Molecular evidence for homologous strains of infectious spleen and kidney necrosis virus (ISKNV) genotype I infecting inland freshwater cultured Asian sea bass (*Lates calcarifer*) in Thailand

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Abstract

Infectious spleen and kidney necrosis virus (ISKNV) is a fish-pathogenic virus belonging to the genus *Megalocytivirus* of the family *Iridoviridae*. In 2018, disease occurrences (40-50% cumulative mortality) associated with ISKNV infection were reported in grown-out Asian sea bass (*Lates calcarifer*) cultured in an inland freshwater system in Thailand. Clinical samples were collected from seven distinct farms located in the eastern and central regions of Thailand. The moribund fish showed various abnormal signs, including lethargy, pale gills, darkened body, and skin hemorrhage, while hypertrophied basophilic cells were observed microscopically in gill, liver, and kidney tissue. ISKNV infection was confirmed on six out of seven farms using virus-specific semi-nested PCR. The MCP and ATPase genes showed 100% sequence identity among the virus isolates, and the virus was found to belong to the ISKNV genotype I clade. Koch’s postulates were later confirmed by challenge assay, and the mortality of the experimentally infected fish at 21 days post-challenge was 50-90%, depending on the challenge dose. The complete genome of two ISKNV isolates, namely KU1 and KU2, was recovered directly from the infected specimens using a shotgun metagenomics approach. The genome length of ISKNV KU1 and KU2 was 111,487 and 111,610 bp, respectively. In comparison to closely related ISKNV strains, KU1 and KU2 contained nine unique genes, including a caspase-recruitment-domain-containing protein that is potentially involved in inhibition of apoptosis. Collectively, this study indicated that inland cultured Asian sea bass are infected by homologous ISKNV strains. This indicates that ISKNV genotype I should be prioritized for future vaccine research.

Introduction

Asian sea bass (*Lates calcarifer* Bloch, 1790), also known as barramundi, is a species native to Thailand and widely distributed in the Indo-West Pacific region from the Persian
Gulf to China, Taiwan, and northern Australia [14]. Asian sea bass is one of the most important marine cultured finfish in Australia and Asian countries including Indonesia, Singapore, Vietnam, and Thailand. Because of its significant economic potential, this fish species is expected by Thai governmental bodies and the private sector to be a major food fish along with tilapia (Oreochromis spp.) and hybrid catfish (Clarias gariepinus × C. batrachus). The total production of the Asian sea bass in Thailand was approximately 39,500 tons in 2018 [9]. Previously, Asian sea bass was cultivated mainly in the coastal area along the Gulf of Thailand and the Andaman Sea, where the fish can grow in open sea cages or earthen ponds with brackish/marine water. Presently, the main practice applied for Asian sea bass grow out has been shifted to inland freshwater pond systems, owing to the catadromous nature of the fish and advancements in the quality of pelleted feed, to align with the continual increase of consumer demand [14]. Asian sea bass farming in inland freshwater in Thailand is usually carried out in intensive systems. Although maximum yield can be expected with high stocking density, this aquaculture system poses a high risk for the emergence of infectious diseases due to the stressful conditions caused by the accumulation of metabolic waste, organic matter, and fluctuation in water quality.

Mortality and morbidity related to the distribution of infectious diseases have been reported to be a major cause of economic losses for the Asian sea bass farming industry worldwide. According to the literature, Asian sea bass are susceptible to numerous pathogens, including bacteria, e.g., Streptococcus iniae, Vibrio alginolyticus, Vibrio harveyi, and Photobacterium damselae subsp. damselae [2, 4, 12] and viruses such as viral nervous necrosis virus, Lates calcarifer herpesvirus, Lates calcarifer birnavirus, and megalocytiviruses [6, 36, 38, 39]. Among the potential viral pathogens, members of the genus Megalocytivirus (family Iridoviridae), such as red sea bream iridovirus (RSIV), turbot reddish body iridovirus (TRBIV), infectious spleen and kidney necrosis virus (ISKNV), and scale drop disease virus (SDDV, a distantly related member of the same genus) have been associated with several disease outbreaks in marine fish in many countries, e.g., red sea bass (Pagrus major), orange-spotted grouper (Epinephelus coioides), olive (Japanese) flounder (Paralichthys olivaceus), and turbot (Scophthalmus maximus). The clinical signs and lesions observed in megalocytivirus-infected fish can be very diverse, depending on pathogenic agent and host species. For example, fish infected with RSIV show lethargy, petechiae on gills, and enlargement of the spleen [26], ISKNV-infected fish exhibit a dark body color with pale gills, and red eyes [11], whereas SDDV infection in Asian sea bass is often associated with extensive scale loss and skin hemorrhage lesions [29, 41]. Megalocytivirus infection (i.e., ISKNV and SDDV) has been also reported to cause high mortality (55-77%) in Asian sea bass in Thailand [29, 48]. However, epidemiological information and molecular characterization of megalocytiviruses in Asian sea bass have been limited to marine environments, since inland freshwater culture is not yet widely practiced on a global scale. To date, only one incidence of coinfection with a pathogenic bacterium (Flavobacterium columnare) and SDDV has been reported in a freshwater system [29].

Recently, disease outbreaks have occurred in inland freshwater-based Asian sea bass grow-out farms located in the eastern and central parts of Thailand, with mortality rates ranging from 40 to 50%. The initial diagnoses indicated that a series of outbreaks may have involved ISKNV infection. Therefore, the aim of this study was to investigate the molecular characteristics of these ISKNV strains and their pathogenic role in Asian sea bass reared in a freshwater system. In addition, the genome sequence of this virus was examined using a metagenomic approach, and this, to our knowledge, is the first ISKNV genome from freshwater-cultured Asian sea bass ever sequenced.

