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Infectious spleen and kidney necrosis virus (ISKNV) spillover from naturally infected tilapia to native free-living fish

Deborah Jacob Freire da Paz

JABOTICABAL – SÃO PAULO
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"We ourselves feel that what we are doing is just a drop in the ocean. But if the drop was not in the ocean, I think the ocean would be less because of the missing drop."

Mother Theresa

DEDICATION

I dedicate this master's degree to my mother Lourdes Jacob, the most beautiful flower in my garden.

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ABSTRACT

Viruses are responsible for high mortality rates in many freshwater and marine fish species in aquaculture. In the Brazilian scenario, the infectious spleen and kidney necrosis virus or ISKNV has been causing problems in the production of Nile tilapia. Diagnoses and research about this pathogen in the country are recent because it is an emerging virus in our territory. We know that this virus is present in most Brazilian tilapia farms, and our hypothesis is that spillover events are occurring between tilapia to native wild fishes, thus facilitating the spread of the virus to the ecosystem, potentially causing a greater permanence of viable viruses in the environment, viral outbreaks in other locations, and more frequently mortality events. This work assessed the health status of Nile tilapia from commercial fish farms and free-living fish near net cages through ectoparasite analysis, histopathology, and molecular diagnosis for ISKNV. The results demonstrated that 63.2% of tilapia were infected by ISKNV, while 1.1% of wild fish were positive for ISKNV. The ISKNV positive fish was a yellow piranha (*Serrasalmus Maculatus).* The results confirmed the infection of tilapia by the virus and confirmed the pathogen spillover hypothesis. This study provides important information about the epidemiology of the ISKNV in Brazil and its possibility to infect native wild Brazilian fish species. Future studies will have to evaluate the impact of ISKNV on wildlife and its epidemiological importance for the maintenance of the disease in Brazilian tilapia farms.

Keywords: Brazilian native fish, iridovirus, tilapia, viral ecology, viral infection.

1. INTRODUCTION

Aquaculture is the fastest-growing food-production sector in the world and its production is dominated by a small number of species, including carp and tilapia (FAO, 2021). The Nile tilapia (*Oreochromis niloticus*) is the second most cultured fish species in the world (followed after carp) and it represents one of the most important species in the aquaculture world, with a global production estimated in 4,500,000 tons in 2018 (FAO, 2020). In Brazil, this specie is the most important for aquaculture and represents 63% (approximately 758.000 tons) of fish production (Peixe BR, 2023). In the last decade, tilapia production increased from 150.000 to 400.000 tons, making Brazil the 4th largest Nile tilapia producer in the world (FAO, 2021). In the southeastern, central-western, and northeastern regions of Brazil, tilapia production is carried out intensively or super intensively, in net cages located in dikes, rivers or river branches, sharing space with other fish species, mainly native.

Consequently, the intensification of Nile tilapia production has negatively affected the sector due to the occurrence of diseases, most importantly viral diseases, such as the infectious spleen and kidney necrosis virus (ISKNV). It is an emerging pathogen that causes high mortality and morbidity in fish worldwide, affecting over 160 species of fish, including tilapia (Jung et al. 2000, Kurita et al. 2002, Girisha et al. 2021, Gibson-Kueh et al. 2003, Jeong et al. 2003, Nakajima et al. 2005, Wang et al. 2007, Song et al. 2008, Wang et al. 2009, Rimmer et al. 2015, de Lucca Maganha et al. 2018, WOAH, 2022). This virus was first identified in tilapia in Brazil in 2019, and since then, this disease has been causing significant economic losses in all states that produce tilapia (Figueiredo et al., 2021; MAPA, 2020).

The Brazilian rivers have a great diversity of fishes that are affected for various natural anthropogenic impacts or both. Amongst these factors, the spillover of disease (transmission of a pathogen from an infected species to another potential host species) negatively affects the population structure and diversity of wild fish (Lovy et al., 2018).

The fish production without the appropriate pathogen screening and quarantine serves to spread of ISKNV to other species (Rimmer et al., 2015). Therefore, we believe that the emergent viral disease ISKNV can be transmitted to wild fish from Nile tilapia production by a series of horizontal transmission mechanisms.

Hence, the objective of the study was to confirm the hypothesis of tilapia naturally infected by ISKNV, can transmit the virus to native wild Brazilian fishes that resides in rivers adjacent to commercial net caged tilapia farms.

2. LITERATURE REVIEW

2.1. Brazilian aquaculture production

Brazilian aquaculture production has increase substantially in the last decades, with an increase of 224.13 tons in the last six year (from 578.800 tons in 2014 to 802.930 tons in 2020) making Brazil the 13th largest fish producer in the World and the 4th largest producer of Nile tilapia (FAO, 2021; Peixe BR, 2021).

In 2022, Brazil produced around 860.355 tons of freshwater fish, with a growth of 2.3% compared to the previous year. The aquaculture in Brazil is dominated by Nile tilapia, tambaqui (*Colossoma macropomum*), pacu (*Piaractus mesopotamicus*), and hybrids of tambaqui with pacu (IBGE, 2022). Nile tilapia is the most important species with an estimated yearly production of 550,060 tons (Peixe Br, 2023). Tilapia is produced due to its favorable organoleptic characteristics, easy reproduction, fast growth, high protein quality, and disease resistance (FAO, 2021). The main states producing this species are Paraná (187.800 t), São Paulo (77.300 t), Minas Gerais (51.700 t), and Santa Catarina (42.500 t) (Peixe BR, 2023).

However, with the increase intensification of tilapia farming in Brazil, the sector has been negatively impacted by infectious diseases (Sebastião et al., 2015, 2017; Chideroli et al., 2017; Delphino et al., 2019) such as infectious spleen and kidney necrosis virus (ISKNV), which was identified for the first time in tilapia in Brazil in 2019 (Viadanna et al., 2019 – unpublished data), confirmed in 2021 by the World Organization of Animal Health (WOAH) and published in 2021 (Figueiredo et al., 2021). In 2021, it is estimated that 219,500 fish died as a result of this disease, which represents a significant direct economic loss (WOAH, 2021).

2.2. Infectious Spleen and Kidney Necrosis Virus (ISKNV): Taxonomy and Epidemiology

ISKNV is a large, double-stranded DNA virus within the genus *Megalocytivirus* (family *Iridoviridae*, subfamily *Alphairidovirinae*), and possesses a circularly permuted and terminally redundant genome (ICTV, 2023). The family *Iridoviridae* is subdivided into two subfamilies: *Alphairidovirinae* including genus *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus*, that causes disease in reptile, amphibian, and fish; and *Betairidovirinae* including genus *Chloriridovirus*, *Decapodiridovirus*, and *Iridovirus* that causes disease in invertebrates (Zhu et al., 2021, ICTV, 2023). The genus *Megalocytivirus* has two species, *Infectious Spleen and Necrosis Virus*, and *Scale Drop Disease Virus* (ICTV, 2023). The genomes of ISKNV strains vary from 110,104 bp to 112,710 bp (Kawato et al. 2020, Shi et al. 2010), with a G+C content ranging from 53% to 55% (ICTV, 2023, Kurita and Nakajima, 2012). Iridovirus species display high genetic density, short intergenic regions, and contain repetitive sequences (ICTV, 2023, Chinchar et al. 2017). ISKNVs have between 104 and 141 predicted open reading frames (ORFs), of which 25 are core genes that are shared by all iridoviruses (Chinchar et al. 2017, Eaton et al. 2007). The major capsid protein (MCP), DNA polymerase (DPOL), and adenosine triphosphatase (ATPase) genes, which are among the core genes, have been used for detecting and analyzing the genetic relationships of ISKNVs (Kurita et al. 1998, He et al. 2001, Sudthongkong et al. 2002, Ballard et al. 2020).

From over 23 closely related ISKNV whole genome strains published, it was possible to phylogenetically classified ISKNV into three main genotypes: ISKNV, red seabream iridovirus (RSIV), and turbot reddish body virus (TRBIV), each of which has two distinct subclades (Song et al. 2008, Koda et al. 2018). It is still unknow why ISKNV evolved into 3 different genotypes, or what are the implications for the virulence, transmission, or host specificity (Nakajima and Kunita, 2005, Fu et al. 2011). It was speculated that for RSIV genotype, the host was mainly marine fish; for ISKNV genotype, the host was mainly freshwater fish; and for TRBIV genotype, the host was mainly flatfish (Fu et al. 2011), but recently studies showed that a single isolate has the ability to infect multiple species (Go et al. 2019), for example, an experimental challenge of a ISKNV genotype ISKNV from freshwater fish (pearl gourami and dwarf gourami) induced lethal disease in a marine fish species rock bream (*Oplegnathus fasciatus*) (Jeong et al. 2008) and murray cod (*Maccullochella peelii peelii*) (Go et al. 2006), and after 10 passages of an ISKNV isolated from diseased mandarin fish in zebrafish, the virus was able to cause disease in the zebrafish

(He et al. 2000, Xu et al. 2008). TRBIV genotype was discovered in ornamental freshwater fishes oscar (*Astronotus ocellatus*) and three spot gourami (*Trichopodus trichopterus*) (Koda et al. 2018), further expanding the TRBIV genotype host range.

