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Detection and Histopathological Characterization of *Megalocytivirus pagrus* 1 (ISKNV) in Nile Tilapia (*Oreochromis niloticus*) under Experimental Infection and Density Stress

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Abstract**—** *The project encompasses the investigation of the presence of Megalocytivirus pagrus 1 (subtype ISKNV) in fish (Oreochromis niloticus), which has serious implications for animal health, public health and agribusiness. We aimed at the prospection and detection of ISKNV with molecular and histological techniques. Viral fish diseases caused by viruses of the family Iridoviridae, to which ISKNV belongs, are distributed worldwide and represent a real threat to the expansion of the global aquaculture system, due to their pathogenicity, which causes significant economic losses. Thus, the improvement and application of existing diagnostic techniques become necessary to control or prevent the advance of this virosis among countries and in Brazil. In addition, studies in our field that aim to detect and characterize them in a more comprehensive way are scarce. We were able to evidence the presence of the virus through clinical signs during the experiment, detection of genetic material by conventional PCR and by the presence of inclusion bodies, identified by histopathological analysis. We observed a higher infection rate in the period of 14 days after infection by intraperitoneal viral inoculation, as well as greater viral proliferation in the treatment submitted to stress, however without resulting in mortality.*

Keywords— Megalocytivirus pagrus 1 (ISKNV), Oreochromis niloticus, Diagnostic techniques.

I. INTRODUCTION

The expansion of aquaculture in recent decades, driven by the increase in global demand for fish, has promoted significant changes in production systems, especially with the increase in stocking density and the intensification of management. In Brazil, tilapia (*Oreochromis niloticus*) stands out as the main cultivated species, representing approximately 42% of national production, which exceeded 700 thousand tons in 2024 (Associação Brasileira de Aquicultura, 2024). On a global scale, aquaculture production intended for human consumption reached around 94 million tons in 2022, evidencing the economic relevance of the sector (FAO, 2024).

However, the intensification of production systems favors the occurrence and dissemination of infectious diseases, since high population densities and controlled environmental conditions may act as predisposing factors for epizootic outbreaks. In this context, viral agents assume a prominent role, especially due to their high transmission capacity and potential to cause expressive economic losses (Wang et al., 2011).

Among the viruses of sanitary importance in aquaculture, members of the family *Iridoviridae* stand out, especially the genus *Megalocytivirus*, responsible for systemic infections in several species of freshwater and marine fish (Koda et al., 2018). *Megalocytivirus pagrus* 1 (subtype ISKNV) is considered an emerging pathogen, with wide geographical distribution

and capacity to infect more than 50 fish species. The infection may result in significant mortality, especially in initial stages of development, such as fingerlings and juveniles.

Clinically, ISKNV infection presents nonspecific signs, including lethargy, anorexia, swimming alterations, hyperpigmentation and cutaneous lesions. From the histopathological point of view, it is characterized by the presence of cytomegalic cells and intracytoplasmic inclusions in multiple organs, such as kidney, spleen, liver and intestine, these alterations being considered important markers for the diagnosis of the disease (Yanong & Waltzek, 2010; Wang et al., 2011).

The detection of ISKNV can be performed by means of different diagnostic approaches, including molecular techniques, such as polymerase chain reaction (PCR), and histopathological analyses.

Given this scenario, the present study aimed to investigate the presence of *Megalocytivirus pagrus* 1 (subtype ISKNV) in Nile tilapia submitted to experimental infection, associated with high-density stress, through the integrated application of molecular and histological techniques (Guo et al., 2025).

General Objective: Improvement and application of molecular and histological diagnostic techniques for the detection and characterization of *Megalocytivirus pagrus* 1 (subtype ISKNV).

Specific Objectives: (1) to perform experimental infection by intraperitoneal inoculation of viral extract in Nile tilapia, (2) to evaluate the presence of viral genetic material after experimental infection by means of the conventional PCR technique, and (3) to perform histopathological analyses by means of the hematoxylin-eosin staining technique (H&E) for the evaluation of post-infection tissue alterations.

II. MATERIAL AND METHODS

The experiment was conducted at the Fisheries and Biological Institutes of São Paulo, in the Multiuser Laboratory for Experimentation and Interinstitutional Aquaculture Health (LISA).

2.1 Ethical Approval

The study was conducted in accordance with the ethical principles of animal experimentation. All procedures were approved by the Institutional Animal Care and Use Committee (Protocol No. [to be specified by the authors]).

2.2 Transport and Acclimatization

The animals were acquired from a fish farm in Vale do Paraíba, in Pindamonhangaba/SP, and transported in 60 L bags containing water and oxygen. Subsequently, they were acclimatized in two 100 L tanks for 30 days.

2.3 Physical and Feeding Management

During the experimental period, the tanks were siphoned daily for the removal of dirt and mortality recording. The fish were fed with feed corresponding to 3% of the biomass, divided into two daily portions.