Materials and methods

Disease history and sample collection

Fish farmers reported the occurrence of unknown diseases in Asian sea bass in seven freshwater grow-out farms located in Samut Sakhon, Samut Songkhram, and Chachoengsao provinces, Thailand, between February and November 2018. The diseased fish ranged from 20 to 30 g in weight. The cumulative mortality was reported by the operators to range from 40 to 50 percent. Moribund fish were euthanized by decerebration, and bacterial isolation was carried out onsite. Internal organs (kidney, spleen, and liver) were collected separately from each individual, preserved in 95% ethanol for PCR testing, and delivered on ice to the laboratory within 4 h. Ethanol-fixed tissues were maintained in a -20 °C freezer until further PCR assay, whereas fresh tissues for virus isolation were preserved at -80 °C. For histopathology, the collected tissue was immersed in 10% neutral buffered formalin at a ratio of 1:10 (w/v) for 24-36 h, followed by replacement with the same volume of 70% ethanol for long-term preservation. Preserved tissue samples were processed for standard histological analysis by dehydration, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) [21]. The H&E-stained tissues were then examined under a light microscope equipped with a digital camera. The negative control used for further assays was an apparently healthy Asian sea bass obtained from a different location. The animal use protocol for this study was approved by the Institutional Animal Care and Use Committee, Faculty of Fisheries, Kasetsart University (permit ID: ACKU61-FIS-055).
Bacterial isolation and identification

Direct isolation from external lesions (gills and skin) and internal organs (spleen, kidney, and liver) was conducted using two different media comprised of (i) tryptic soy agar (TSA, Himedia, India) supplemented with 5% sheep blood using a generalized medium and (ii) Anacker and Ordal agar (AOA, tryptone 0.5g/L, yeast extract 0.5g/L, sodium acetate 0.2 g/L, beef extract 0.2 g/L, and agar 10 g/L) supplemented with 1 μg mL⁻¹ tobramycin (Sigma-Aldrich, Singapore) using as a selective medium for Flavobacterium sp. [44]. Streaked plates were delivered to the laboratory and incubated at 28 °C until bacterial colonies were visible (24-48 h). Colonies were sub-cultured using the same kind of medium until a pure colony was obtained. Pure colonies of bacteria grown on either TSA or AOA medium were subjected to preliminary identification using Gram staining and primary biochemical assays including oxidase, catalase, oxidation-fermentation, and motility tests. Bacterial taxonomy was determined to the genus level based on Cowan and Steel’s manual [7].

Identification of ISKNV infection from tissue samples

DNA extraction from fish tissues

Screening of ISKNV infection was carried out for each individual fish collected in this study (n = 26). To extract DNA, the tissue sample was removed from the ethanol and homogenized using disposable polypropylene pestles. DNA was extracted using a Tissue Genomic DNA Extraction Mini Kit (Geneaid, Taiwan) according to the manufacturer’s instructions. The DNA concentration was determined using a NanoDrop analyzer (Titertek Berthold, Germany) and stored at -20 °C.

Identification of ISKNV by PCR

ISKNV-specific primers (Table 1) were used for screening by one-tube semi-nested PCR (snPCR) [11]. Each 25-µL PCR mixture contained 1X master mix (Go-Taq®-Green, Promega USA), 10 nM each working primer, and 100 ng of DNA template. The thermal cycling conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis followed by staining with Red Safe (Chembio, UK) and visualized under UV light. The expected PCR products from ISKNV-positive samples were either a single amplicon of 164 bp, two amplicons of 517 and 164 bp, or three amplicons of 754, 517, and 164 bp, representing light, moderate, and heavy infections, respectively, as described previously [11]. As a positive control, we used a DNA template extracted from an ISKNV-infected sample that was kindly provided by Centex, Mahidol University. Healthy Asian sea bass and nuclease-free water without DNA template served as the internal and negative control, respectively.

Sequence analysis of viral MCP and ATPase genes

DNA sequencing

Asian sea bass DNA extracts giving positive PCR results for ISKNV screening based on the one-tube snPCR were used for phylogenetic analysis. ISKNV-positive samples were

Table 1 Primers used in this study

| Primer name      | Primer sequence (5’–3’)                  | Feature                                      | Product size (bp) | Reference |
|------------------|------------------------------------------|----------------------------------------------|-------------------|-----------|
| ISKNV screening  |                                          |                                              |                   |           |
| Megalo-F         | AGATGATGGCATGCGCAGCG                     | Semi-nested PCR targeting the MCP gene        | 754               | [11]      |
| Megalo-1R        | TTGGACAGGCGCGGTAGT                      |                                              |                   |           |
| Megalo-2Rsn      | TACACGGGACTGCGCGC                       |                                              | 517               |           |
| Megalo-3Rsn      | CTGAAATGGAGCCAGCGGC                   |                                              | 164               |           |
| ISKNV sequencing |                                          |                                              |                   |           |
| MMCP-F           | ATGTCTCGCATCTCAGT                      | Sequencing of the MCP gene                    | 1362              | [20]      |
| MMCP-R           | TYACAGGATAGGGAAGCTGT                    |                                              |                   |           |
| MATPase-F        | ATGGAAATCMAAGTTGTCYTGT                  | Sequencing of the ATPase gene                 | 720               |           |
| MATPase-R        | TTACRCCACGCCACCGTTGTA                  |                                              |                   |           |
| ISKNV qPCR       |                                          |                                              |                   |           |
| Meg-MCP160F      | TCACAAAACAGACTGCGCATG                  | qPCR targeting the MCP gene                   | 190               | [27]      |
| Meg-MCP349R      | TAAATGACACCGACACCTCCTC                 |                                              |                   |           |
| Meg-MCP239P      | 6-FAM-FAM-TGGGCGCTGGTGTTA AGA          |                                              |                   |           |

TCCCCCTCCA-BHQ-1
selected randomly (only one sample per farm), and DNA was extracted from the liver. Two ISKNV genes, encoding for MCP and ATPase, were targeted for sequence analysis. Details about each primer set are shown in Table 1. The PCR conditions were described previously [20]. After agarose gel electrophoresis, the amplicons were purified using a Universal DNA Purification Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The purified DNA fragments were ligated into pGEM-T Easy Vector (Promega, WI, USA), and the construct was used to transform Escherichia coli JM109 competent cells as described by Russell and Sambrook [40]. Transformants were selected using Luria-Bertani agar (LB, Oxoid, UK) containing selective antibiotics and grown in LB broth prior to plasmid isolation using a NucleoSpin® Plasmid MiniPrep Kit (Macherey-Nagel, Germany). The extracted plasmid was submitted to a sequencing laboratory service (1st BASE Pte Ltd., Malaysia) for Sanger sequencing using pUC/M13 primers as described in Promega’s pGEM-T Easy Vector manual.