Little is known about the ISKNV replication in the membrane of the cells and the cellular metabolism. Studies suggest that ISKNV infection induces glutaminolysis for the efficient replicate of the virus in the beginning of the infection and to promote the enzymes expression of glucose metabolism (Fu et al., 2017; Guo et al., 2019), it also, may be involved in the oxidative stress activated the virus (Fu et al., 2022).

Recent research indicates that when a chronically infected fish with ISKNV is exposed to a hypoxic environment, it can trigger a new outbreak (He et al., 2020). Therefore, dissolved oxygen is an important environmental factor for the prediction of disease outbreaks.

2.3. Clinical signs and diagnostic

The ISKNV infection causes non-specific clinical signs and may include scale protrusion, petechial hemorrhages, lethargy, anorexia, abnormal swimming patterns, increased respiratory effort, darkening or lightening of body color, celomic distension due to ascites, enlarged spleen, kidney, and liver, and pale viscera (Gibson-Kueh et al. 2003, Johan et al. 2020, WOAH 2023). ISKNV infections induce stereotypical microscopic lesions characterized by enlargement of affected cells with basophilic cytoplasmic inclusions, known as inclusion body-bearing cells or megalocytes, in various parts of the body (Gibson-Kueh et al. 2003). ISKNV is mesotheliotropic, and thus, megalocytes are often present near the endothelium of blood vessels (Gibson-Kueh et al. 2003, Chinchar et al. 2009).

Megalocytes can be observed in the spleen, kidney, intestines, eye, pancreas, connective tissue surrounding the ovarian follicles, liver, heart, gills, and brain (Gibson-Kueh et al. 2003). Smaller numbers of megalocytes can also be observed in the liver, heart, gills, and brain. The presence of megalocytes is frequently associated with degenerative and necrotic alterations, with or without hemorrhagic lesions, particularly affecting the spleen, kidney, and intestine (Gibson-Kueh et al. 2003). Hypertrophic cells are increased due to the large number of viral particles present in the cytoplasm (Zhu et al., 2020). The main diagnostic methods for virus infections are based on PCR and viral isolation, with characteristic histopathological findings of the disease being a complement to the diagnosis (WOAH 2019, Johnson et al., 2019).

Outbreaks of ISKNV are mainly seen in juvenile fish (He et al. 2002). Horizontal transmission can occur through contaminated water, cannibalism/necrophagy, and cohabitation (He et al., 2002). Studies indicate that vertical transmission can also happen (Suebsing et al., 2016) with the transition from undue (infected) parents to offspring. Mortalities vary according to possible secondary bacterial infections (Alonso et al., 2005, Ciulli et al., 2015; Zhu et al., 2021). Outbreaks of ISKNV can result in severe acute disease, which can lead to mortality rates as high as 100%, or chronic systemic disease, that leads to low-grade mortality over several months (Dong et al. 2015, Subramaniam et al. 2012, Hick et al. 2016).

Experimental infections using member viruses of each ISKNV genotype (ISKNV, RSIV, TRBIV) have resulted in little or no clinical change in cold water temperatures (±20°C). In water with higher temperatures (+25°C), moribund fish and cumulative mortality of up to 100% were observed (He et al., 2002; Jun et al., 2009; Koda et al., 2018).

2.4. ISKNV in tilapia in Brazil and the World

In 2019, ISKNV was diagnosed and notified to WOAH for the first time in Brazil, in juveniles of Nile tilapia (n=23) in Goiás State (Figueiredo et al. 2021). The clinical signs observed in the sampled fish were anorexia, melanosis, mucus hypersecretion, integument hemorrhage, ascites, and gill pallor. At necropsy, was observed hepatomegaly, splenomegaly, hemorrhagic viscera, friable muscle, and presence of liquid in the celomatic cavity. The histopathological analysis revealed several tissues with the presence of megalocytes, often in large numbers in the kidney and splenic parenchyma, and in smaller numbers in the hepatic parenchyma, cardiac endothelia, glomerulus of the posterior kidney, gills, and the intestinal and gastric lamina propria (Figueiredo et al. 2021). Numerous cells containing electron-dense bodies in the cytoplasm were observed in the spleen (transmission electron microscopy analyses) with numerous diffusely spread icosahedral virions with 160 nm diameter with an external double membrane and a central electron dense core (Figueiredo et al. 2021). All collected fish were positive for RSVID/ISKNV in a PCR analysis, and bacteriological examination demonstrated co-infection of the virus with bacteria *Streptococcus agalactiae* (n = 6 fish), *Edwardsiella tarda* (n = 7) and *Aeromonas hydrophila* (n=2). The phylogenetic analysis of the major capsid protein (MCP) gene showed that the ISKNV strain found in Brazil belongs to the ISKNV subclade 1 (Figueiredo et al., 2021).

Recent studies indicates that outbreaks of ISKNV in tilapia might have been happening in Brazil much before 2019 (Fonseca et al. 2022).

ISKNV was first reported in the USA in 2012 (Subramaniam, 2016), but it is strongly suggested that the 1998 iridovirus outbreak in tilapia in Florida, USA (McGrogan et al., 1998) was caused by ISKNV (Subramaniam, 2016). Subsequently, it was detected in Thailand (Dong et al., 2015; Suebsing et al., 2016), Ghana (Ramírez-Paredes et al., 2021, Ayiku et al., 2022), and most recently in Brazil (Figueiredo et al., 2021, Fonseca et al. 2022).

2.5 ISKNV host range and spill-over

Another aggravating factor for the productive sector and the environment is that a large part of tilapia is reared in intensive floating net cages installed in rivers or reservoirs, that have a rich diversity of fishes, which may become infected by the ISKNV through spillover events, affecting the population structure and diversity, and potentially causing acute mortality among wild fish population (Swaminathan et al., 2022). Since iridoviruses, such as ranaviruses, have such low host specificity, affecting fish, reptiles, and amphibians, it is possible to hypothesize about spillover events involving different species of fish shedding ISKNV into the environment.

ISKNV infections have already been reported in several species of Brazilian ornamental fish such as guppy (*Poecilia reticulata*), goldfish (*Carassius auratus*) and native fish, such as red piranha (*Pygocentrus nattereri*), freshwater angelfish (*Pterophyllum scalare*) and spotted catfish (*Pseudoplatystoma corruscans*) (Maganha et al., 2018, Kawato et al., 2020, Fonseca et al., 2022).

The increase of livestock-wildlife interactions enhances the opportunities for pathogen transmission, through spillover events, and risk of emerging diseases in livestock and wildlife (Jori et al. 2021). Pathogen spillover is a critical process in the prediction of emergence of infectious diseases (Power and Mitchell, 2004). Pathogen spillover itself occurs as a result of a series of events, usually involving exposition of a high-density population, shedding a high concentration of the virus to a second vulnerable population, with enough particles to cause disease (Plowright et al. 2017). For aquatic systems, Shea et al. (2020) showed that salmon farms served as a potential reservoir for several infectious pathogens, increasing the risk of exposure to wild salmon and other fish species that share adjacent marine environment.

Current data does not provide enough evidence that ISKNV from diseased cage-farmed tilapia can or is being active transmitted to wild native fishes. Since ISKNV can affect so many different fish species, and cross infection has been shown multiple times (Jeong et al. 2008, Go et al. 2006), the hypothesis of spillover of ISKNV from tilapia to wild native fish represents a novel threat to the environment, the production of native Brazilian fishes, and potential perpetuation of the virus in the environment, producing enzootic cycles between tilapia and native wild fishes.

3. OBJECTIVES

3.1 General

- Identify ISKNV spillover between tilapia produced in floating net cages located in Paraná River and wild native Brazilian fishes located adjacent to fish net cages by testing the farmed tilapia and wild fish against ISKNV.

3.2 Specific

- Identify farmed tilapia and wild fishes that are positive for ISKNV through molecular diagnostic and evaluate fish health trough ancillary test, such as clinical signs, necropsy, parasitology, water quality, and histopathology.

4. MATERIAL AND METHODS

4.1. Fish Collection

Apparently healthy *Oreochromis niloticus* (tilapia) (n=57) were collected from ten floating net cages (18 m^3) in the Paraná River, São Paulo State, Brazil on November 25, 2022, and March 30, 2023.The tilapia were collected with a fish net. Concurrently, it was collected 40 wild native Brazilian fishes on areas adjacent to the net-cages using hook and line. Additionally, it was collected 51 more wild native Brazilian fishes on May 23, 2023. All collected wild fish species were identified using morphometric characteristics. We also collected weight and length information of each fish. The collected fishes were transported live in plastic bags containing water from the river to the Microbiology and Parasitology of Aquatic Organisms Laboratory (LAPOA), São Paulo State University (UNESP), Jaboticabal, São Paulo, where they were processed and analyzed, or the fish were necropsied *in locus*.

