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First report of infectious spleen and kidney necrosis virus (ISKNV) in two native cichlids cultured in Brazil

Primeiro relato do vírus da necrose infecciosa do baço e do rim (ISKNV) em dois ciclídeos nativos cultivados no Brasil

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Highlights

Detection of ISKNV in two native Brazilian cichlids.
High sequence identity of ISKNV of native cichlids with previously detected strains.
A conventional PCR assay with an ISKNV detection rate equivalent to nested PCR assays.

Abstract

Peacock bass (syn.: tucunaré, *Cichla ocellaris*) and the pearl cichlids (syn.: acará, *Geophagus brasiliensis*) are South American cichlids that are highly valued in both the ornamental and sport fish industries. Since 2017, a number of outbreaks of infectious spleen and kidney necrosis virus (ISKNV) have been reported on Brazilian food and ornamental fish farms. In this study, we detected ISKNV in farmed peacock bass

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and pearl cichlid by PCR and sequence analysis of the partial major capsid protein (MCP) gene. Moribund peacock bass (n=10) and pearl cichlids (2) from a farm experiencing elevated mortality among juveniles and adults of these species, were submitted for bacteriological and molecular diagnostics. Spleen, liver, brain, and kidney tissues were cultured on 5% sheep blood agar and cystine heart agar with 1% glucose and bovine haemoglobin. No bacteria were isolated from the 12 fish. Additionally, DNA extracts from the liver and spleen of all animals were tested for ISKNV using two conventional polymerase chain reaction (cPCR) assays and two nested PCR (nPCR) assays. ISKNV DNA was amplified in all 12 fish DNA extracts tested, in two or more of the PCR assays. Selected ISKNV amplicons were confirmed by Sanger sequencing. The nucleotide sequences derived from these animals were identical to ISKNV strains previously detected in food (e.g., tilapia and carp) and ornamental species, including strains previously detected in fish from Brazil. To the authors' knowledge, this is the first report of ISKNV in these native Brazilian cichlids.
Key words: Aquaculture. Fish disease. Molecular diagnosis. Peacock bass. Pearl cichlid. Iridovirus.

Resumo

Tucunaré (*Cichla ocellaris*) e acará (*Geophagus brasiliensis*) são ciclídeos sul-americanos muito valorizados tanto na indústria de peixes ornamentais como esportivos. Desde 2017, vários surtos do vírus da necrose infecciosa do baço e do rim (ISKNV) foram relatados em produções brasileiras de peixes de espécies alimentares e ornamentais. Neste estudo, relata-se a detecção de ISKNV em tucunarés e acarás cultivados por PCR e análise de sequência parcial do gene principal proteína do capsídeo (*major capsid protein* - MCP). Tucunarés (n=10) e acarás (2) moribundos provenientes de uma piscicultura com elevada mortalidade entre juvenis e adultos dessas espécies, foram submetidos ao diagnóstico bacteriológicas e molecular. Os tecidos do baço, fígado, cérebro e rim foram semeados em ágar sangue de carneiro a 5% e ágar cistina coração com 1% de glicose e hemoglobina bovina. Nenhuma bactéria foi isolada dos 12 peixes. Além disso, o DNA extraído do fígado e baço de todos os animais foram testados para ISKNV usando dois ensaios convencionais de reação em cadeia da polimerase (cPCR) e dois ensaios de nested PCR (nPCR). O DNA de ISKNV foi amplificado em todos os 12 extraídos de DNA dos peixes testados, em dois ou mais ensaios de PCR. Os *amplicons* de ISKNV selecionados foram confirmados pelo sequenciamento Sanger. As sequências de nucleotídeos derivadas desses animais eram idênticas às cepas de ISKNV previamente detectadas em espécies alimentares (ex. tilápias e carpas) e espécies ornamentais, incluindo cepas previamente detectadas em peixes do Brasil. Acredita-se que este é o primeiro relato de ISKNV nesses ciclídeos nativos brasileiros.
Palavras-chave: Aquicultura. Doenças em Peixes. Diagnóstico Molecular. Tucunaré. Acará. Iridovírus.