Phylogenetic analysis

Low-quality bases were trimmed manually based on a chromatogram of the raw sequences. The trimmed sequences were then assembled into contigs using ContigExpress. A homology search was performed using Megablast against NCBI’s nucleotide database for alignment and comparison to the other ISKNV strains. The MCP and ATPase genes of the ISKNV isolates from this study (n = 6, one sample per farm) were compared to those of other members of the genus Megalocytivirus, including ISKNV (n = 11-22), RSIV, (n = 22-43), and TRBIV (n = 7-14). The nucleotide sequences were aligned using ClustalW, and a phylogenetic tree was constructed using the maximum-likelihood method with the GTR+G+I substitution model and 1000 bootstrap replicates. Multiple sequence alignment and phylogenetic reconstruction were carried out using MEGA-X software [31].

Genome analysis

Library preparation and next-generation sequencing

Two samples with possibly severe ISKNV infection (one-tube snPCR yielded three distinct amplicons, as described in the previous section) from Samut Sakhon province were used for metagenomic shotgun sequencing. A Nextera XT Library Preparation Kit (Illumina, CA, USA) was used to construct a paired-end library from the extracted genomic DNA according to the manufacturer’s instructions, and high-throughput sequencing was performed using an Illumina HiSeq system with a 150-bp read length. Library construction and sequencing were carried out using the service provided by Novogene (Beijing, China).

ISKNV genome reconstruction and annotation

Processing of raw reads and de novo assembly were conducted as described in our previous publication [28]. The adaptor sequences and low-quality reads were filtered out from raw reads using Trimmomatic v0.39 [3]. Then, host-derived reads were discarded by mapping trimmed reads against an Asian sea bass reference genome sequence (NCBI assemblies accession no. GCA_001640805.1) using the -x function in the Bowtie2 program [33]. The remaining non-host reads were subjected to de novo metagenome assembly using MEGAHIT v 1.2.9 with a minimum length of output contigs of 1000 bp [34]. The generated assemblies were submitted to the web server version of the Kaiju program to predict the taxonomic identity of each contig [37]. Contigs assigned to ISKNV were annotated using Prokka v1.14.0 with Viruses Annotation mode, and the complete ISKNV reference genome sequence (GenBank accession no. NC_003494.1) was selected as the annotation template. The names “ISKNV KU1” and “ISKNV KU2” were assigned to the ISKNV-like contigs observed in our two samples. Visualization of the ISKNV KU1 and KU2 sequences as circular genomes was performed by uploading the annotated genomes to the web server version of CGview (http://cgview.ca/) [16].

Phylogenomic analysis and genome distance

The ISKNV KU1 and KU2 sequences were aligned to the ISKNV reference genome sequence using MAUVE progressive alignment [8]. The genome segments were rearranged manually to correspond to the ISKNV reference genome. To determine genetic distance, a multiple alignment of the new sequences with those of other megalocytiviruses was performed using the FFT-NS-i method in the MAFFT v7 online service [25], and MEGA X was used to calculate the distance based on the maximum-composite likelihood model. A phylogenomic network was generated using SplitsTree4 based on the alignment of the megalocytivirus genome sequences [22].

Identification of orthologous groups

OrthoFinder [13] was used to identify possible orthologs among four ISKNV genome sequences, including two ISKNV strains from this study (KU1 and KU2) and two closely related strains (RSIV-Ku and the ISKNV reference strain). The coding sequences (CDSs) of strain RSIV-Ku and the reference genome were obtained from GenBank under the accession nos. KT781098 and NC_003494, respectively. The OrthoFinder pipeline automatically
categorizes the proteins from tested subjects into orthologous groups (also called orthogroups) based on sequence similarity. In this study, proteins of ISKNV KU1 and KU2 predicted as ‘non-orthologous’ comparing to the reference strain and RSIV-Ku were subjected for further protein BLAST analysis.

**Propagation of ISKNV using the GF cell line**

ISKNV isolate KU1 was used for virus isolation. One gram of liver and spleen preserved at -80°C was pooled and homogenized in 10 ml of L15 medium (Gibco, CA, USA), followed by centrifugation at 9600 × g for 30 min at 4 °C. After centrifugation, the supernatant was collected and filtered using a sterile 0.22-µm membrane filter. The filtrate (0.5 mL) was then inoculated onto a monolayer grunt fin (GF) cells in a 5-mL flask for 2 hours, and the medium was replaced with fresh L15 medium supplemented with 10% fetal bovine serum (Gibco, CA, USA). The flasks were then incubated at 25 °C and observed under a microscope daily for 10 days to monitor the cytopathic effect (CPE). The virus was harvested by centrifugation of the cell culture supernatant at 1000 × g for 5 minutes at 4°C. Cell debris was discarded, and the supernatant containing ISKNV was collected and preserved in a -80 °C freezer until used in the challenge experiment.

The viral copy number of ISKNV in viral suspension was determined using a qPCR assay specific for ISKNV [27]. One hundred forty microlitres of the supernatant was used for DNA extraction by the phenol-chloroform method. The qPCR reaction consisted of 1X iTaq Universal Probes Supernatix (Bio-Rad), 3 µL of DNA template, 900 nM each forward and reverse primer (Meg-MCP160F and Meg-MCP349R) and 250 nM Meg-MCP239P probe in a total volume of 20 µL. The qPCR conditions included initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 64 °C for 1 min. Both virus detection protocols were performed using a Bio-Rad CFX Connect RealTime PCR System, and the product size was 190 bp.

**Pathogenicity test**

Healthy Asian sea bass (n = 150) were used in the experimental challenge test to fulfill Koch’s postulates. The fish, with an average weight of 22 g, were purchased from a local grow-out farm. Five fish were sampled randomly to verify their ISKNV-free status, using splenic tissue in a virus-specific one-tube snPCR as described above. ISKNV-free fish were used for pathogenicity tests after a week of acclimatization in a 3,000-L tank. Fish were divided into three groups (50 per group) comprising one control group and two challenge groups. The control group received sterile cell culture medium, whereas the challenge groups were injected intraperitoneally with 0.2 ml of virus suspension (low dose [10^{-2} dilution] or high dose [undiluted]). Injected fish was transferred to aerated 500-L tanks (50 per tank) with the water temperature and dissolved oxygen maintained at 30-32 °C and 5-7 ppm, respectively. Freshly dead and moribund fish were removed from the tank as soon as they were noticed. The liver, spleen, and kidney of affected fish were collected for histopathological investigation and ISKNV screening using one-tube snPCR. The daily mortality was recorded for 21 days, and a Kaplan-Meier survival curve with a logrank statistical test was made using the IBM SPSS Statistics 25 program.