2.4 Experimental Design

The experiment lasted 14 days. The viral inoculum was prepared from tissue samples (25 mg) previously positive for *Megalocytivirus pagrus* 1 (subtype ISKNV), presenting Ct 24. The material was macerated in 500 µL of sterile PBS under laminar flow and subsequently centrifuged at 16,000 × g, for 10 min, at 4 °C, with the supernatant being used as inoculum.

Before the experimental infection, the fish were anesthetized using a eugenol solution prepared with 8 mL of eugenol diluted in 50 mL of alcohol. Then, the animals were inoculated intraperitoneally and transferred to their respective experimental aquariums.

The experimental design was completely randomized, composed of four treatments with two simultaneous replicates:

- **T1** — negative control (without inoculation);
- **T2** — control inoculated with PBS;

- **T3** — fish infected with ISKNV;
- **T4** — fish infected with ISKNV (high density).

All groups were subjected to the condition of high-density stress. Aquariums of 12 L were used, with random distribution of the fingerlings. In treatments T1, T2 and T3, 12 fish per aquarium were housed, while in T4, 28 fish per aquarium were maintained. Additionally, treatment T3 had an extra replicate intended for histological sampling, totaling 104 fish in the experiment (Figure 1).

All sampled and surviving animals were anesthetized with eugenol (75 mg L^{-1}), euthanized by spinal cord section, autoclaved and properly discarded. The materials used and the aquarium water were disinfected with chlorine at 10 ppm for 24 h.

In the first collection, 12 fish were sampled, six from T3 and six from T4 (three from each replicate) to confirm the experimental infection. In the second collection, 42 fish were sampled for PCR analysis, six from T1, four from T2, eight from T3 and 24 from T4, in addition to 24 fish intended for histopathological analysis. The organs collected were kidney and spleen, subsequently fixed in 10% buffered formalin for seven days for histopathology or stored frozen in 1.5 mL microtubes for subsequent molecular analysis by PCR.

2.5 Conventional PCR

DNA extraction was performed using the commercial Wizard® Genomic DNA Purification kit (Promega®, 2023), following the manufacturer's instructions.

Viral detection was conducted by conventional PCR using the GoTaq® G2 Master Mix (Promega®) and the primers MCP-uni332-F3 (5'-AGGTGTCGGGCGCAAAG-3') and MCP-uni1108-R8 (5'-TCTCAGGCATGCTGGGCGCAAAG-3'), described by Kurita and Nakajima (2012), which amplify a 777 bp fragment of the MCP gene. The amplification conditions consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with final extension at 72 °C for 5 min.

The amplified products were submitted to electrophoresis in 2.4% agarose gel, using Nucleic Acid Stain (Cellco®) for staining, and visualized in a Gel Doc™ XR+ system (Bio-Rad®) through the Image Lab software. The analyses were performed using spleen and kidney samples collected at 7 and 14 days post-infection, with pooled extraction being performed when necessary.

2.6 Histological Technique (Hematoxylin-Eosin – H&E)

The samples were processed at the Laboratory for Investigation in Aquaculture Health (LISA) according to the protocol described by Michalany (1980), including the steps of dehydration in increasing series of alcohols, clearing, paraffin embedding, microtomy and staining by the hematoxylin-eosin technique (H&E).

2.7 Histopathological Scoring

For semi-quantitative analysis, histopathological lesions were scored using a standardized system: 0 = absent, 1 = mild, 2 = moderate, 3 = severe. Lesions evaluated included inclusion bodies, inflammatory infiltrate, glomerulotubulonephrosis, tubular vacuolization, melanomacrophage centers, and hemorrhage. Scores were compared between treatments and sampling periods.

2.8 Biometry and Acclimatization

The animals had a mean body weight of 9.28 ± 3.2 g, and the mean water temperature during the experimental period was 24.93 ± 1.52 °C.

2.9 Clinical Signs

Infected fish showed clinical signs characterized by erratic swimming, melanosis, anorexia, lethargy, continuous stress, ascites, cutaneous hemorrhagic lesions, and friable kidneys (Figure 1). In the groups subjected to high-density stress, the formation of more cohesive schools was observed, indicating a behavioral change associated with the stress condition. In contrast, the control groups (T1 and T2) showed no relevant clinical changes throughout the experimental period.