On November 25, 2022, it was collected *Serrasalmus geryi* (Géry's pirambeba) (n=1, 222.4 g; 25.5 cm) (Figure 1A), *Serrasalmus maculatus* (yellow piranha) (n=15; 259.44±46.10 g; 24.5±1.53 cm) (Figure 1B), and tilapia (n=31; 28.67±6.64 g; 13.27±1.25 cm). On March 30, 2023, it was captured Géry's pirambeba (n=1; 315g; 21cm), *Serrasalmus marginatus* (white piranha) (n=1; 76.6 g; 16 cm) (Figure 1C), yellow piranha (n=22; 280.01±78.24g; 21.36±1.86 cm) and tilapia (n=26, 22.27±6.53g; 9.84±0.99 cm). On May 23, 2023, it was captured, Géry's pirambeba (n=19, 329.15±99.63 g; 35.71±54.35 cm), yellow piranha (n=29 ; 340.08±111.50 g; 22.8±1.90 cm) and *Cichla ocellaris* (peacock bass) (n=3 ; 431.47±375.84 g; 34.5±5.26 cm) (Figure 1D).

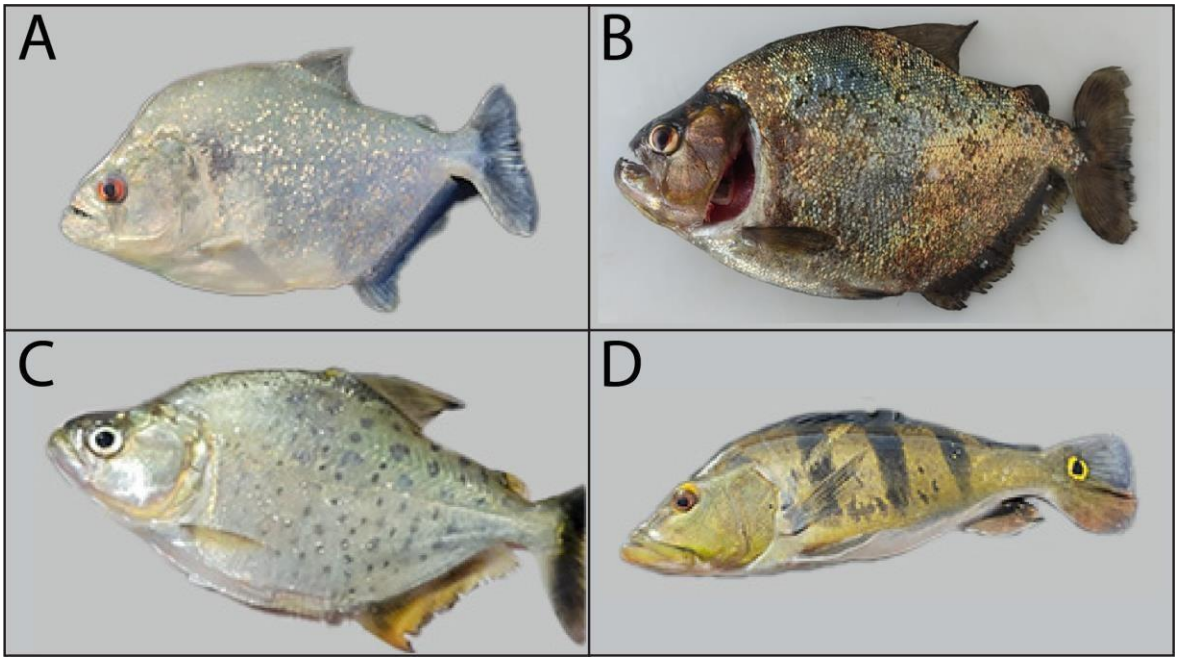

Figure 1. Collected Brazilian native fish species from Paraná River, in areas adjacent to tilapia net-cages, São Paulo, Brazil. A) *Serrasalmus geryi* (Géry's pirambeba); B) *Serrasalmus maculatus* (yellow piranha); C) *Serrasalmus marginatus* (white piranha); D) *Cichla ocellaris* (Peacock bass).

4.2. Necropsy and parasitological analysis

Fishes were euthanized by deepening the anesthetic plane with benzocaine (0.1 g L^{-1}), and the skin and gills were examined.

After the euthanasia, skin and gill mucus was collected by gently scrapping off their body and branchial surfaces with a glass slide and evaluated under light microscopy for external parasites identification (Amato et al. 1991, Martins & Souza, 1997). Additionally,

gills were collected and fixed in 1:4000 formalin solution for preservation of parasites and 10% formalin solution for histopathological evaluation.

The necropsy of the animals was performed, and fragments of spleen, liver, and kidney were collected and fixed in a 10% formalin solution for histopathological analysis and in absolute ethanol solution for subsequent DNA extraction and molecular analysis. Samples were pooled into groups up to three specimens, according to the collection date and species. To lower contamination risk between different specimens, all instruments and gloves were changed between every collection of pooled of samples, and the working place was cleaned with 10% bleach solution for 30 minutes between each sampling.

Fixed tissues in 10% formalin solution were submitted to Jose Luiz de Souza – Histotechnology laboratory, Ribeirão Preto, São Paulo, Brazil for conventional histopathological processing, which includes serial dehydration in alcohol, diaphanization in xylol, embedding in paraffin, 5 µm thick histological sections, and staining of slides with hematoxylin and eosin (H&E). The histopathological slides were analyzed using a light microscope (Nikon, Eclipse E200).

4.3. DNA extraction and molecular identification of ISKNV

Liver, kidney, and spleen preserved in absolute ethanol were minced, and combined with samples from the same pool, providing a total of 25 mg of tissue per pool sample. The combined tissue was DNA extracted using the DNeasy Blood and Tissue Kit (Qiagen®) extraction kit, as per the manufacturer's instructions. Briefly, 25 mg of tissue was combined with 180 µL of Buffer ATL and 30 µL of proteinase K, latter being incubated for one hour at 56°C. The solution of vortexed and centrifuged, and transferred to a new tube containing 200 µL of Buffer AL and 200 µL of 100% ethanol. After mixing it, it was transferred again to the Qiagen DNeasy Mini spin columns, where it was centrifugated and discarded the remaining solution. Later, the DNA was washed using a step containing 500 µL of buffer AW1, and a second step containing 500 µL of buffer AW2. The DNA was eluted in buffer AE, and the DNA quality control and concentration was measured using NanoDrop OneC (Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer). For the quality control of the extraction a mock sample (extraction negative control) was used for every set of extraction.

The conventional Polymerase Chain Reaction (PCR) technique was performed for the detection of ISKNV. We used the primers and protocols developed and established by

the WOAH (WOAH, 2019). The primers used were the RSIV-1F (CTCAAACACTCTGGCTCATC) and RSIV-1R (GCACCAACACATCTCCTATC), in which amplify the partial laminin-like protein and phosphatase gene, which amplify a product of 570 bp. For the reaction, we used 25µL of solution containing: 5µL of 5x Buffer, 0.5µL DNTP, 0.5µL of reverse and forward primers each, 0.2 µL Platinum 2 Taq Hot-Start DNA Polymerase (Thermo Fisher®), 1 µL extracted DNA sample and 17.3 µL Ultrapure water. Samples were subjected to initial DNA denaturation step at 94°C for 2 minutes, followed by 40 cycles of annealing and extension steps at 94 ºC for 15 seconds, 60 ºC for 15 seconds, and 68 ºC for 15 seconds, and final extension at 72 °C for 10 minutes. Aliquots of 10 µl of each PCR product sample were electrophoresed in a 1.5% agarose gel containing 1 µL of Nancy-520 DNA Gel Stain®, and amplified DNA was visualized on a UV transilluminator. We used an ISKNV positive DNA sample as our PCR positive sample (positive control). This sample was provided by Dr. Miguel Frederico Fernandez Alarcon from Pathovet laboratory.

Three positive samples derived from two pooled tilapia sample (sample DJ1 and DJ3), and one yellow piranha (DJ2), were purified and subjected for Sanger sequencing at the Center for Biological and Biological Resources Genomics (CREBIO), FACV-UNESP, Jaboticabal, São Paulo, Brazil. For the sample DJ2, we re-amplified the sample using the same PCR assay in order to obtain a higher DNA yield and better sequencing result. The obtained nucleotide sequence was assembled using BioEdit v. 7.7.1. The primer regions were removed, and the resulted sequences were subjected to nucleotide BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to finds regions of similarity between different available sequences to confirm the infection by ISKNV. To avoid over-estimation of ISKNV infection among wild fishes, pools derived from this fishes that were PCR positive for ISKNV were re-extracted individually and subjected for a new molecular evaluation for ISKNV.

4.4. Phylogenetic Analysis

Maximum Likelihood (ML) phylogenetic trees were constructed based on the partial laminin-like protein and phosphatase gene alignments of strains DJ1, DJ2, and DJ3 to 23 other ISKNV strains. The 34 whole genome sequences were retrieved from GenBank (Table 1) and aligned with the DJ1, DJ2, and DJ3 sequences in Geneious Prime v2019.2.1 using MAFFT with default settings (Katoh et al. 2013). ML trees were constructed in IQ-TREE

with 1000 non-parametric standard bootstraps performed to test the robustness of the clades (Nguyen et al. 2015).