**Results**

**Clinical manifestations in naturally infected fish**

The observed losses in grow-out Asian sea bass varied from 40 to 50% within 1-2 weeks after the disease was noticed. The moribund fish showed clinical signs such as lethargy and anorexia, while external lesions including darkened body color, pale gills, skin hemorrhage, and ascites were also observed. Internally, swollen spleen and hemorrhage of the liver and trunk kidney were the most abundant lesions (Supplementary Fig. S1). Histopathological manifestations included severe hemorrhage, inflammatory cell infiltration, accumulation of melanocytes, extensive necrosis of the skin and gills, and moderate tubular degeneration in the trunk kidney (Fig. 1A). Interestingly, the typical microscopic lesions associated with viral infection, namely, eosinophilic inclusion, and the hallmark histological changes associated with ISKNV infection, i.e., basophilic hypertrophied cells, were also detected in gill, liver, and kidney tissue.

Bacterial isolation was attempted from necrotic gills and skin lesions using the Flavobacterium-selective medium AOA, whereas generalized medium, TSA, was used for internal organs with apparent lesions. Bacterial colonies grown on AOA medium were yellowish in color and showed rhizoid morphology. Details about the collected fish samples and the observed lesions, together with the diagnostic results, are summarized in Supplementary Table S1.

**Identification of ISKNV**

Primary screening for ISKNV infection in Asian sea bass samples was done using a one-tube snPCR targeting the MCP gene. Out of 26 fish collected in this study, 20 were positive for ISKNV (6 out of 7 farms). Despite the fact that the samples were from the same farm, the degree of infection, as interpreted by the number of amplicons, varied among the positive samples. The electrophoresis
According to the original article describing the semi-quantitative nature of the one-tube snPCR [11], relatively high, moderate, and low viral copy numbers would result in three, two, and one amplicon, respectively, appearing in agarose gel electrophoresis.

**Phylogenetic analysis based on the MCP and ATPase genes**

The MCP and ATPase genes were amplified from six ISKNV-positive samples (one per farm) (Supplementary Fig. S3). The nucleotide sequences of the almost complete MCP (1,275 bp) and complete ATPase (720 bp) genes were identical among the ISKNV isolates of this study. Therefore, we submitted the MCP and ATPase gene sequences from only a single ISKNV isolate to the GenBank database under the accession nos. MW269579 and MW269580, respectively. BLAST analysis of the MCP and ATPase gene sequences indicated that the ISKNV isolate from this study was identical to several ISKNV strains available in NCBI’s nucleotide sequence database, including strains AFIV-16 (MK689685) and RSIV-Ku (KT781098). Phylogenetic trees based on the MCP and ATPase genes of ISKNV, RSIV, and TRBIV (members of the genus *Megalocytivirus*) are shown in Figure 2. In the MCP-based tree, the ISKNV isolates clustered into two different subclades, corresponding to genotypes I and II, and the isolates from this study were all in genotype I. In contrast, there was no clear sub-clustering among the ISKNV isolates in the ATPase-based tree, which could be due to the smaller number of sequences.

**Genome features**

Asian sea bass samples from farms E and F ($n = 2$) in Samut Sakhon province with a relatively high viral load (as shown by one-tube snPCR) were selected for metagenomic shotgun sequencing. Non-host reads were assembled *de novo* into 1,482 and 1,030 contigs, respectively. Taxonomic identification using the Kaiju web server indicated that, from each sample, only the longest contig was identified as ISKNV. The length (111 kb) and GC content (54.8%) of these contigs were almost identical to those of the ISKNV reference genome sequence. These virus
strains were named ‘ISKNV KU1’ and ‘ISKNV KU2’, and a summary of their genomic characteristics is shown in Table 2. Both ISKNV genome sequences were submitted to the GenBank database under the accession numbers MT128666 and MT128667. Their genome map is shown in Figure 3.

Phylogenomic analysis

The neighbor-net network analysis was able to differentiate 15 members of the genus Megalocytivirus into three distinct clusters, namely, the RSIV, TRBIV, and ISKNV groups (Fig. 2C). The genome sequences of ISKNV KU1 and KU2 were almost identical to those of the ISKNV reference strain and the RSIV-Ku strain, with minimal genome distances of 0.02% and 0.06%, respectively. In contrast, the genome distance between distinct clusters can be as high as 5.5-9.4%. A reticulated pattern was clearly observed among the ISKNV and RSIV groups, which indicated possible genetic recombination within this cluster.

Orthology between ISKKNV KU1, KU2, and reference strains

OrthoFinder categorized a total of 499 genes, obtained from four distinct ISKNV strains (KU1, KU2, RSIV-Ku, and reference strain), into 124 orthogroups. Among these, 88 orthogroups (70.9%) were presented in all ISKNV strains. The RSIV-Ku and reference strains possessed 13 and 18 genes that were not assigned to any orthogroup (unique genes). Seven orthogroups were found exclusively in the strains KU1 and KU2 but were absent in the other two ISKNV strains. The strain KU1 also carried two genes that are unique to its genome. Most of these KU1- and KU2-specific genes were predicted to encode hypothetical proteins and were similar to genes of other viruses in the genus Megalocytivirus, including angelfish iridovirus AFIV-16, scale drop disease virus, Banggai cardinalfish iridovirus, and red sea bream iridovirus. There was one protein from the KU1 (QQZ00456) and KU2 (QQZ00673) strains that were almost identical to the caspase recruitment domain (CARD) containing protein of angelfish iridovirus AFIV-16. Details about the nine genes present only in strains KU1 and KU2 are shown in Table 3, together with the BLAST protein analysis results.