Introduction

Peacock bass (syn.: tucunaré, *Cichla ocellaris*) and the pearl cichlid (syn.: acará, *Geophagus brasiliensis*) are two South American cichlids that are highly sought-after by anglers and aquarists alike (Rodrigues et al., 2020). Peacock bass is recognized as a premier game fish due to its aggressive strikes, aerial acrobatics once hooked, and unmatched fighting spirit. In 2021, Brazil produced approximately 155

tons of peacock bass to support this industry (Instituto Brasileiro de Geografia e Estatística [IBGE], 2024). The pearl cichlid is a popular aquarium fish and an important game fish as well (Azevedo et al., 2006). However, the optimal production of these fishes has been hindered by knowledge gaps in fish husbandry, nutrition, and health (Valladão et al., 2018). To date, several parasites have been reported in peacock bass and pearl cichlid, including cestodes, digeneans, monogeneans, nematodes, coccidia, and copepods (Békési & Molnár, 1991; Azevedo et al., 2012; Januário et al., 2019; Lacerda et al., 2018; Madi & Ueta 2009; Pavanelli et al., 2018; Pozza et al., 2018; Rassier et al., 2015; Rocha et al., 2015; Scholz et al., 1996).

Infectious spleen and kidney necrosis virus (ISKNV) is a double-stranded DNA virus that is a member of the family *Iridoviridae,* subfamily *Alphairidovirinae,* genus *Megalocytivirus.* Megalocytiviruses negatively impacting aquaculture include ISKNV, red sea bream iridovirus (RSIV), turbot reddish body iridovirus (TRBIV), and scale drop disease virus (International Committee on Taxonomy of Viruses [ICTV], 2022). Since 2017, there have been an increasing number of ISKNV outbreaks on tilapia farms in Brazil (Figueiredo et al., 2022; Fonseca et al., 2022). ISKNV has also been reported in the country in native species including red piranha (*Pygocentrus nattereri*), pintado (*Pseudoplatystoma corruscans*), and several ornamental species (Lucca Maganha et al., 2018; Fonseca et al., 2022).

The clinical signs of ISKNV are non-specific and include lethargy, anorexia, irregular swimming, pallor of the gills or body, and coelomic distension due to ascites (Dong et al., 2015; Johan & Zainathan, 2020; Subramaniam et al., 2016). Molecular assays targeting the major capsid protein (MCP) gene are widely used for the detection of ISKNV (Kurita & Nakajima, 2012). In this study, we detected ISKNV in moribund peacock bass and pearl cichlids on a Brazilian farm by PCR and Sanger sequencing.

Materials and Methods

Outbreak description

From April to May 2021, a fish farm in the state of Rio de Janeiro, Brazil reported an increase in mortality within their pond-reared peacock bass. The farm owner reported the number of dead animals had increased from six animals to more than 200 per day, including both juveniles and adults. The dammed river system where the farm is located belongs to a sports club and contains native fish species including *Astyanax* spp., trahira (*Hoplias* spp.), and *Leporinus* spp., as well as farmed Nile tilapia (*Oreochromis niloticus*) reared in cages. These native species appeared normal, except the Nile tilapia that experienced elevated mortality during the same period. Nile tilapia samples were not processed due to their advanced state of decomposition.

Bacterial isolation and identification

In May 2021, 12 moribund fishes (ten peacock bass and two pearl cichlids) were packed in insulated boxes on ice and sent for diagnostic evaluation. Transport between the farm and the laboratory took eight hours. The fish were immediately necropsied, and the spleen, liver, brain, and kidney tissues were

cultured on 5% sheep blood agar and cystine heart agar with 1% glucose and bovine hemoglobin. The plates were incubated at 28 °C for 4 days and checked daily for bacterial growth. Then, liver and spleen tissues were also collected from each fish and frozen at -20 °C for molecular diagnostics.