Viral strain designation	Abbreviation	Host	GenBank accession no.
Angelfish iridovirus AFIV-16	AFIV-16	Freshwater angelfish *Pterophyllum scalare*	MK689685
Banggai cardinalfish iridovirus isolate BCIV/WVL17393/2012	BCIV/2012	Banggai cardinalfish *Pterapogon kauderni*	MN432490
Banggai cardinalfish iridovirus isolate BCIV/2017	BCIV/2017	Banggai cardinalfish *Pterapogon kauderni*	MT926123
Giant seaperch iridovirus isolate GSIV-K1	GSIV-K1	Asian seabass *Lates calcarifer*	KT804738
Infectious spleen and kidney necrosis virus	ISKNV	Mandarin fish *Siniperca chuatsi*	AF371960
Infectious spleen and kidney necrosis virus isolate Bali/Hybrid-grouper/2016/SVC-18-009	ISKNV 18-009	Hybrid grouper *E. fuscoguttatus* x *E. polyphekadion*	MW464172
Infectious spleen and kidney necrosis virus isolate EFIV-2018	EFIV-2018	Albino rainbow shark *Epalzeorhynchos frenatus*	MW273353
Infectious spleen and kidney necrosis virus isolate EFIV-2019	EFIV-2019	Albino rainbow shark *Epalzeorhynchos frenatus*	MW273354
Infectious spleen and kidney necrosis virus isolate KU1	ISKNV KU1	Asian seabass *Lates calcarifer*	MT128666
Infectious spleen and kidney necrosis virus strain RSIV-Ku	RSIV-Ku	Red seabream *Pagrus major*	KT781098
Infectious spleen and kidney necrosis virus strain tilapia iridovirus 2020	TIV-2020	Tilapia *Oreochromis niloticus*	ON212400
Large yellow croaker iridovirus	LYCIV	Large yellow croaker *Pseudosciaena crocea*	AY779031
Orange-spotted grouper iridovirus	OSGIV	orange-spotted grouper *Epinephelus coioides*	AY894343
Pompano iridovirus isolate PIV2010	PIV2010	Florida pompano *Trachinotus carolinus*	MK098185
Pompano iridovirus isolate PIV2014a	PIV2014a	Florida pompano *Trachinotus carolinus*	MK098186
Pompano iridovirus isolate PIV2016	PIV2016	Florida pompano *Trachinotus carolinus*	MK098187
Red sea bream iridovirus strain Ehime-1	RSIV Ehime-1	Red sea bream *Pagrus major*	AB104413
Red seabream iridovirus isolate KagYT-96	RSIV KayYT-96	Japanese amberjack *Seriola quinqueradiata*	MK689686
Red seabream iridovirus strain RIE12-1	RSIV RIE12-1	Red sea bream *Pagrus major*	AP017456
Rock bream iridovirus isolate RBIV-C1	RBIV-C1	Rock bream *Oplegnathus fasciatus*	KC244182
Rock bream iridovirus strain RBIV-KOR-TY1	RBIV-KOR-TY1	Rock bream *Oplegnathus fasciatus*	AY532606
South American cichlid iridovirus	SACIV	Keyhole cichlid *Cleithracara maronii*	MG570131
Three spot gourami iridovirus	TSGIV	Three spot gourami *Trichopodus trichopterus*	MG570132
Turbot reddish body iridovirus	TRBIV	Turbot *Scophthalmus maximus*	GQ273492
Infectious spleen and kidney necrosis virus DGIV 2010	DGIV 2010	Dwarf gourami *Trichogaster lalius*	MW883604
Infectious spleen and kidney necrosis virus HGIV-Cantik1-2014	HGIV-Cantik1-2014	Hybrid grouper, cantik *E. fuscoguttatus x E. polyphekadion*	MW883596

Viral strain designation	Abbreviation	Host	GenBank accession no.
Infectious spleen and kidney necrosis virus LakeVolta_BF-2	LakeVolta BF-2	Tilapia *Oreochromis niloticus*	OQ513807
Infectious spleen and kidney necrosis virus OGIV-PW-2018	OGIV-PW	Giant gourami *Osphronemus goramy*	MW883606
Infectious spleen and kidney necrosis virus TLIV-2011	TLIV	Pearl gourami *Trichopodus leerii*	MW883593
Infectious spleen and kidney necrosis virus TLIV-2015	TLIV	Dwarf gourami *Trichogaster lalius*	MW883601
Infectious spleen and kidney necrosis virus TTIV-2011	TTIV-2011	Blue/gold gourami *Trichopodus trichopterus*	MW883594
Infectious spleen and kidney necrosis virus TTIV-2015	TTIV-2015	Three spot gourami *Trichopodus trichopterus*	MW883599
Infectious spleen and kidney necrosis virus XHIV-2015	XHIV-2015	Green swordtail *Xiphophorus hellerii*	MW883600
Infectious spleen and kidney necrosis virus XMIV-2015	XMIV-2015	Southern platyfsh *Xiphophorus maculatus*	MW883602
Infectious spleen and kidney necrosis virus DJ1 2023	DJ1 2023	Tilapia *Oreochromis niloticus*	This study
Infectious spleen and kidney necrosis virus DJ2 2023	DJ2 2023	Yellow piranha *Serrasalmus maculatus*	This study
Infectious spleen and kidney necrosis virus DJ3 2023	DJ3 2023	Tilapia *Oreochromis niloticus*	This study

Table 1. Viral strain designation, abbreviation, host, and GenBank accession number (no.) of 34 publicly available ISKNV strains and three unpublished sequences from this study used in the phylogenetic and comparative analysis.

4.5. Water quality

Water samples from the Paraná River were collected from each fish net-cage using 1L bottles, which were properly identified and stored at 4 °C. These samples were later analyzed at the Aquaculture Center of UNESP, Water Analysis Laboratory for nitrate, nitrite, and total phosphorus concentration. Dissolved oxygen, pH, and temperature were measured *in locus*, using a YSI 55 Oximeter digital device (YSI Inc., Yellow Springs, United States), and YSI pH 100 pHmeter (YSI Inc., Yellow Springs, United States).

5. RESULTS

5.1. Fish collection

We collected a total of 57 farmed tilapia, 67 yellow piranha, 20 Géry's pirambeba, 3 peacocok bass, and 1 white piranha. While the tilapia were on average 11.55 cm long, the native fish were much bigger, with an average of 23. 5 cm (Table 2).

Fish species	Total length (cm)	Weight (grams)

Farmed tilapia	9.73±1.04	21.07±5.98
Yellow piranha	22.82±2.3	309.36±101.12
Géry's pirambeba	35.3±52.92	330.47±94.2
Peacocok bass	34.5±5.26	431.47±375.84
White piranha	16	76.6

Table 2. Summary of the collected fish from November 25, 2022, to March 30, 2023, from a fish farm and adjacent areas on the Paraná River, São Paulo, Brazil.

5.2. Necropsy and parasitological analysis

All fish collected appeared healthy and had no macroscopical lesion. The parasitological examination revealed that Monogenean were the most prominent parasite group found (Table 3). *Dolops* sp. was found in one yellow piranha (Figure 2), while *Trichodina* sp. was found in tilapia and peacock bass concurrently with Monogenean.

	Gills									
Host species	Monogenea	(%)	Trichodina	(%)	Dolops	(%)	No parasite	(%)	not collected	(%)
Farmed tilapia	26	44.1	2	3.4	0	0.0	0	0.0	31	52.5
Yellow piranha	50	74.6	0	0.0	1	1.5	1	1.5	15	22.4
Géry's pirambeba	18	90.0	0	0.0	0	0.0	1	5.0	1	5.0
Peacocok bass	3	75.0	1	25.0	0	0.0	0	0.0	0	0.0
White piranha	1	100.0	0	0.0	0	0.0	0	0.0	0	0.0

	Skin					
Host species	Monogenea	(%)	No parasite	(%)	not collected	(%)
Farmed tilapia	21	35.0	8	13.3	31	51.7
Yellow piranha	49	73.1	3	4.5	15	22.4
Géry's pirambeba	0	0.0	19	95.0	1	5.0
Peacocok bass	0	0.0	3	100.0	0	0.0
White piranha	0	0.0%	1	100.0%	0	0.0%

Table 3. Prevalence of ectoparasites per species found in caged farmed tilapia and adjacent wild fishes collected from the Paraná River, São Paulo, Brazil.

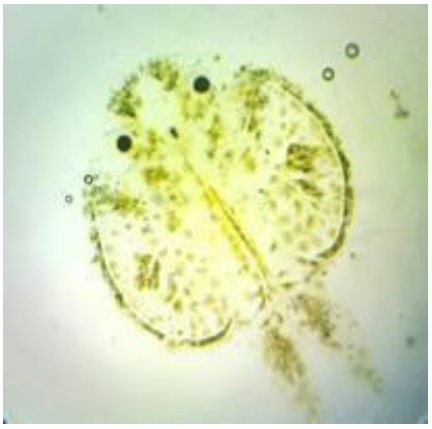

Figure 2. Ectoparasite *Dolops* sp. found in the gills of yellow piranha (40x).