Viral pathogenicity assay

Healthy Asian sea bass reared in freshwater were challenged intraperitoneally with GF-cell-grown ISKNV at a dosage of $2.9 \times 10^5$ (high dose) or $2.9 \times 10^3$ (low dose) copies/fish. The onset of mortality for the high-dose and low-dose groups occurred at 5- and 8-days post-challenge (dpc), respectively. The daily mortality in both groups was 1-3 fish per day, except at 18 and 19 dpc, when 5-8 fish in the high-dose group died. In the low-dose group, no more deaths occurred after 19 dpc. The cumulative mortality at the end of the experiment (21 dpc) was 90% for the high-dose group (45 out of 50 fish), which was significantly higher than for the low-dose group (50%, 25 out of 50 fish [$p < 0.05$]). The experimentally infected fish tested positive in the one-tube snPCR. These infected fish samples yielded three specific amplicons, suggesting a relatively high viral load in the liver (Supplementary Fig. S5). Two fish in the control group (4%) died a day after mock infection, which could have been due to injection injury, since the one-tube snPCR showed negative results. The survival curve is shown in Figure 5.

The experimentally infected fish showed clear pathological changes similar to those observed in naturally infected fish. Externally, moribund fish exhibited a darkened body color and with pale gills. Microscopically, hematopoietic tissues showed apoptosis (pyknotic nuclei) and cytoplasmic inclusion bodies, tubular degeneration was found in the kidney, and gill lamella contained showed hypertrophic basophilic cells (Fig. 1C and D).

Discussion

In this study, ISKNV was identified in diseased Asian sea bass cultured in an inland freshwater system. The most common histopathological lesions in ISKNV-infected Asian sea bass collected in this study, i.e., severe necrosis and the appearance of basophilic inclusion bodies in gills, liver, and kidney, was consistent with the pathognomonic lesions of
megalocytivirus infection (hypertrophy/megalocytosis in gill and liver) reported previously [32, 45]. In this study, the histopathological manifestations in the spleen, kidney, and liver also suggested viral tropism for these hematopoietic organs which, to some extent, may result in immune incompetence/suppression and increased susceptibility to opportunistic infections. Coinfection with Flavobacterium columnare and SDDV, another member of the genus Megalocytivirus, in grown-out Asian sea bass was observed in our previous investigation [29]. Considering that coinfection with ISKNV and potential bacterial pathogens such as Aeromonas sp., Streptococcus sp., and Flavobacterium sp. was identified in three out of seven disease incidences in this study, it is feasible that simultaneous infection is relatively common in cases of natural disease in Asian sea bass. Simultaneous infections, also called concurrent infections, occur frequently in farmed fish and might be more common than single infections [10]. In our case, we speculated that simultaneous infection by ISKNV and bacteria could influence the large diversity of clinical appearances, which range from inapparent to severe, e.g., extensive hemorrhage. In this study, it was not clear whether ISKNV or a bacterial pathogen was the primary pathogen, and their relative contributions in vivo require further investigation.

In addition to Asian sea bass, ISKNV has been reported in farmed Nile tilapia and ornamental fish in Thailand as well [1, 10, 47]. The clinical appearances of these ISKNV-infected fishes also similar to those observed in this study. The ISKNV from ornamental fish were genetically classified as genotype I [1] based on the MCP gene sequence, similar to the ISKNV strains in this study. In previous studies, disease outbreaks were observed in the same region (central Thailand) where the ISKNV-positive Asian sea bass samples were collected in this study. Therefore, the possibility of cross-species transmission between these freshwater-farmed fishes, although not yet documented officially, should be of concern (particularly in the area with a history of outbreaks), since sharing of water resources among farms cannot be easily avoided.

ISKNV has also been identified in various marine fish species in other Southeast Asian countries, including Indonesia, Vietnam, Malaysia, and Singapore [11, 23, 39, 46]. Most of the ISKNV isolates found in these countries were genotype I, like in this study, indicating that this ISKNV genotype is widespread in Southeast Asia. According to the original article describing ISKNV genotyping [15], ISKNV can be classified into three genotypes (I, II, and III) based on the diversity of the MCP gene. These genotypes were later assigned to three clusters comprising the RSIV, ISKNV, and TRBIV groups in the same virus species, as described by the International Committee on Taxonomy of Viruses (ICTV, https://talk.ictvonline.org/ictv-reports/ictv_online_report/dddna-viruses/w/iridoviridae/615/genus-megalocytivirus).

To date, ISKNV genotype II has been reported in orbiculate batfish (Platx orbicolor), Banggai cardinalfish (Pterapogon kauderni), and marble goby (Oxyeleotris marmorata) in Indonesia, Japan, the USA, and China [32, 43, 49], while Asian sea bass mortality associated with ISKNV genotype II infection has been reported in Southern China and Vietnam [11, 50]. According to the phylogenetic analysis conducted in this study, the genetic diversity among the current ISKNV isolates was rather low, as all of the samples were classified as genotype I and shared 100% sequence identity in both the MCP and ATPase genes. The MCP, ATPase, and DNA polymerase genes are generally used to determine genetic relationships between megalocytivirus due to their evolutionary conservation [30, 32]. However, a relationship between genetically similar strains cannot be inferred, at least for the current collection of virus isolates, relying on these conserved genes alone. To date, in-depth epidemiological information regarding the ISKNV genotype distribution in Thailand and neighboring countries in Southeast Asia is scarce. Thus, an efficacious ‘regional’ vaccine against ISKNV in Southeast Asia cannot be developed unless more epidemiological data are collected. For further investigations, other molecular markers, such as the four ankyrin repeat domains [30], could be added to the phylogenetic comparisons which would allow intra-genotype diversity to be analyzed on a finer scale.

Isolation of ISKNV from infected Asian sea bass specimens using the GF cell line was successful in this study. Application of GF cells for the propagation of ISKNV simultaneously with nervous necrosis virus was described in our previous publication [24]. However, the results of this study were slightly inconsistent with those of another publication in terms the onset of CPE [48]. In the previous study, CPE was not observed clearly until 14 dpi, whereas it was observed already at 5 dpi in this study and was quite pronounced at 7 dpi. This discrepancy might have been due to the difference in the initial inoculation dose, since, in this study, we intentionally selected a specimen with a potentially high viral titer (as indicated by one-tube snPCR). Recently, the alternative cell line GS-1, originating from orange-spotted grouper (Epinephelus coioides) fibroblasts, was reported to be susceptible to ISKNV infection, allowing the titer to reach 10^{5.2} TCID_{50}/ml within 7 days. However, a
direct comparison of the ISKNV replication kinetics in these two cell lines has not been reported [19].