Detection of ISKNV DNA

DNA extraction of liver and spleen tissue pools from each fish was performed using a PureLink™ Genomic DNA Mini Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA). DNA extracts from all animals were then screened for ISKNV using two conventional polymerase chain reaction (cPCR) assays (Kurita & Nakajima, 2012; Kurita et al., 1998) and two nested PCR (nPCR) assays (Pattanayak et al., 2020; Rimmer et al., 2012). The cycling conditions were in house standardized to optimize the assays (Tables 1 and 2). The cycling conditions of the PCR assays matched those previously reported (Table 1) with minor modifications. As example, there was necessary perform a temperature gradient tests of primers in PCR reaction of Kurita and Nakajima (2012), also used in Pattanayak et al. (2020) work, due the presence of unspecific bands. Thus, temperature ranging used was 58 to 61 ºC (0.5 ºC increments) and the better result with clear and single band was observed with annealing temperature of 60.5 ºC and was therefore chosen. PCR products were subjected to electrophoresis in a 1% agarose gel stained with SyBR Safe DNA (Invitrogen® Life Technologies, Carlsbad, CA, USA).

Table 1
Thermocycler conditions for the conventional and nested PCR assays used to detect ISKNV in the fish tissue DNA extracts

PCR protocols	Molecular assays	Initial denaturation step	Amplification step			Number of cycles	Final extension step
			Denaturation	Annealing	Extension		
Kurita et al., 1998	PCR	94 ºC, 5 m	94 ºC, 30s	58 ºC, 1m	72 ºC, 1m	30	72 ºC, 5m
Kurita and Nakajima, 2012	PCR	95 ºC, 5 m	95 ºC, 1m	60.5 ºC, 1m	72 ºC, 1m	35	72 ºC, 5m
Rimmer et al., 2012	PCR	95 ºC, 5 m	95 ºC, 30s	55 ºC, 30s	72 ºC, 1m	30	72 ºC, 5m
	Nested	95 ºC, 5 m	95 ºC, 30s	55 ºC, 30s	72 ºC, 1m	30	72 ºC, 5m
Pattanayak et al., 2020	PCR	95 ºC, 5 m	95 ºC, 1m	57 ºC, 1m	72 ºC, 1m	35	72 ºC, 5m
	Nested	95 ºC, 5 m	95 ºC, 1m	60.5 ºC, 1m	72 ºC, 1m	35	72 ºC, 5m

Table 2
Primer sequences, amplification techniques, and amplified product sizes used for the identification of ISKNV in fish tissue DNA extracts

Primer name	Sequence (5'-3')	Molecular technique	Amplified product sizes (bp)	Protocol reference
1-F	CTCAAACACTCTGGCTCATC	cPCR	570	Kurita et al., 1998
1-R	GCACCAACACATCTCCTATC			
MCP-specI465-F3	GGTGGCCGGCATCACCAACGGC	cPCR	415	Kurita and Nakajima 2012
MCP-specI879-R3	CACGGGGTGACTGAACCTG			
C1105	GGGTTCATCGACATCTCCGCG	PCR	1075	Pattanayak et al., 2020
MCP-uni1108-R8	TCTCAGGCATGCTGGGCGCAAAG			
MCP-specI465-F3	GGTGGCCGGCATCACCAACGGC	nPCR	415	
MCP-specI879-R3	CACGGGGTGACTGAACCTG			
C1105	GGGTTCATCGACATCTCCGCG	PCR	430	Rimmer et al., 2012
C1106	AGGTCGCTGCGCATGCCAATC			
C1073	AATGCCGTGACCTACTTTGC	nPCR	167	
C1074	GATCTTAACACGCAGCCACA			

cPCR: conventional PCR; nPCR: nested PCR.