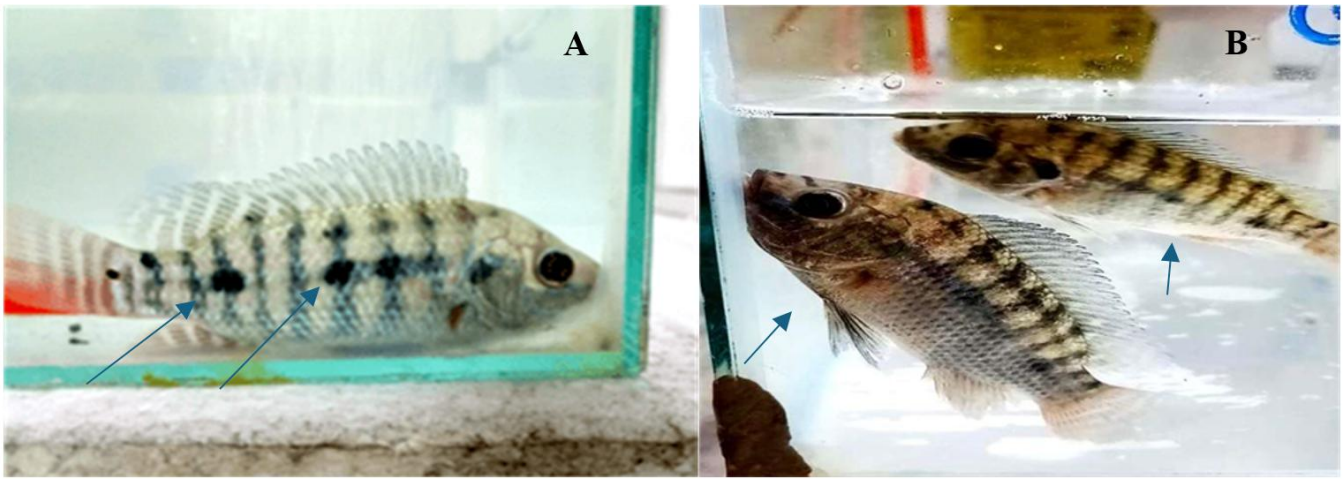

FIGURE 1: A. Melanosis near the lateral line (long arrow). B. Erratic swimming and cutaneous lesions (short arrow).

2.10 Statistical Analysis

All statistical analyses were performed using [specify software, e.g., GraphPad Prism or SPSS]. Differences in PCR positivity rates between treatments and sampling periods were analyzed using Fisher's exact test. Histopathological scores were compared using the Mann-Whitney U test (for two groups) or Kruskal-Wallis test followed by Dunn's post-hoc test (for multiple groups). Significance was set at $p < 0.05$. Data are presented as mean ± standard deviation where applicable.

III. RESULTS

3.1 PCR

Of the 32 samples analyzed, 43.75% (14/32) tested positive in groups T3 and T4, whereas all six samples from the control groups (T1 and T2) were negative.

A higher number of positive samples was observed on day 14 compared with day 7 post-infection. On day 7, 2 out of 12 samples (16.7%) were positive, while on day 14, 12 out of 20 samples (60.0%) were positive (Fisher's exact test, $p < 0.05$). Additionally, the high-density stress group (T4) showed a higher positivity rate (11/18, 61.1%) compared to the infected group without stress (T3) (3/14, 21.4%) on day 14 (Fisher's exact test, $p = 0.04$).

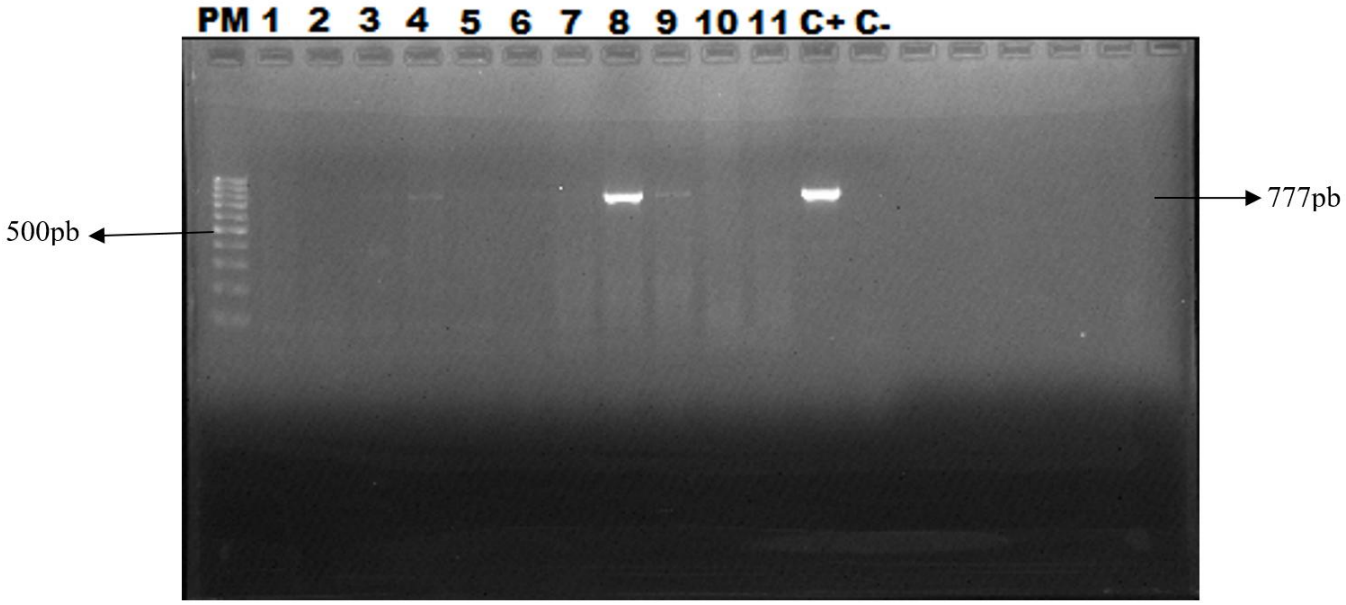

FIGURE 2: Electrophoresis of PCR-amplified DNA products for the amplification of a fragment of the MCP region of *Megalocytivirus pagrus* 1 (subtype ISKNV) on days 7 and 14. PM:

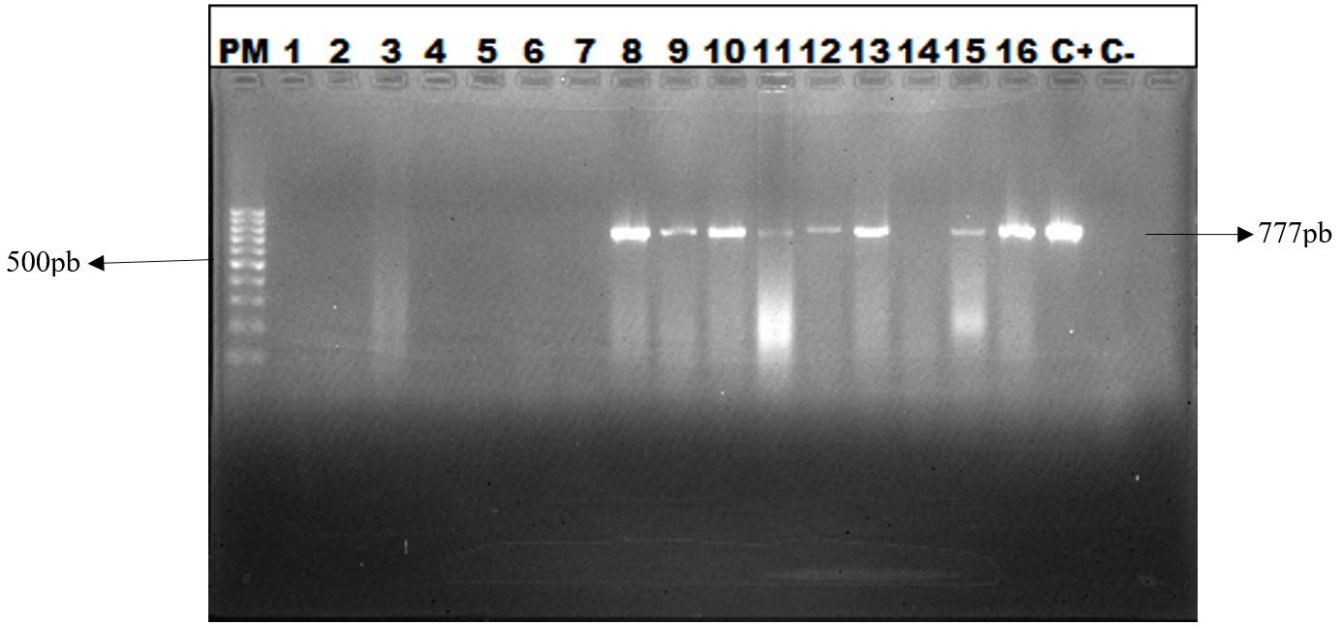

FIGURE 3: Electrophoresis result of PCR-amplified DNA products for the amplification of a fragment of the MCP region of *Megalocytivirus pagrus* 1 (subtype ISKNV) on day 14. PM: molecular weight marker (100 bp DNA Ladder, Celco®); lanes 1 to 6: control samples (T1 and T2); lanes 7 to 16: infected DNA samples (T3 and T4); C+: positive control; C−: negative control.

TABLE 1
CONVENTIONAL PCR RESULTS BY TREATMENT ON DAY 7

Sample	Treatment	Aquarium	PCR
1	T3	Infected	Negative
2	T3	Infected	Negative
3	T3	Infected	Positive
4	T3	Infected	Positive
5	T3	Infected	Negative
6	T3	Infected	Negative
7	T4	Infected	Negative
8	T4	Infected	Negative
9	T4	Infected	Negative
10	T4	Infected	Negative
11	T4	Infected	Negative
12	T4	Infected	Negative