5.3. Molecular analysis (PCR)

The conventional PCR for ISKNV revealed that 36 of 57 tilapia (63.2%) and 1 of 91 Brazilian native fish species (1.1 %) (Table 4) were positive for ISKNV. The only ISKNV positive Brazilian native fish was a yellow piranha, collected on November 25, 2022, and the gel band showed a mildly faint 570 bp band (Figure 3). The sanger sequence of the positive ISKNV sample DJ1 and DJ3 from tilapia, and sample DJ2 from yellow piranha, revealed that samples DJ1, DJ2, and DJ3 were 100% identical to each other. The recovered DNA sequence from sample DJ1 was partial, missing 9 nt, while DJ2 and DJ3 were recovered fully. The nucleotide BLASTN analysis of samples DJ1, DJ2, and DJ3 were 100% identical to several strains of ISKNV clade, including strain TIV-2020 that was collected from a tilapia from Brazil. Sample DJ1, DJ2, and DJ3 was 99.62% identical to ISKNV from tilapia from Ghana.

Host species	ISKNV result	(%)
Farmed tilapia	36	63.2
Yellow piranha	1	1.5
Géry's pirambeba	0	0
Peacocok bass	0	0
White piranha	0	0

Table 4. Conventional PCR results for the detection of ISKNV from caged farmed tilapia and adjacent wild fishes collected from the Paraná River, São Paulo, Brazil. Percentage derived from each individual species.

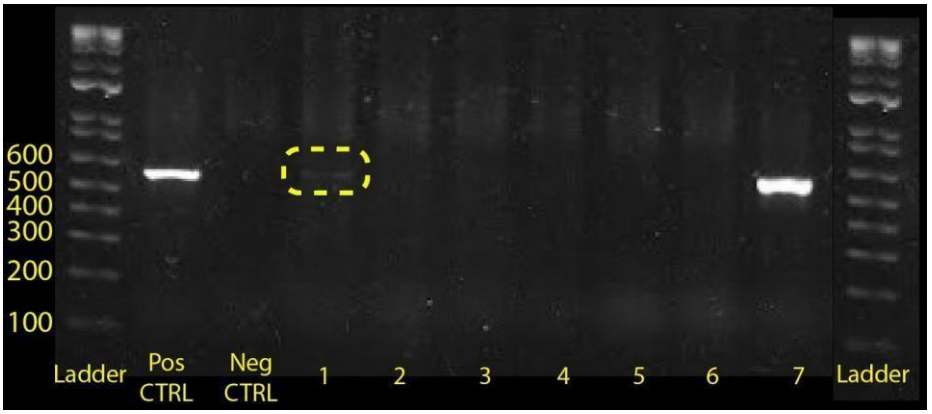

Figure 3. Selected samples amplification of ISKNV conventional PCR assay, showing 100bp ladder (L), positive control (CTRL), negative extraction control (CTRL), mildly positive yellow piranha sample (1), highlighted in yellow dashed line, series of negative samples from other collected yellow piranha (2-6), positive tilapia sample (7). All these samples were collected on November 25, 2022.

5.4. Phylogenetic analysis

The amplified partial laminin-like protein and phosphatase gene phylogenetic analysis revealed that among 34 other strains of ISKNV, the samples DJ1, DJ2, and DJ3 grouped in the ISKNV clade (Figure 13). The partial genes were not able to discriminate the 37 strains into ISKNV subclades.

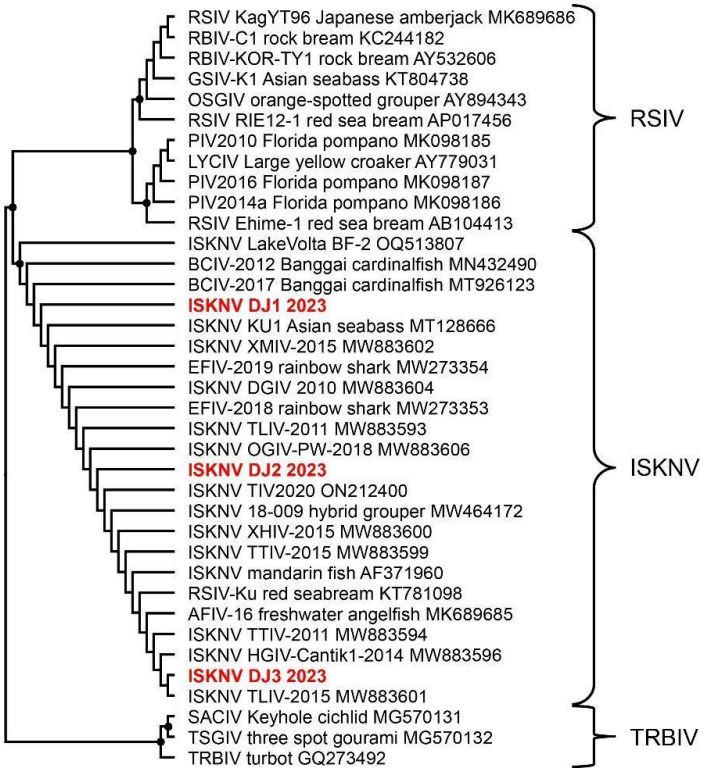

Figure 4. Maximum Likelihood Cladogram depicting the relationship of infectious spleen and kidney necrosis virus (ISKNV) strain DJ1, DJ2, and DJ3, to 34 other ISKNV based on their aligned partial laminin-like protein and phosphatase gene. All nodes supported by bootstrap values > 80 are represented by black circles. See Table 1 for virus abbreviations. Strain DJ1, DJ2, and DJ3 are bolded in red.

5.5. Histopathology

The histopathological analysis did not reveal any suggestive lesion of ISKNV infection, including kidney, liver, spleen necrosis, presence of megalocytes in parenchymatous organs and endothelial lining, or enteritis.

5.6. Water quality

The limnological analysis showed that nitrate was between 16.25± 0.2 to 76.97± 0.73 µg/L, nitrite was between 2.62± 0.2 to 3.39± 0.21 µg/L, and phosphorus was between 100.29 ± 0.2 to 110.81 ± 9.44 µg/L, which is within the optimal range for raising tropical fishes (Noga, 2010). The dissolved oxygen value varied between 2.5 to 4 mg/L which was low for raising tilapia, while temperature 28 to 31.1 ºC, and pH 7.73 to 8.15 was appropriate for tropical fishes (Noga, 2010).

6. DISCUSSION

The expanding dispersion of ISKNV in the World can be attributed to the overwhelming capacity of this virus to infect multiple different species of fish, including tilapia (Jung et al. 2000, Kurita et al. 2002, Girisha et al. 2021, Gibson-Kueh et al. 2003, Jeong et al. 2003, Nakajima et al. 2005, Wang et al. 2007, Song et al. 2008, Wang et al. 2009, Rimmer et al. 2015, de Lucca Maganha et al. 2018, WOAH, 2022). This ability also contributes to the establishment of the virus in new locations as well, such as seen in other Iridovirus, such as ranavirus (Ahne et al. 1997, Chinchar 2002, Williams et al. 2005, Brunner et al. 2015). Brazil has seen an uprise of cases of ISKNV cases in ornamental, native Brazilian, and tilapia fishes (de Lucca Maganha et al. 2018, Figueiredo et al. 2021, Fonseca et al. 2022). Our work is the first to show infection of a native Brazilian fish (yellow piranha) collected from the wild, adjacent to a tilapia caged farm that was experiencing an active outbreak of asymptomatic ISKNV disease. More importantly, the partial genome sequence

revealed that both strains (tilapia and piranha) are identical, suggesting that horizontal transmission between these species has happened.

Since it was discovered a much higher number of tilapia samples (63.2%) positive for ISKNV, when compared to wild fishes (1.1%), we hypothesize that spillover infection from tilapia to wild fish has happened. As other Iridoviruses, the dispersion of ISKNV can be achieved by contact fish to fish, contaminated water, and predation/cannibalism/necrophagy (He et al. 2002, Brunner et al. 2015, Fusianto et al. 2019, Kawato et al. 2023). The tilapia from our study were raised in fish nets, which significantly decreases the possibility of dispersion by contact from wild fish to tilapia and vice-versa. The farmer did not have a history of wild fish getting into the fish net, nor tilapia escaping it. If water was the main dispersion factor, we would have a higher number of wild fishes infected with the virus, as seen by Kawato et al. (2023). The physic of dispersion of particulates dictates that the flux of particles goes from the higher concentration to the lower concentration, therefore, for wild fishes to transmit the virus by water to tilapia, it would have to have a higher number of infected wild fish than what we found. Lastly, the dispersion of the virus through predation/cannibalism/necrophagy represents the most logical and probable way of transmission of ISKNV in an ecological setting between cultivated and wild fishes. Sick or dead animals harbor the highest amount of virus in their organs (Brunner et al. 2015). Tilapia that dies from ISKNV might end-up eaten by other tilapia from the same cage or might be collected and released back in the river, or due to autolysis, break-it down to sizes that might pass though the net cage. Regardless, wild fauna will consume it, and if they consume it enough, they might become infected. We cannot confirm what was the final destiny of dead fish from this farm, but since it is a common practice among farmers to dispose dead fish back into the river, we assume that this behavior significantly increases the chances of infection in wild fishes. To add to this hypothesis, the collected wild fish were larger than the tilapia, with a piscivore behavior that includes necrophagy. The impact of wild fishes harboring ISKNV is still unpredictable, and further studies will need to address this issue.