Sequence analysis

Four ISKNV amplicons of the expected size (415 bp) generated by the cPCR assay of Kurita and Nakajima (2012) were purified using a PureLink™ Quick Gel Extraction & PCR Combo Kit (Invitrogen® Life Technologies, Carlsbad, CA, USA) and the concentration of the purified samples was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Direct Sanger sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA) in both directions, using the primers described above on a 3500 Genetic Analyzer. The sequence data were assembled and edited (including the removal of primer sequences) using MEGA version 7.0.26 (Kumar et al., 2016). BLASTN analyses were performed using the edited sequences (https://blast.ncbi.nlm.nih.gov/). A Maximum likelihood phylogenetic analysis was performed using the Kimura-2 model (Kimura, 1980) in MEGA version.7.0.26, with 1000 non-parametric standard bootstraps to test the robustness of the clades (Efron et al., 1996).

Results and Discussion

No bacteria were isolated from the 12 fish (ten peacock bass and two pearl cichlids). ISKNV DNA was identified in all liver and spleen tissues pools analyzed in two or more PCR assays (Table 3). Different results (negative or positive amplification) were observed among the four PCR assays used in this study. Although the cPCR assay

developed by Kurita and Nakajima (2012) and the nPCR assay described by Rimmer et al. (2012) detected ISKNV DNA in all evaluated fish samples, the cPCR protocol developed by Kurita et al. (1998) did not amplify the ISKNV gene target in any of the evaluated tissue extracts. The nPCR protocol developed by Pattanayak et al. (2020) amplified ISKNV DNA in all peacock bass samples, but only one of the two pearl cichlid samples.

Table 3
Test results of the sampled fish by the ISKNV conventional PCR (cPCR) and nested PCR (nPCR) assays

PCR protocol	A.ssay	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	A1	A2
Kurita et al., 1998	cPCR	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Kurita and Nakajima, 2012	cPCR	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
Rimmer et al., 2012	nPCR	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
Pattanayak et al., 2020	nPCR	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg	pos

cPCR: conventional PCR; nPCR: nested PCR
T1-T10: peacock bass; A1-A2: pearl cichlids.

Sanger sequencing of the amplicons (n=4) generated by the Kurita and Nakajima (2012) assay was performed to confirm the presence of ISKNV DNA in the fish tissues. After sequence editing and primer removal, the resulting sequences (354 bp) from the peacock bass and pearl cichlids were found to be identical. The ISKNV two sequences amplified from pearl cichlids (BRA/RJ-Acar1/2021; accession no. OQ875186 and BRA/RJ-Acar2/2021; OQ875187) and two sequences amplified from peacock bass (BRA/RJ-Tucunar4/2021; OQ875188 and BRA/RJ-Tucunar6/2021; OQ875189) were submitted to GenBank. BLASTN analysis of the 354 bp ISKNV sequence revealed it was identical to 90 ISKNV sequences, including strains previously detected in cultured Nile tilapia (BRA/PR-Til1/2021, BRA/PR-Til3/2021, BRA/PR-Til4/2021) and common carp (BRA/PR-Carpe1/2021) in different regions of Brazil (unpublished data) as well as strains originating from ornamental fishes (data not shown). The maximum likelihood analysis supported the Brazilian ISKNV strain, amplified from peacock bass and pearl cichlid tissues, as a member of the ISKNV genotype (Figure 1).