TABLE 2
CONVENTIONAL PCR RESULTS BY TREATMENT ON DAY 14

Sample	Treatment	Aquarium	PCR
1	T1	Control	Negative
2	T1	Control	Negative
3	T1	Control	Negative
4	T1	Control	Negative
5	T1	Control	Negative
6	T1	Control	Negative
7	T2	Control	Negative
8	T2	Control	Negative
9	T2	Control	Negative
10	T2	Control	Negative
11	T3	Infected	Positive
12	T3	Infected	Negative
13	T3	Infected	Negative
14	T3	Infected	Negative
15	T3	Infected	Positive
16	T3	Infected	Negative
17	T3	Infected	Positive
18	T3	Infected	Positive
19	T4	Infected	Positive
20	T4	Infected	Positive
21	T4	Infected	Positive
22	T4	Infected	Positive
23	T4	Infected	Positive
24	T4	Infected	Positive
25	T4	Infected	Positive
26	T4	Infected	Positive
27	T4	Infected	Positive
28	T4	Infected	Positive
29	T4	Infected	Positive
30	T4	Infected	Positive
31	T4	Infected	Negative
32	T4	Infected	Positive
33	T4	Infected	Positive
34	T4	Infected	Positive
35	T4	Infected	Positive
36	T4	Infected	Negative
37	T4	Infected	Negative
38	T4	Infected	Negative
39	T4	Infected	Negative
40	T4	Infected	Negative
41	T4	Infected	Negative
42	T4	Infected	Negative

3.2 Histopathology

Histopathological analysis revealed significant alterations in the kidney and spleen of the infected groups. In the kidney, lesions were characterized by glomerulotubulonephrosis, tubular vacuolization and hyalinization, subcortical hemorrhage, enlargement of Bowman's space, inflammatory infiltrate, and the presence of scattered melanomacrophages, as well as an increase in melanomacrophage centers.

Inclusion bodies were identified in medullary and cortical regions in treatments T3 and T4. In treatment T3, these structures were observed in both sampling periods, at 7 and 14 days post-infection (Figures 4 and 5). Histopathological scores were significantly higher in infected groups compared to controls ($p < 0.05$), with higher scores observed on day 14 compared to day 7 (Mann-Whitney U test, $p < 0.05$).

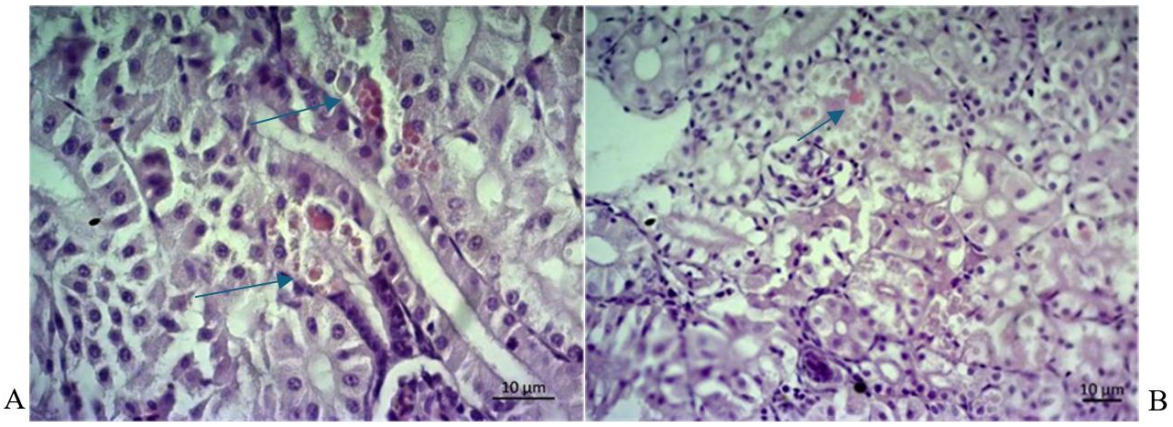

FIGURE 4: Photomicrograph of kidney tissue from Nile tilapia (*Oreochromis niloticus*) experimentally infected with *Megalocytivirus pagrus* 1, subtype ISKNV, at 7 days of experimentation. T3: infected animals without stress. A – Arrows indicate inclusion bodies in the medullary region of the histological section, monolymphocytic glomerulotubulonephritis, and tubular vacuolization in the cortical regions. HE staining; 630× magnification. B – Arrows indicate inclusion bodies near the cortical regions of the histological section and monolymphocytic glomerulotubulonephritis in the central region. HE staining; 400× magnification.