There was apparently no difference in the prevalence of parasites between net caged tilapia and wild fish. Both groups were infected with mainly Monogenetic ectoparasites. The parasitology collection was a limitation factor of our work, since we have more than 50% of analyzed tilapia that was not investigated for parasites. Nevertheless, poor water renewal can

lead to the proliferation of ectoparasites such as Monogenetic (Martins et al., 2010, Pouder et al., 2011).

The water quality examination revealed that the oxygen level was low. Hypoxia is connected to outbreaks of ISKNV (He et al 2022) in susceptible fishes. We do not have information about the mortality rate of this farm. The water temperature was between 28 to 31.1 ºC, which is not associated with outbreak of ISKNV disease in tilapia (He et al. 2002). In experimental setting, the mortality rate of infected tilapia with ISKNV was higher at 23 than 28 ºC (Viadanna personal communication). The higher water temperature could also explain the lack of clinical signs and microscopic lesions of ISKNV-positive tilapia and yellow piranha. Outbreak of ISKNV in rainbow shark revealed that even in a high mortality event, histopathological lesions can be mild to non-existent (Koda et al., 2018).

Phylogenetic analysis supported the strains DJ1, DJ2, and DJ3 as ISKNV clade ISKNV, as seen in ISKNV outbreaks of tilapia worldwide (Figueiredo et al. 2021, Ramírez-Paredes, 2021). Clade ISKNV has been reported in native brazilian fishes, such as freshwater angelfish (*Pterophyllum scalare*) (Kawato et al. 2021), red piranha (*Pygocentrus nattereri*) (Maganha et al. 2017), and spotted catfish (*Pseudoplatystoma corruscans*) (Fonseca et al. 2022). Since all members of this clade are closely related phylogenetically, it is possible that they can infect all these hosts interchangeably, as demonstrated by He et al. (2002).

In conclusion, we confirmed that there is a spillover event on this farm. The causes for this spillover could be due to the break of biosecurity rules, such as proper removal of carcasses and correct disposal of them. The implications of such event is still unknown, and more studies will need to address this in the epidemiological cycle of ISKNV disease. The phylogenetic work corroborated with our hypothesis, as well the descriptive analysis of prevalence. Lastly, water quality parameters, such as water temperature and oxygen level are important for the prediction of outbreaks of ISKNV disease, as such, lower temperatures (23-25 °C) and hypoxic environments, will potentialize the mortality events related to ISKNV, increase virus shedding, and increase the dispersion and maintenance of the virus into the environment, producing a continued cycle of the disease.

We highlight the urgency of vaccines against ISKNV for tilapia, to prevent mortality events, and to lower the virus shedding to susceptible species. We also highlight the importance of biosecurity measures, such as quarantine, proper disposal of carcasses, and routine health assessments. All these measures are vital to a sustainable fish production.

7. REFERENCES

Ahne, W., Bremont, M., Hedrick, R. P., Hyatt, A. D., & Whittington, R. J. Special topic review: Iridoviruses associated with epizootic haematopoietic necrosis (EHN) in aquaculture. **World Journal of Microbiology and Biotechnology**, 1997, 13, 367–373. doi.org/10.1023/A:1018563930712.

Alonso, M. C., Cano, I., Garcia-Rosado, E., Castro, D., Lamas, J., Barja, J. L., & Borrego, J. J. Isolation of lymphocystis disease virus from sole, *Solea senegalensis* Kaup, and blackspot sea bream, *Pagellus bogaraveo* (Brunnich). **Journal of Fish Diseases**, 2005, 28(4), 221–228. doi.org/10.1111/j.1365-2761.2005.00621.x.

Amato, J. F. R., Boeger, W. A., & Amato, S.B. Protocolo para laboratório: coleta e processamento de parasitas do pescado. Rio de Janeiro: **Imprensa Universitária UFRRJ**, 1991, 81.

Ayiku, A. N. A., Adelani, A. A., Appenteng, P., Nkansah, M., Ngoi, J. M., Morang'a, C. M., Paley, R., Cudjoe, K. S., Verner-Jeffreys, D., Quashie, P. K., & Duodu, S. Molecular epidemiology of Infectious spleen and kidney necrosis virus (ISKNV) in Ghanaian cultured tilapia. **bioRxiv: Cold Spring Harbor Laboratory**, 2022. doi.org/10.1101/2022.11.15.516701.

Ballard, D. R., Davis, A. J., Fuller, R. B., Garner, A. R., Mileham, A. D., Serna, J. D., Brue, D. E., Harding, C. M., Dodgen, C. D., Culpepper, W., Piatt, B., Rosario, S. E., & Duffus A. L. J. An examination of the Iridovirus core genes for reconstructing *Ranavirus* phylogenies. **FACETS**, 2020, 5, 523–533. doi.org/10.1139/facets-2020-0009.

Brunner, J. L., Storfer, A., Gray, M. J., & Hoverman, J. T. *Ranavirus* ecology and evolution: from epidemiology to extinction. In: Gray, M.J., Chinchar, V.G. (Eds.), **Ranaviruses**, Springer, 2015, 71–104. doi.org/10.1007/978-3-319-13755-1_4.

Chinchar, V. G. Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers. **Archives of virology**, 2002, 147(3), 447–470. doi.org/10.1007/s007050200000.

Chinchar, V. G., Hick, P., Ince, I. A., Jancovich, J. K., Marschang, R., Qin, Q., Subramaniam, K., Waltzek, T. B., Whittington, R., Williams, T., & Zhang, Q. Y. ICTV Virus taxonomy profile: *Iridoviridae*. **Journal of General Virology**, 2017, 98, 890–891. doi.org/10.1099/jgv.0.000818.

Chinchar, V. G., Hyatt, A., Miyazaki, T., & Williams, T. Family *Iridoviridae*: Poor viral relations no longer. **Lesser Known Large dsDNA Viruses**. Springer, 2009, 123–170. doi.org/10.1007/978-3-540-68618-7_4.

Ciulli, S., Pinheiro, A. C. de A. S., Volpe, E., Moscato, M., Jung, T. S., Galeotti, M., Stellino, S., Farneti, R., & Prosperi, S. Development and application of a real-time PCR assay for the detection and quantitation of lymphocystis disease virus. **Journal of Virological Methods**, 2015, 213, 164–173. doi.org/10.1016/j.jviromet.2014.11.011.

Delphino, M. K., Leal, C. A., Gardner, I. A., Assis, G. B., Roriz, G. D., Ferreira, F. & Gonçalves, V. S. Seasonal dynamics of bacterial pathogens of Nile tilapia farmed in a Brazilian reservoir. **Aquaculture**, 2019, 498, 100-108. doi.org/10.1016/j.aquaculture.2018.08.023.

Dong, H.T., Nguyen, V.V., Le, H.D., Sangsuriya, P., Jitrakorn, S., Saksmerprome, V., Senapin, S., & Rodkhum, C. Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis Niloticus*) Farms. **Aquaculture**, 2015, 448, 427–435. doi.org/10.1016/j.aquaculture.2015.06.027.

Eaton, H. E., Metcalf, J., Penny, E., Tcherepanov, V., Upton, C. & Brunetti, C. R. Comparative genomic analysis of the family *Iridoviridae*: re-annotating and defining the core set of iridovirus genes. **Virology Journal**, 2007, 4, 11. doi.org/10.1186/1743-422X-4-11.

FAO, 2020. **The State of World Fisheries and Aquaculture 2020**. In brief. Sustainability in action. Rome. doi.org/10.4060/ca9229en.

FAO, 2021**. The State of World Fisheries and Aquaculture 2021**. Making agrifood systems more resilient to shocks and stresses. Rome. doi.org/10.4060/cb4476en.

Figueiredo, H. C. P., Tavares, G. C., Dorella, F. A., Rosa, J. C. C., Marcelino, S. A. C., Pierezan, F., & Pereira, F. L. First report of infectious spleen and kidney necrosis virus in Nile tilapia in Brazil. **Transboundary and Emerging Diseases**, 2021**,** 1-8. doi.org/10.1111/tbed.14217.

Fonseca, A. A., Laguardia-Nascimento, M. J., Ferreira, A. P. S., Pinto, C. A., Freitas, T. R. P., Rivetti, A. V. J., Homem, V. S. F., & Camargos, M. F. Detection of megalocytivirus in *Oreochromis niloticus* and *Pseudoplatystoma corruscans* in Brazil. **Diseases of aquatic organisms**, 2022, 149, 25–32. doi.org/10.3354/dao03657.