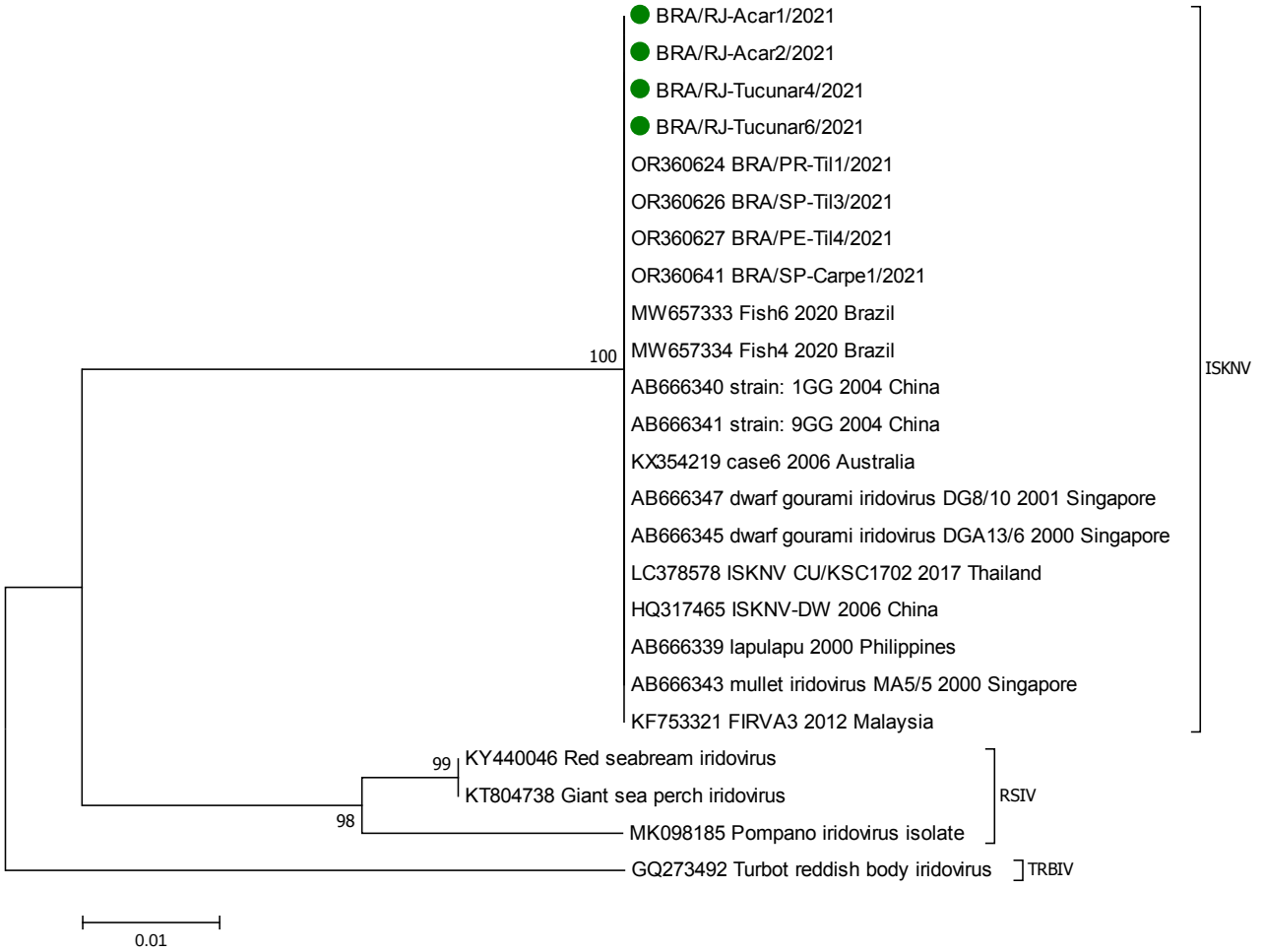

Figure 1. Maximum likelihood phylogenetic analysis performed on the partial (354 nt) major capsid protein gene alignment of 24 sequences including representatives from each of the three ISKNV genotypes (ISKNV [20 sequences], red seabream iridovirus [RSIV: 1 sequence], giant sea perch iridovirus [GSIV-K1: 1 sequence], pompano iridovirus [PIV: 1 sequence], and turbot reddish body iridovirus [TRBIV: 1 sequence]). The evolutionary history was inferred by using the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The peacock bass (BRA/RJ-Tucunar4/2021 and BRA/RJ-Tucunar6/2021) and pearl cichlid (BRA/RJ-Acar1/2021 and BRA/RJ-Acar2/2021) viral sequences were supported as members of the ISKNV genotype. The peacock bass (BRA/RJ-Tucunar4/2021 and BRA/RJ-Tucunar6/2021) and pearl cichlid (BRA/RJ-Acar1/2021 and BRA/RJ-Acar2/2021) sequences generated in this study are marked with green filled circles.