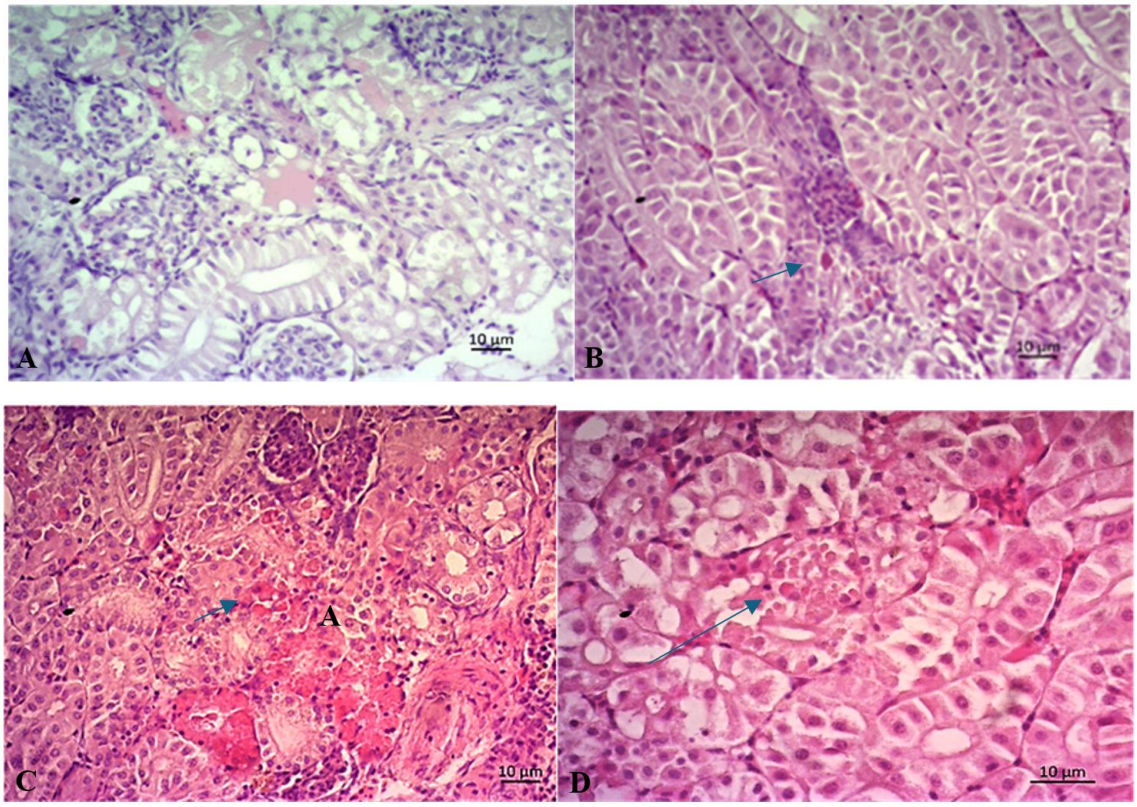

FIGURE 5: Photomicrograph of kidney tissue from Nile tilapia (*Oreochromis niloticus*) experimentally infected with *Megalocytivirus pagrus* 1, subtype ISKNV, at 14 days of experimentation. T4: infected animals subjected to stress; and T3: infected animals without stress. A – Hyalinized and/or vacuolated glomeruli and tubules. HE staining; 400× magnification. B – Arrows indicate inclusion bodies in the medullary and cortical regions of the section. HE staining; 400× magnification. C – Tubular hyalinization with the presence of melanomacrophages and structures suggestive of inclusion bodies. HE staining; 400× magnification. D – Arrow indicates structures suggestive of inclusion bodies in the central region of the section. HE staining; 630× magnification.

In the spleen, the alterations observed in treatments T3 and T4 included subcapsular hemorrhage, vascular congestion, hemosiderin deposition, increased numbers of melanomacrophages, and lymphoid hypoplasia. Additionally, inclusion bodies were identified in splenic parenchyma (Figure 6).