Fu, X., Hu, X., Li, N., Zheng, F., Dong, X., Duan, J., Lin, Q., Tu, J., Zhao, L., Huang, Z., Su, J., & Lin, L. Glutamine and glutaminolysis are required for efficient replication of infectious spleen and kidney necrosis virus in Chinese perch brain cells. **Oncotarget**, 2017, 8(2), 2400–2412. doi.org/10.18632/oncotarget.13681.

Fu, X., Li, K., Niu, Y., Lin, Q., Liang, H., Luo, X., Liu, L., & Li, N. (2022). The mTOR/PGC-1α/SIRT3 pathway drives reductive glutamine metabolism to reduce oxidative stress caused by ISKNV in CPB Cells. In: Jones, C. J. (Eds.), **Microbiology Spectrum**, American Society for Microbiology, 2022, 10. doi.org/10.1128/spectrum.02310-21.

Fu, X., Li, N., Liu, L., Lin, Q., Wang, F., Lai, Y., Jiang, H., Pan, H., Shi, C., & Wu, S. Genotype and host range analysis of infectious spleen and kidney necrosis virus (ISKNV). **Virus Genes**, 2011, 42(1), 97-109. doi.org/10.1007/s11262-010-0552-x.

Fusianto, C., Hick, P.M., & Becker, J.A. Stability of Infectious spleen and kidney necrosis virus and susceptibility to physical and chemical disinfectants. **Aquaculture**, 2019, 506, 104–111. doi.org/10.1016/j.aquaculture.2019.03.024.

Gibson-Kueh, S., Netto, P., Ngoh-Lim, G. H., Chang, S. F., Ho, L.L., Qin, Q. W., Chua, F. H., Ng, M. L., & Ferguson, H.W. The pathology of systemic iridoviral disease in fish. **Journal of Comparative Pathology**, 2003, 129(2-3), 111–119. doi.org/10.1016/s0021-9975 (03)00010-0.

Girisha, S. K., Kushala, K. B., Nithin, M. S., Puneeth, T. G., Naveen Kumar, B. T., Vinay, T. N., Suresh, T., Ajay, S. K., Venugopal, M. N., & Ramesh, K. S. First report of the infectious spleen and kidney necrosis virus (ISKNV) infection in ornamental fishes in India. **Transboundary and Emerging Diseases**, 2021, 68(2), 964–972. doi.org/10.1111/tbed.13793.

Go, J., & Whittington, R. Australian bass *Macquaria novemaculeata* susceptibility to experimental megalocytivirus infection and utility as a model disease vector. **Disease of Aquatic Organisms**, 2019, 133(2), 157-174. doi.org/10.3354/dao03340.

Go, J., & Whittington, R. Experimental transmission and virulence of a megalocytivirus (Family *Iridoviridae*) of dwarf gourami (*Colisa lalia*) from Asia in Murray cod (*Maccullochella peelii peelii*). **Australian Aquaculture**, 2006, 258, 140−149. doi.org/10.1016/j.aquaculture.2006.04.033.

Guo, X., Wu, S., Li, N., Lin, Q., Liu, L., Liang, H., Niu, Y., Huang, Z., & Fu, X. Accelerated metabolite levels of aerobic glycolysis and the pentose phosphate pathway are required for efficient replication of Infectious spleen and kidney necrosis virus in Chinese Perch Brain Cells. **Biomolecules**, 2019, 9(9), 440. doi.org/10.3390/biom9090440.

He, J. H., Xia, Q., Weng, S., He, J., & Xu, X. Identification of infectious spleen and kidney necrosis virus (ISKNV)-encoded microRNAs. **Virus Genes**, 2020, 56(6), 724-733. doi.org/10.1007/s11262-020-01798-6.

He, Jian., Yang, Yu., Zhi-Min, Li., Zhi-Xuan, Liu., Shao-Ping, Weng., Chang-Jun, Guo., & Jian-Guo, He. Hypoxia triggers the outbreak of infectious spleen and kidney necrosis virus disease through viral hypoxia response elements. **Virulence**, 2022, 13(1), 714-726. doi.org/ 10.1080/21505594.2022.2065950.

He, J. G., Wang, S. P., Zeng, K., Huang, Z. J., & Chan, S. M. Systemic disease caused by an iridovirus-like agent in cultured mandarinfish, *Siniperca chuatsi* (Basilewsky), in China. **Journal of Fish Diseases**, 2000, 23, 219-222. doi.org/10.1046/j.1365-2761.2000.00213.x.

He, J., Zeng, K., Weng, S., & Chan, S. M. Experimental transmission, pathogenicity and physical–chemical properties of infectious spleen and kidney necrosis virus (ISKNV). **Aquaculture**, 2002, 204(1-2), 11–24. doi.org/10.1016/s0044-8486(01)00639-1.

He, J. G., Deng, M., Weng, S. P., Li, Z., Zhou, S. Y., Long, Q. X., Wang, X. Z., & Chan, S. M. Complete genome analysis of the mandarin fish Infectious spleen and kidney necrosis iridovirus. **Virology**, 2001, 291, 126–139. doi.org/10.1006/viro.2001.1208.

Hick, P., Becker, J., & Whittington, R. Iridoviruses of fish. In: Kibenge, F. S. B., Godoy M. G. (Eds.), **Aquaculture Virology**, Academic Press, 2016, 127–152. doi.org/10.1016/B978-0-12-801573-5.00008-5.

IBGE, **Produção da Pecuária Municipal** 2021; Brasil: IBGE, 2022.

International committee on taxonomy of viruses (ICTV), 2023. Retrieved from https://ictv.global/taxonomy/.

Jeong, J. B., Cho, H. J., Jun, L. J., Hong, S. H., Chung, J., & Jeong, H. D. Transmission of iridovirus from freshwater ornamental fish (*pearl gourami*) to marine fish (*rock bream*). **Disease of Aquatic Organisms**, 2008, 82, 27−36. doi.org/10.3354/dao01961.

Jeong, J. B., Jun, L. J., Yoo, M. H., Kim, M. S., Komisar, J. L., & Jeong, H. D. Characterization of the DNA nucleotide sequences in the genome of red sea bream iridoviruses isolated in Korea. **Aquaculture**, 2003, 220, 119–133. doi.org/10.1016/S0044-8486(02)00538-0.

Johnson, S. J., Hick, P. M., Robinson, A. P., Rimmer, A. E., Tweedie, A., & Becker, J. A. The impact of pooling samples on surveillance sensitivity for the *Megalocytivirus* Infectious spleen and kidney necrosis virus. **Transboundary and Emerging Diseases**, 2019, 66, 2318–2328. doi.10.1111/tbed.13288.

Jori, F., Hernandez-Jover, M., Magouras, I., Dürr, S., & Brookes, V. J. Wildlife–livestock interactions in animal production systems: what are the biosecurity and health implications?. **Animal Frontiers**, 2021, 11(5), 8–19. doi.org/10.1093/af/vfab045.

Jun, L. J., Jeong, J. B., Kim, J. H., Nam, J. H., Shin, K. W., Kim, J. K., Kang, J. C., & Jeong, H. D. Influence of temperature shifts on the onset and development of red sea bream iridoviral disease in rock bream *Oplegnathus fasciatus*. **Diseases of aquatic organisms**, 2009, 84(3), 201–208. doi.org/10.3354/dao02041.

Jung, S. J., & Oh, M. J. Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula. **Journal of Fish Diseases**, 2000, 23, 223—226. doi.org/10.1046/j.1365-2761.2000.00212.x.

Katoh, K., & Standley, D. M. MAFFT Multiple sequence alignment software version 7: Improvements in Performance and Usability. **Molecular Biology and Evolution**, 2013, 30, 772–780. doi.org/10.1093/molbev/mst010.

Kawato, Y., Mohr, P. G., Crane, M. S. J., Williams, L. M., Neave, M. J., Cummins, D. M., Dearnley, M., Crameri, S., Holmes, C., Hoad, J., & Moody, N. J. G. Isolation and characterisation of an ISKNV-genotype megalocytivirus from imported angelfish *Pterophyllum scalare*. **Diseases of aquatic organisms**, 2020, 140, 129–141. doi.org/10.3354/dao03499.

Kawato, Y., Takada, Y., Mizuno, K., Harakawa, S., Yoshihara, Y., Nakagawa, Y., Kurobe, T., Kawakami, H., & Ito, T. Assessing the transmission risk of red sea bream iridovirus (RSIV) in environmental water: insights from fish farms and experimental settings. In: Kibenge, F. S. B. (Eds.), **Microbiology Spectrum**, American Society for Microbiology, 2023, 11(5). doi.org/10.1128/spectrum.01567-23.

Koda, S. A., Subramaniam, K., Francis-Floyd, R., Yanong, R. P., Frasca, S. J., Groff, J. M., Popov, V. L., Fraser, W. A., Yan, A., Mohan, S., & Waltzek, T. B. Phylogenomic characterization of two novel members of the genus *Megalocytivirus* from archived ornamental fish samples. **Diseases of Aquatic Organisms,** 2018, 130(1), 11–24. doi.org/10.3354/dao03250.