The pathogenicity assay conducted in this study was able to fulfill Koch’s postulates, showing that ISKNV is pathogenic to freshwater-reared Asian sea bass. The results suggested that the pathogenicity of ISKNV and onset of mortality depend on the infection dose (Fig. 5). The cumulative mortality rates were similar to those seen in previous investigations (90, 77, and 85.89%), as was the onset of mortality (5-9 dpc) [48, 50]. It should be mentioned that the fish examined in this study (juvenile, 22 g weight) were larger than those in the previous report (fingerling, 3.5 g weight) [48], indicating that ISKNV is able to cause high mortality in Asian sea bass at various life stages. Regarding clinical signs, it is worth mentioning that the experimental animals exhibited only the typical darkened body and pale gills, in agreement with those described in the recent study [50]. Other obvious external lesions, such as scale loss, muscle necrosis, and hemorrhage, were observed only in naturally infected fish. This difference in clinical manifestations could be due to coinfection with bacterial pathogens in natural cases (Supplementary Table S1). This emphasizes that diagnosis of field outbreaks should be performed with caution, and multiple approaches, including pathogen isolation, PCR, and histopathology, should be applied when possible to get a comprehensive understanding of the disease scenario.

Recently, we showed that metagenomic shotgun sequencing was able to produce a draft genome sequence of SDDV directly from the infected specimen, without a need for culturing the virus [28]. In this study, the same analytical approach was implemented, and the complete genome sequences of ISKNV KU1 and KU2 were reconstructed.

| Genome features of ISKNV KU1 and KU2 compared to those of the reference genome |
|---------------------------------------------------------------|
| **GenBank accession number** | MT128666 | MT128667 | NC_003494 |
| **Genome length** | 111,487 bp | 111,610 bp | 111,362 bp |
| **Number of contigs (coverage depth)** | 1 (47x) | 1 (56x) | 1 (na) |
| **GC content** | 54.8% | 54.8% | 54.8% |
| **Number of genes** | 122a | 123a | 125 |
| **Host** | Asian sea bass (Lates calcarifer) | Asian sea bass (Lates calcarifer) | Mandarin fish (Siniperca chuatsi) |
| **Nucleotide sequence identity compared to the reference genome** | 99.98% | 99.98% | na |

*a number of genes predicted using Prokka v 1.14.0
na, not applicable
Table 3 Proteins of ISKNV strains KU1 and KU2 that are non-orthologous to those of ISKNV RSIV-Ku and the reference strain

| Protein ID | Length (aa) | Best protein BLAST hit | Accession number | Query coverage, Identity (%) | E-value |
|------------|-------------|------------------------|------------------|-----------------------------|---------|
| QQZ00456.1 | 101         | Caspase recruitment domain-containing protein [angelfish iridovirus AFIV-16] | QIQ54447.1       | 100, 99.01                  | 5.00E-68 |
| QQZ00464.1 | 80          | Hypothetical protein [scale drop disease virus] | QL60734.1        | 86, 37.84                   | 5.00E-06 |
| QQZ00514.1 | 122         | Hypothetical protein [Banggai cardinalfish iridovirus] | QO77200.1        | 100, 100                    | 6.00E-84 |
| QQZ00518.1 | 97          | Not found              | -                | -                           | -       |
| QQZ00523.1 | 158         | ORF068 [angelfish iridovirus AFIV-16] | QIQ54512.1       | 100, 100                    | 1.00E-115 |
| QQZ00536.1 | 34          | Not found              | -                | -                           | -       |
| QQZ00568.1 | 95          | Not found              | -                | -                           | -       |
| QQZ00479.1 | 316         | ORF025 [angelfish iridovirus AFIV-16] | QIQ54469.1       | 100, 61.59                  | 3.00E-37 |
| QQZ00505.1 | 63          | Hypothetical protein ORF050 [red seabream iridovirus] | -                | -                           | -       |

*aCategorization of the orthologous group was carried out using the OrthoFinder program*

Fig. 4 Photomicrograph of a grunt fin (GF) cell monolayer inoculated with filtrate extracted from the tissue of ISKNV-infected (right panel) Asian sea bass. Control GF cells (left panel) were cultured under conditions identical to those of ISKNV-infected cells. Arrows indicate cytopathic effect (vacuolization) observed in the ISKNV-infected cells at 7 days post-inoculation.

with an acceptable coverage depth of 47-56x. A genome-scale phylogenetic network (Fig. 2C) showed a reticular pattern within the ISKNV and RSIV groups, implying that genetic recombination had occurred between the members of these groups. In fact, one of the ISKNV group members, RSIV-Ku, was similar to GSIV-K1 in 7% of its genome,
sugest suggesting that this strain is an ISKNV/RSIV recombinant [42]. Here, possible recombination between ISKNV KU1/KU2 and other megalocytiviruses was screened using the RDP4 program [35], but no evidence of recombination was found in their genomes (data not shown). Orthology analysis showed a surprisingly large number of unassigned orthogroups (unique genes) among the genomes of ISKNN KU1/KU2, RSIV-Ku, and the reference strain. It is predicted that strain RSIV-Ku, a natural recombinant virus, may possess numerous unique genes, since its genome has a 7.8-kb-long region resembling RSIV genotype II rather than ISKNV [42]. In the case of the ISKNV reference strain, 18 genes were not assigned to any orthologous groups, although the core genome similarity in comparison to ISKNV KU1/KU2 was as high as 99.98%. This could be explained by differences in the genome annotation methods used for CDS prediction in the ISKNV reference strain and KU1/KU2 genomes. Protein-encoding sequences of the ISKNV reference strain were identified by querying sequences through a protein domain database [17], whereas a program based on an unsupervised machine learning algorithm (Prokka) was employed in the case of ISKNV KU1/KU2. Among the non-orthologous genes present in ISKNV KU1/KU2, a caspase recruitment domain (CARD)-containing protein was identified. This protein was highly similar to those found in angelfish iridovirus AFIV-16, which also belongs to the ISKNV genotype I [27], isolated from the angelfish Pterophyllum scalare in Southeast Asia. CARDs are well-known interaction motifs involved in regulation of inflammation and apoptosis [18]. CARD proteins have been demonstrated in vitro to have inhibitory effects on apoptosis in grouper iridovirus, a member of the genus Ranavirus of the family Iridoviridae [5]. However, the role of the CARD protein in the molecular pathogenesis of ISKNV and whether it is involved in inhibition of apoptosis, remain to be elucidated.