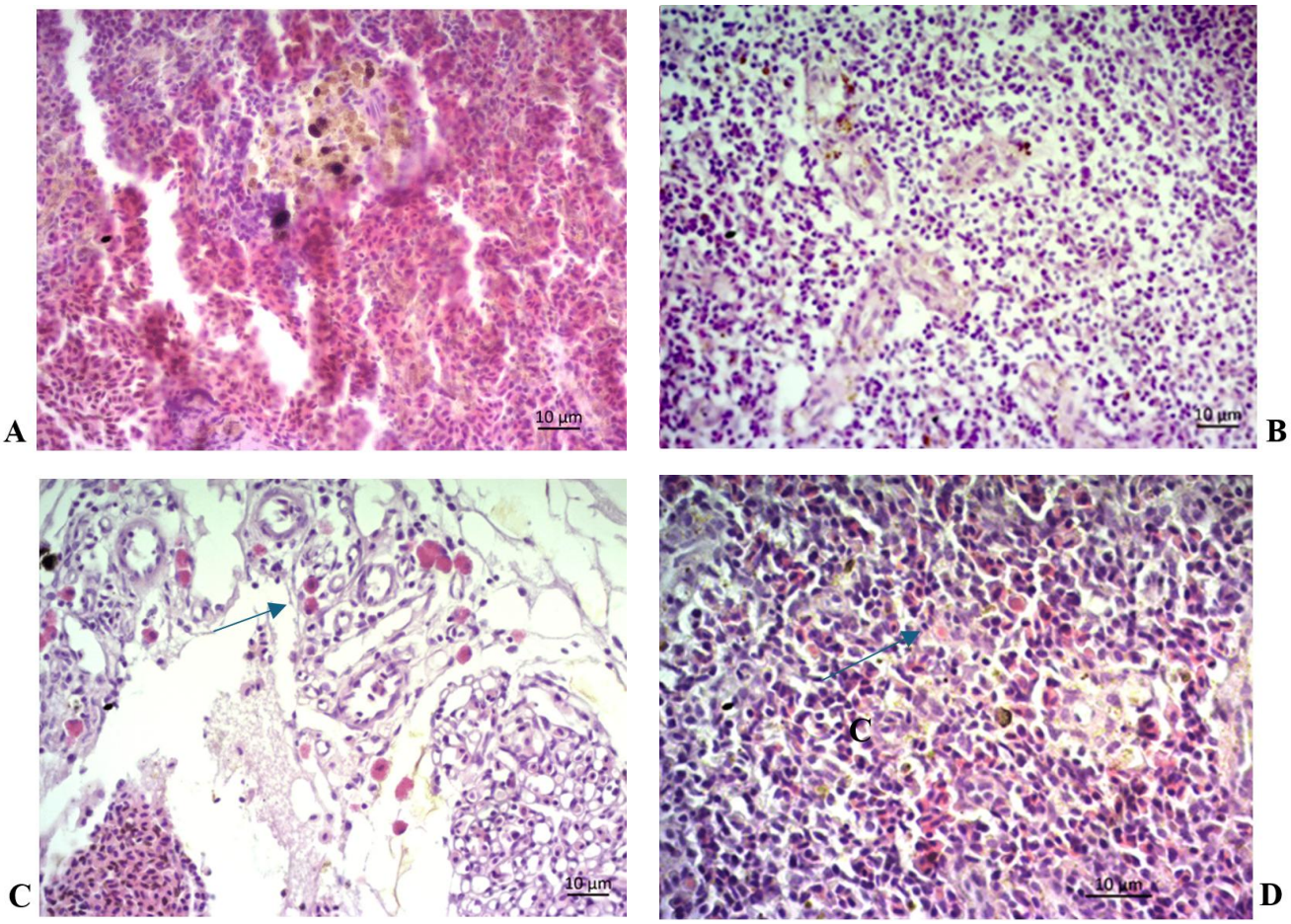

FIGURE 6: Photomicrograph of splenic tissue from Nile tilapia (*Oreochromis niloticus*) experimentally infected with *Megalocytivirus pagrus* 1, subtype ISKNV, at 7 and 14 days of experimentation. T4: infected animals subjected to stress; and T3: infected animals without stress. A – Melanomacrophage center in the medullary region, congested erythrocytes in the cortical regions, and lymphoid follicle hypoplasia near the white pulp. H&E staining; 630× magnification. B – Splenic hypoplasia throughout the section parenchyma and focal melanomacrophages in the medullary region of the histological section. H&E staining; 400× magnification. C – Red arrows indicate multiple eosinophils and a congested area in the pulp with hemosiderin deposition. H&E staining; 400× magnification. D – Arrows indicate an inclusion body in the splenic parenchyma. H&E staining; 630× magnification.

Inclusion bodies were identified at 7 and 14 days post-infection. On day 14, a greater extent of lesions was observed, associated with an increase in inflammatory infiltrate (Figures 5 and 6). Additionally, all infected samples showed renal lesions, indicating kidney involvement during the experimental infection (Figures 4 and 5).

TABLE 3
SUMMARY OF HISTOPATHOLOGICAL FINDINGS

Lesion Type	T1 (Control)	T2 (PBS)	T3 (ISKNV)	T4 (ISKNV + Stress)
Inclusion Bodies	Absent	Absent	Present (Day 7 & 14)	Present (Day 7 & 14)
Glomerulotubulonephrosis	Absent	Absent	Moderate (Day 7) / Severe (Day 14)	Moderate (Day 7) / Severe (Day 14)
Tubular Vacuolization	Absent	Absent	Mild-Moderate	Moderate-Severe
Inflammatory Infiltrate	Absent	Absent	Mild (Day 7) / Moderate (Day 14)	Moderate (Day 7) / Severe (Day 14)
Melanomacrophage Centers	Minimal	Minimal	Increased	Markedly Increased
Splenic Lesions	Absent	Absent	Present	Present

IV. DISCUSSION

The results obtained in this study confirm the occurrence of infection by *Megalocytivirus pagrus* 1 (ISKNV) in tilapia subjected to experimental infection, as evidenced by molecular detection using PCR and corroborated by the histopathological findings. The identification of cytomegalic cells and intracytoplasmic inclusions in organs such as the kidney and spleen is consistent with the classical pattern described for megalocytivirus infections in different fish species (Dong et al., 2008; He et al., 2001; Wang et al., 2011; Subramaniam et al., 2016; Liu et al., 2019), reinforcing the biological consistency of the experimental model employed.

The higher frequency of viral detection on day 14 (60.0%) compared with day 7 (16.7%) indicates progression of the infection throughout the experimental period, suggesting an increase in viral load in the analyzed tissues ($p < 0.05$, Fisher's exact test). This temporal pattern is compatible with the viral replication dynamics previously described for ISKNV (Maganha et al., 2018) and indicates that the experimental period was sufficient to establish a detectable systemic infection.