Kurita, J. U. N., Nakajima, K., Hirono, I., & Aoki, T. Complete genome sequencing of Red Sea Bream Iridovirus (RSIV). **Fisheries Science**, 2002, 68(2), 1113–1115. doi.org/10.2331/fishsci.68.sup2_1113.

Kurita, J., & Nakajima, K. Megalocytiviruses. **Viruses**, 2012, 4(4), 521-538. doi.org/10.3390/v4040521.

Kurita, J., Nakajima, K., Hirono, I., & Aoki, T. Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV). **Fish Pathology**, 1998, 33(1), 17–23. doi.org/10.3147/jsfp.33.17.

Maganha, S. R. L., Cardoso, P. H. M., de Carvalho Balian, S., de Almeida-Queiroz, S. R., Fernandes, A. M., & de Sousa, R. L. M. Molecular detection and phylogenetic analysis of *Megalocytivirus* in Brazilian ornamental fish. **Archives of Virology**, 2018, 163(8), 2225–2231. doi.org/10.1007/s00705-018-3834-6.

Martins, M. L., & Souza, V. N. Henneguya piaractus n.sp. (Myxozoa: *Myxobolidae*), a gill parasite of *Piaractus mesopotamicus* Holmberg (*Osteichthyes: characidae*), in Brazil. **Revista Brasileira de Biologia**, 1997, 57 (2), 239-245.

McGrogan, D., Ostland, V. E., & Ferguson, H. W. Systemic disease involving an iridovirus-like agent in cultured tilapia, *Oreochromis niloticus* L. – a case report. **Journal of Fish Diseases,** 1998, 21(2), 149–152. doi.org/10.1046/j.1365-2761.1998.00082.x.

Nakajima, K., & Kunita, J. Red sea bream iridoviral disease. **Japanese Association of Virology**, 2005, 55(1), 115–25. doi.org/10.2222/jsv.55.115.

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. **Molecular Biology and Evolution**, 2015, 32, 268–274. doi.org/10.1093/molbev/msu300.

Noga, E. J. Diagnosis and Treatment, Second Edition. Wiley. **Journal of Fish Disease,** 2010, 13-48, 143-147, 375-420. doi.org/10.1002/9781118786758.

Peixe Br, Anuário Brasileiro da Piscicultura Peixes BR 2023. **Associação Brasileira da Piscicultura**, 2023.

Plowright, R. K., Parrish, C. R., McCallum, H., Hudson, P. J., Ko, A. I., Graham, A. L., & Lloyd-Smith, J. O. Pathways to zoonotic spillover. **Nature Reviews Microbiology**, Springer, 2017, 15 (8), 502–510. doi.org/10.1038/nrmicro.2017.45.

Pouder, D. B., Curtis, E. W., & Yanong, R. P. E. Common freshwater fish parasites pictorial guide: **Sessile ciliates,** 2011.

Power, A. G., & Mitchell, C. E. Pathogen spillover in disease epidemics. **The American Naturalist**, 2004, 164(5), 79–89. doi.org/10.1086/424610.

Ramjrez-Paredes, J. G., Paley, R. K., Hunt, W., Feist, S. W., Stone, D. M., Field, T. R., Haydon, D. J., Ziddah, P. A., Nkansa, M., Guilder, J., Gray, J., Duodu, S., Pecku, E. K.,

Awuni, J. A., Wallis, T. S., & Verner-Jeffreys, D. W. First detection of infectious spleen and kidney necrosis virus (ISKNV) associated with massive mortalities in farmed tilapia in Africa. **Transboundary and Emerging Diseases**, 2020, 68 (3), 1550–1563. doi.org/10.1111/tbed.13825.

Rimmer, A. E., Becker, J. A., Tweedie, A., Lintermans, M., Landos, M., Stephens, F., & Whittington, R. J. Detection of dwarf gourami iridovirus (Infectious spleen and kidney necrosis virus) in populations of ornamental fish prior to and after importation into Australia, with the first evidence of infection in domestically farmed Platy (*Xiphophorus maculatus*). **Preventive Veterinary Medicine,** 2015, 122 (1-2), 181–194. doi.org/10.1016/j.prevetmed.2015.09.008.

Sebastião, F. A., Furlan, L. R., Hashimoto, D. T., & Pilarski, F. Identification of bacterial fish pathogens in Brazil by direct colony PCR and 16S rRNA gene sequencing. **Advances in Microbiology**, Scientific Research Publishing, 2015, 05 (06), 409–424. doi.org/10.4236/aim.2015.56042.

Shea, D., Bateman, A., Li, S., Tabata, A., Schulze, A., Mordecai, G., Ogston, L., Volpe, J. P., Frazer, L. N., Connors, B., Miller, K. M., Short, S., & Krkošek, M. Environmental DNA from multiple pathogens is elevated near active Atlantic salmon farms. **Proceedings of the Royal Society,** Biological Sciences, 2020, 287 (1937). doi.org/10.1098/rspb.2020.2010.

Shi, C. Y., Jia, K. T., Yang, B., & Huang, J. Complete genome sequence of a *Megalocytivirus* (family *Iridoviridae*) associated with turbot mortality in China. **Virology Journal**, Springer, 2010, 7 (1). doi.org/10.1186/1743-422x-7-159.

Song, J. Y., Kitamura, S. I., Jung, S. J., Miyadai, T., Tanaka, S., Fukuda, Y., Kim, S. R., & Oh, M. J. Genetic variation and geographic distribution of megalocytiviruses. **Journal of Microbiology**, 2008, 46(1), 29–33. doi.org/10.1007/s12275-007-0184-6.

Subramaniam, K., Gotesman, M., Smith, C., Steckler, N., Kelley, K., Groff, J., & Waltzek, T. *Megalocytivirus* infection in cultured Nile tilapia *Oreochromis niloticus*. **Diseases of Aquatic Organisms**, 2016, 119(3), 253–258. Doi.org/10.3354/dao02985.

Subramaniam, K., Shariff, M., Omar, A. R., & Hair-Bejo, M. *Megalocytivirus* infection in fish. **Reviews in Aquaculture**, 2012, 4 (4), 221-233. doi.org/10.1111/j.1753-5131.2012.01075.x.

Sudthongkong, C., Miyata, M., & Miyazaki, T. Viral DNA sequences of genes encoding the ATPase and the major capsid protein of tropical iridovirus isolates which are pathogenic to fishes in Japan, South China Sea and Southeast Asian countries. **Archives of Virology**, 2002, 147, 2089– 2109. doi.org/10.1007/s00705-002-0883-6.

Suebsing, R., Pradeep, P. J., Jitrakorn, S., Sirithammajak, S., Kampeera, J., Turner, W. A., Saksmerprome, V., Withyachumnarnkul, B., & Kiatpathomchai, W. Detection of natural infection of infectious spleen and kidney necrosis virus in farmed tilapia by hydroxynapthol blue-loop-mediated isothermal amplification assay. **Journal of Applied Microbiology**, 2016, 121, 55–67. doi.org/10.1111/jam.13165.

Swaminathan, T. R., Johny, T. K., Nithianantham, S. R., Sudhagar, A., Pradhan, P. K., Ramachandra, K. S., Nair, R. R., & Sood, N. A natural outbreak of infectious spleen and kidney necrosis virus threatens wild pearlspot, *Etroplus suratensis* in Peechi Dam in the Western Ghats biodiversity hotspot, India. **Transboundary and Emerging Diseases**, 2022, 69 (5). doi.org/10.1111/tbed.14494.

Wang, C. S., Chao, S. Y., Ku, C. C., Wen, C. M., & Shih, H. H. PCR amplification and sequence analysis of the major capsid protein gene of megalocytiviruses isolated in Taiwan. **Journal of Fish Diseases**, 2009, 32, 543–550. doi.org/10.1111/j.1365-2761.2009.01043.x.

Wang, Y. Q., Lü, L., Weng, S. P., Huang, J. N., Chan, S.-M., & He, J. G. Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis virus-like (ISKNV-like) virus. **Archives of Virology**, 2007, 152(4), 763–773. doi.org/10.1007/s00705-006-0870-4.

Williams, T., Barbosa-Solomieu, V., & Chinchar, V. G. A decade of advances in iridovirus research. **Advances in virus research**, 2005, 65, 173–248. doi.org/10.1016/S0065-3527(05)65006-3.

WOAH, Chapter 2.3.7. Red Sea Bream Iridoviral Disease, World Organization for Animal Health, 2022. Available online at: https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_rsbid.pdf (accessed February 23, 2023).

Xu, X., Zhang, L., Weng, S., Huang, Z., Lu, J., Lan, D., Zhong, X., Yu, X., Xu, A., & He, J. A zebrafish (*Danio rerio*) model of infectious spleen and kidney necrosis virus (ISKNV) infection. **Virology**, 2008, 376(1), 1-12. doi.org/10.1016/j.virol.2007.12.026.

Zhu, Z., Duan, C., Li, Y., Huang, C., Weng, S., He, J., & Dong, C. 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**, 2021, 534, 736326. doi.org/10.1016/j.aquaculture.2020.