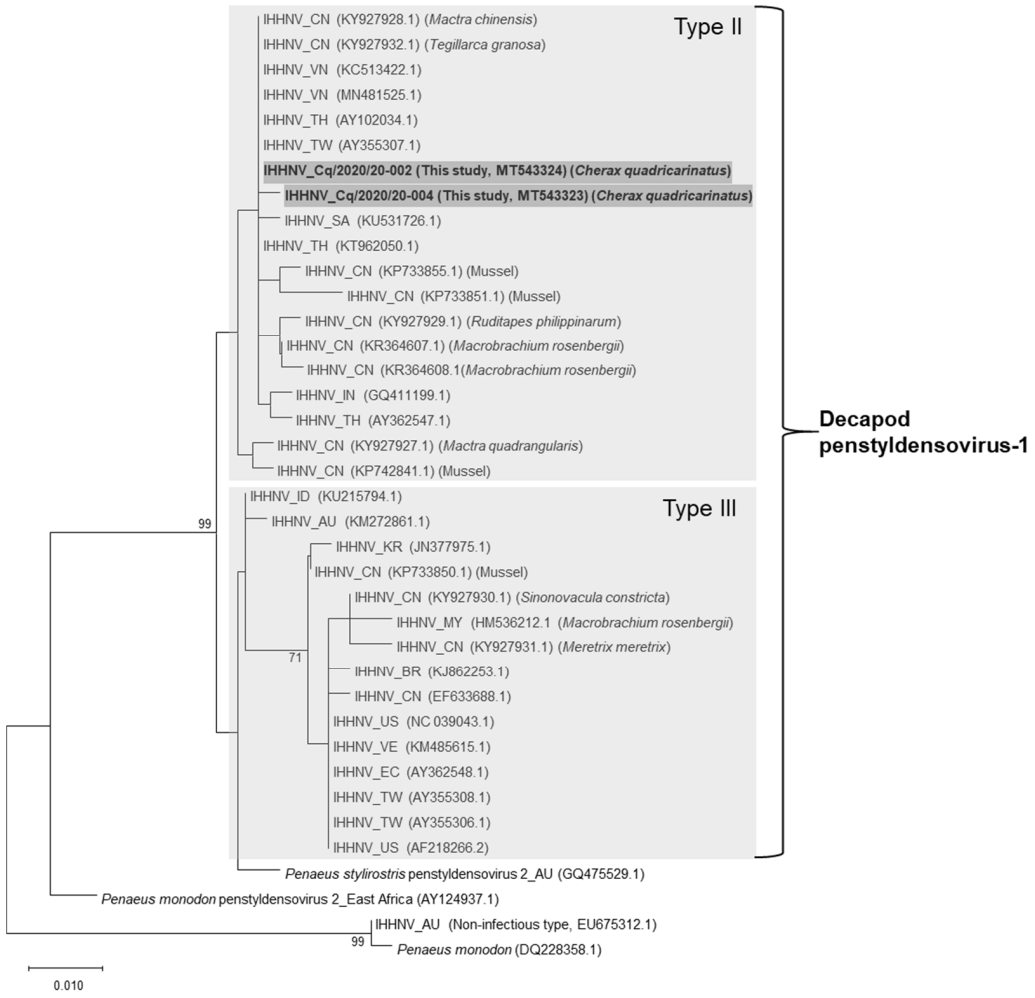


**Figure 2.** Phylogenetic tree based on the nucleotide sequences of the partial nonstructural protein-coding region (~380 bp) in the representative IHHNV strains including the strain detected in two batches of Cherax quadricarinatus (Sample 20-002; MT543324; Sample 20-004, MT543323) imported from Indonesia. The maximum-likelihood tree also included non-infectious endogenous IHHNV types (EU675312.1 and DQ228358.1), which were used as an outgroup. Bootstrap values were calculated from 1000 replicates. The scale bar (0.01) represents 1 substitution per 100 nucleotides.

The detection of IHHNV in the imported commodity red claw crayfish indicates that this virus poses a threat to crustacean aquaculture industries in South Korea [34,35], particularly as viruses can remain viable in frozen shrimp tissue and be transmitted via feeding of infected tissue to other crustaceans [36]. Stricter controls and monitoring of imported crayfish for crustacean viruses are warranted. In addition, an experimental challenge of *C. quadricarinatus* with IHHNV is necessary to determine its histopathological and mortality characteristics.

## 4. Conclusions

Recently, crayfish have become economically important crustaceans in the aquaculture industry for food and ornamental purposes. However, transboundary movements can cause significant environmental disturbance and provide opportunities for inadvertent translocation of disease-causing pathogens to new locations. Although infection by IHHNV was recently confirmed in the red swamp crayfish *P. clarkii*, it remains unknown whether red claw crayfish (*C. quadricarinatus*) are susceptible to IHHNV infection. Here, we detected IHHNV infection in *C. quadricarinatus* by identifying the viral DNA in tissue samples of the commodity imported into South Korea as the IHHNV type II strain. These results indicate that the red claw crayfish could be a potential carrier of the infectious IHHNV and suggest the need for stricter monitoring of imported crayfish crustacean viruses that may impact cultured and wild populations of crustacean in South Korea.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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