In summary, homologous strains of ISKNV genotype I were identified as causative agents of mass mortality in freshwater-cultured Asian sea bass in eastern and central Thailand by a combination of histopathology, molecular analysis, and pathogenicity assays. The complete genome sequences of two ISKNV isolates, KU1 and KU2, were determined using a metagenomics approach. The genome information, as well as the virus archive collected in this study, could be useful for evolutionary analysis and selection of potential genotype I vaccine candidates for the sustainable prevention of ISKNV outbreaks in the future.

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Author contributions  Pattarawit Kerdee: Carried out the experiment, wrote the manuscript. Nguyen Dinh-Hung: Performed analysis (disease diagnosis). Ha Thanh Dong: Performed the analysis (histopathology, cell culture and qPCR). Ikuo Hirono: Contributed analysis tool (GF cell line). Chayanit Soontara: Performed analysis (disease diagnosis and experimental challenge). Nontawith Areechon: Conceived of overall direction and planning. Prapanusk Srisapoome: Collected the samples from natural outbreaks. Pattanapon Kayansamruaj: Designed experiment, wrote the manuscript.

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References

1. Baoprasertkul P, Kaenchan N (2019) Distribution and detection of megalocytivirus in ornamental fish in Thailand. J Fish Environ 43:11–24
2. Barnette P, Labella A, Alonso C, Mancho D, Castro D, Borrero J (2009) The first isolation of Photobacterium damselae subsp. damselae from Asian seabass Lates calcarifer. Fish Pathol 44:47–50
3. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120
4. Bromage ES, Thomas A, Owens L (1999) Streptococcus iniae, a bacterial infection in barramundi Lates calcarifer. Dis Aquat Org 36:177–181
5. Chen C-W, Wu M-S, Huang Y-J, Lin P-W, Shih C-J, Lin F-P, Chang C-Y (2015) Iridovirus CARD protein inhibits apoptosis through intrinsic and extrinsic pathways. PLoS ONE 10:e0129071
6. Chen J, Toh X, Ong J, Wang Y, Teo KH, Lee B, Wong PS, Khor D, Chong SM, Chee D, Wee A, Wang Y, Ng MK, Tan BH, Huangfu T (2019) Detection and characterization of a novel marine birnavirus isolated from Asian seabass in Singapore. Virol J 16:71
7. Cowan ST, Steel KJ, Barrow GI, Feltham RKA (1993) Cowan and Steel’s manual for the identification of medical bacteria, 3rd edn. Cambridge University Press, Cambridge
8. Darling AE, Mau B, Perna NT (2010) progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS ONE 5:e11147
9. DOF (2018) Fisheries statistics of Thailand 2018. In: Fisheries development policy and strategy division. https://www.fisheries.go.th/strategy-stat/themeWeb/books/2559/1/yearbook_2559.pdf. Accessed Jan 2021
10. Dong HT, Nguyen VV, Le HD, Sangsuriya P, Jitrakorn S, Saksmerprome V, Senapin S, Rodkhum C (2015) Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (Oreochromis niloticus) farms. Aquaculture 448:427–435
11. Dong HT, Jitrakorn S, Kayansamruaj P, Pirarat N, Rodkhum C, Rattanarojpong T, Senapin S, Saksmerprome V (2017) Infectious spleen and kidney necrosis disease (ISKNV) outbreaks in farmed barramundi (Lates calcarifer) in Vietnam. Fish Shellfish Immunol 68:65–73
12. Dong HT, Taengphu S, Sangsuriya P, Charoensapsri W, Phiw saiya K, Sornwatana T, Khunrae P, Rattanarojpong T, Senapin S (2017) Recovery of Vibrio harveyi from scale drop and muscle necrosis disease in farmed barramundi, Lates calcarifer in Vietnam. Aquaculture 473:89–96
13. Emms DM, Kelly S (2019) OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol 20:238
14. FAO (2021) Cultured Aquatic Species Information Programme. Lates calcarifer. cultured aquatic species information programme. Text by Rimmer, M.A. In: FAO Fisheries Division [online]. Rome. Updated. [Cited 25 March 2021]. http://www.fao.org/fishe ry/culturedspecies/Lates_calcarifer/en. Accessed Jan 2021
15. Fu X, Li N, Liu L, Lin Q, Fang W, Lai Y, Jiang H, Pan H, Shi C, Wu S (2011) Genotype and host range analysis of infectious spleen and kidney necrosis virus (ISKNV). Virus Genes 42:97–109
16. Grant JR, Stothard P (2008) The CGView Server: a comparative genomics tool for circular genomes. Nucleic Acids Res 36:W181–184
17. He JG, Deng M, Weng SP, Li Z, Zhou SY, Long QX, Wang XZ, Chan SM (2001) Complete genome analysis of the mardarin fish infectious spleen and kidney necrosis iridovirus. Virol 291:126–139
18. Hofmann K, Bucher P, Tschopp J (1997) The CARD domain: a new apoptotic signalling motif. Trends Biochem Sci 22:155–156
19. Huang S-M, Kuo S-T, Kuo H-C, Chang S-K (2018) Assessment of fish iridoviruses using a novel cell line GS-1, derived from the spleen of orange-spotted grouper Epinephelus coioides (Hamilton) and susceptible to ranavirus and megalocytivirus. J Vet Med Sci 80:1766–1774
20. Huang SM, Tu C, Tseng CH, Huang CC, Chou CC, Kao HC, Chang SK (2011) Genetic analysis of fish iridoviruses isolated in Taiwan during 2001–2009. Arch Virol 156:1505–1515
21. Humason GL (1979) Animal tissue techniques, 4th edn. W.H. Freeman, San Francisco. p 661. https://doi.org/10.5962/bhl.title.5890
22. Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267
23. Jeong JB, Kim HY, Jun LJ, Lyu JH, Park NG, Kim JK, Jeong HD (2008) Outbreaks and risks of infectious spleen and kidney necrosis virus disease in freshwater ornamental fishes. Dis Aquat Org 78:209–215
24. Jitrakorn S, Gangnonngiw W, Bunnontae M, Manajit O, Rattanarojpong T, Chaiwisuthangkura P, Dong HT, Saksmerprome V (2020) Infectious cell culture system for concurrent propagation and purification of Megalocytivirus ISKNV and nervous necrosis virus from Asian Sea bass (Lates calcarifer). Aquaculture 520:734931
25. Katoh K, Rozewicki J, Yamada KD (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20:1160–1166
26. Kawakami H, Nakajima K (2002) Cultured fish species affected by infectious spleen and kidney necrosis virus disease (ISKNV) outbreaks in farmed barramundi (Lates calcarifer) in Vietnam. Fish Shellfish Immunol 68:65–73
27. Kawato Y, Mohr PG, Crane MSJ, Williams LM, Neave MJ, Cummins DM, Dearnley M, Crameri S, Holmes C, Hoad J, Moody...
NJG (2020) Isolation and characterisation of an ISKNV-genotype megalocytivirus from imported angelfish *Pterophyllum scalare*. Dis Aquat Org 140:129–141. https://doi.org/10.3354/dao03499