Herein, we report the first detection of ISKNV in two native fish species (peacock bass and pearl cichlids) in Brazil. The virus has previously been reported in Brazil in Nile tilapia, red piranha, pintado, and ornamental fishes (Figueiredo et al., 2022; Fonseca et al., 2022). In this study, we employed four different PCR assays including two cPCR and two nPCR assays. The cPCR assay developed by Kurita et al. (1998) did not detect ISKNV DNA in any tissue samples, while the cPCR assay developed by Kurita and Nakajima (2012) was able to identify ISKNV in all the tissue samples. This discrepancy may be explained by the lower sensitivity of the Kurita et al. (1998) cPCR assay as previously reported (Rimmer et al., 2012). The apparent lower sensitivity of the Kurita et al. (1998) cPCR assay may be a function of the lower number of cycles (30) performed when using this cPCR assay. It is also possible that the Brazilian ISKNV strain detected in this study possesses mutations resulting in poor binding of the primers employed in the Kurita et al. (1998) cPCR assay. Importantly, the Kurita et al. (1998) protocol is recommended by the World Organization for Animal Health (WOAH). Therefore, our study reinforces the need to update the WOAH-recommended diagnostic protocol for the detection of ISKNV in order to improve the identification of infected animals.

On the same dam where the farm is located, an increase in mortality of Nile tilapia was noted at roughly the same time as the outbreak experienced on the farm. However, the etiology of the tilapia mortality was not investigated due to logistical limitations and the advanced state of degradation of the tilapia specimens. It is known that native fish seek food remains in aquaculture facilities and tilapia can escape from fish farming tanks (Azevedo-Santos et al., 2011; Casimiro et al., 2018). These interactions may be responsible for the transfer of pathogens between species and, even in some cases, for the introduction of exotic pathogens into native fauna (Costa et al., 2021). Both species in this study, peacock bass and pearl cichlids, are members of the family Cichlidae which also includes Nile tilapia. This genetic relatedness may facilitate transmission of pathogens like ISKNV between cichlid species. Although there were native fish from other fish families present in the dammed river where the fish farm is located, none showed clinical signs of disease. It is not clear whether the Nile tilapia mortality was due to ISKNV and whether future ISKNV outbreaks as reported here in two Brazilian endemic cichlids might pose a risk to other native species. However, ISKNV is known to exhibit low host specificity resulting in disease in many orders of freshwater and marine fishes, including Brazilian catfish (*Pseudoplatystoma corruscans*) (Fonseca et al., 2022).

The partially characterized ISKNV strain identified in native cichlids in this study was identical to previous strains identified in cultured tilapia and carp in Brazil, suggesting that viral transmission between these species may be possible (Swaminathan et al., 2022). Our findings underscore the importance of implementing proper biosecurity measures in Brazilian aquaculture to both control the impact and spread of endemic diseases as well as to prevent the entry of exotic pathogens.

Conclusions

In this study, the presence of ISKNV was detected in two native cichlid species during a period of elevated mortality on a farm in Brazil. To the authors' knowledge, this is the first report of the ISKNV in these species. Additional studies are needed to determine the relative risk ISKNV poses to wild and farmed fish populations in Brazil.

Ethics Committee

This study was approved by the Londrina State University (UEL) Institutional Ethical Committee of Animal Care and Use (CEUA/UEL Protocol Number 053.2020).

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Data Availability Statement

Data available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest concerning the research, authorship, and/or publication of this article.

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