28. Kayansamruaj P, Soontara C, Dong HT, Phiwaiya K, Senapin S (2020) Draft genome sequence of scale drop disease virus (SDDV) retrieved from metagenomic investigation of infected barramundi, *Lates calcarifer* (Bloch, 1790). J Fish Dis 43:1287–1298

29. Kerddee P, Dong HT, Chokmangmeepisarn P, Rodkhum C, Srisapoome P, Areechon N, Del-Pozo J, Kayansamruaj P (2020) Simultaneous detection of scale drop disease virus and *Flavobacterium columnare* from diseased freshwater-reared barramundi *Lates calcarifer*. Dis Aquat Org 140:119–128

30. Kim KL, Lee ES, Do JW, Hwang SD, Cho M, Jung SH, Jee BY, Kwon WI, Do Jeong H (2019) Genetic diversity of *Megalocytivirus* from cultured fish in Korea. Aquaculture 509:16–22

31. Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549

32. Kurita J, Nakajima K (2012) Megalocytiviruses. Viruses 4:521–538

33. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359

34. Li D, Liu CM, Luo R, Sadakane K, Lam TW (2015) MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31:1674–1676

35. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B (2015) RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol 1:vey003

36. Meemetta W, Domingos JA, Dong HT, Senapin S (2020) Development of a SYBR Green quantitative PCR assay for detection of *Lates calcarifer* herpesvirus (LCHV) in farmed barramundi. J Virol Methods 285:113920

37. Menzel P, Ng KL, Krogh A (2016) Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun 7:11257

38. Parameswaran V, Kumar SR, Ahmed VPI, Hameed ASS (2008) A fish nodavirus associated with mass mortality in hatchery-reared Asian sea bass, *Lates calcarifer*. Aquaculture 275:366–369

39. Sah Putra B, Hick PM, Hall E, Whittington RJ, Kharirul R,varianti N, Becker JA (2020) Prevalence of infectious spleen and kidney necrosis virus (ISKNV), nervous necrosis virus (NNV) and ectoparasites in juvenile *Epinephelus* spp. farmed in Aceh, Indonesia. Pathogens 9:578

40. Sambrook J, Russell RW (2001) Molecular cloning: A laboratory manual, 3rd edn, 3rd edn. Cold Spring Harbor, Cold spring Harbor Laboratory Press

41. Senapin S, Dong HT, Meemetta W, Gangnonngiw W, Sangsuriya P, Vanichviriyakit R, Sonthi M, Nuangsang B (2019) Mortality from scale drop disease in farmed *Lates calcarifer* in Southeast Asia. J Fish Dis 42:119–127

42. Shiu JY, Hong JR, Ku CC, Wen CM (2018) Complete genome sequence and phylogenetic analysis of megalocytivirus RSIV-Ku: A natural recombination infectious spleen and kidney necrosis virus. Arch Virol 163:1037–1042

43. Sriwanayos P, Francis-Floyd R, Stidworthy MF, Petty BD, Kelley K, Waltzek TB (2013) Megalocytivirus infection in orbiculate batfish *Platax orbicularis*. Dis Aquat Org 105:1–8

44. Starliper C (2008) General and specialized media routinely employed for primary isolation of bacterial pathogens of fishes. J Wild Dis 44:121–132

45. Subramaniam K, Shariff M, Omar AR, Hair-Bejo M (2012) Megalocytivirus infection in fish. Rev Aquac 4:221–233

46. Subramaniam K, Shariff M, Omar AR, Hair-Bejo M, Ong BL (2014) Detection and molecular characterization of infectious spleen and kidney necrosis virus from major ornamental fish breeding states in Peninsular Malaysia. J Fish Dis 37:609–618

47. Suebsing R, Pradeep PJ, Jitrakorn S, Sirithammajak S, Kampeera J, Turner WA, Saksmerprome V, Wityachumnarnkul B, Kiathoomchat W (2016) Detection of natural infection of infectious spleen and kidney necrosis virus in farmed tilapia by hydroxynaphthol blue-loop-mediated isothermal amplification assay. J Appl Microbiol 121:55–67

48. Thanasaikiri K, Takano R, Fukuda K, Chaweepack T, Wongtavatchai J (2019) Identification of infectious spleen and kidney necrosis virus from farmed barramundi *Lates calcarifer* in Thailand and study of its pathogenicity. Aquaculture 500:188–191

49. Wang Q, Zeng WW, Li KB, Chang OQ, Liu C, Wu GH, Shi CB, Wu SQ (2011) Outbreaks of an iridovirus in marbled sleepy goby, *Oxyeleotris marmoratus* (Bleeker), cultured in southern China. J Fish Dis 34:399–402

50. Zhu Z, Duan C, Li Y, Huang C, Weng S, He J, Dong C (2021) Pathogenicity and histopathology of infectious spleen and kidney necrosis virus genotype II (ISKNV-II) recovering from mass mortality of farmed Asian seabass, *Lates calcarifer*, in Southern China. Aquaculture 534:736326

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