Despite confirmation of infection and the presence of clinical signs and evident histopathological alterations, no mortality was observed in the infected group subjected to high-density stress. This finding contrasts with studies reporting high mortality rates associated with ISKNV, particularly in early developmental stages (Subramaniam et al., 2016; Wang et al., 2011). This discrepancy suggests that factors such as the viral dose used, strain virulence, observation period (14 days), or physiological characteristics of the animals may have influenced the course of infection in the present study. It is possible that a longer observation period or higher viral dose would have resulted in mortality. The absence of mortality may also reflect the use of a less virulent strain or the age of the fish (juveniles may be more resistant than fingerlings).

Although high-density stress did not result in increased mortality up to day 14, a higher frequency of viral detection was observed in the groups subjected to this condition (T4: 61.1% vs. T3: 21.4% on day 14; $p = 0.04$, Fisher's exact test). This pattern suggests a possible increase in viral dissemination, potentially associated with greater contact among individuals and continuous exposure to contaminated water. Behavioral changes, such as the formation of more cohesive schools in the stressed groups, may have contributed to this scenario, since they increase proximity among individuals and favor horizontal transmission of pathogens (Rodrigues et al., 2021). In addition, high-density stress may impair the immune response of fish, favoring infection progression (Molico, 2013). In this context, the absence of mortality does not indicate absence of biological impact, but rather a possible modulation of infection severity under the experimental conditions evaluated.

The presence of clinical signs, such as lethargy, melanosis, and swimming abnormalities, associated with consistent histopathological alterations, indicates that the infection was biologically relevant. These findings agree with previous descriptions of megalocytivirus infection (Maganha et al., 2018; Silva, 2022) and reinforce the importance of an integrated assessment combining clinical signs, histopathology, and molecular methods.

The histopathological alterations observed in this study are compatible with infection by *Megalocytivirus*, as described by Zhu et al. (2021) and Martins et al. (2015, 2022). However, they differ from experimental models using *Danio rerio*, in which more extensive tissue necrosis has been described (Xu et al., 2008). This difference may reflect variations in susceptibility among species, viral strain virulence, or the experimental conditions employed. The detection of inclusion bodies in the analyzed tissues, especially more evident on day 14, suggests intensification of viral replication over time, corroborating the molecular findings. The semi-quantitative histopathological scoring confirmed that lesion severity increased from day 7 to day 14 ($p < 0.05$), with higher scores observed in the high-density stress group (T4) compared to the infected group without stress (T3).

Detection of viral DNA by PCR demonstrated high sensitivity, confirming its applicability as a diagnostic tool for ISKNV, as widely documented (Kurita & Nakajima, 2012; WOAH, 2025; Joahan et al., 2023; Saksmerprome et al., 2025). The use of conventional PCR with primers targeting the MCP gene proved effective for detecting the virus in both kidney and spleen tissues.

Limitations of the Study: The study did not perform quantitative PCR (qPCR), which would have allowed quantification of viral load and more precise assessment of viral replication dynamics. The observation period was limited to 14 days, which may have been insufficient to observe mortality. Stress indicators (e.g., cortisol, glucose) were not measured to confirm the physiological impact of high-density stress. The viral dose was not quantified (only tissue homogenate volume was specified). Future studies should address these limitations by incorporating qPCR, longer observation periods, stress biomarker measurements, and viral dose quantification.

Taken together, the results demonstrate that the integrated approach using PCR and histopathology was effective in detecting ISKNV infection. The study contributes to the understanding of ISKNV infection dynamics in tilapia and highlights the potential role of density stress in modulating viral dissemination.

V. CONCLUSION

The results of this study confirm that experimental infection with *Megalocytivirus pagrus* 1 (ISKNV) was successfully established in Nile tilapia, as detected by PCR and corroborated by characteristic histopathological alterations, especially in the kidney and spleen. The integrated diagnostic approach combining PCR and histopathology proved effective for detecting viral infection.

The absence of mortality, even in the groups subjected to high-density stress, indicates that, under the experimental conditions evaluated, the infection did not progress to lethal outcomes during the analyzed period, despite the presence of clinical signs and evident tissue lesions. This finding suggests that factors such as exposure time, viral load, and environmental conditions may influence the expression of ISKNV pathogenicity. A longer observation period or higher viral dose may be necessary to induce mortality.

A trend toward greater viral dissemination was observed in the groups subjected to density stress (T4: 61.1% positivity vs. T3: 21.4% on day 14; $p = 0.04$), indicating that intensive farming conditions may act as modulators of infection dynamics, favoring viral transmission even in the absence of increased mortality. High-density stress may impair the immune response and increase contact among individuals, facilitating viral spread.

Thus, the results obtained contribute to the understanding of ISKNV infection dynamics in tilapia and reinforce the importance of combined diagnostic strategies, especially in intensive production systems, where stress factors may influence the dissemination of pathogenic agents.

Limitations: The study did not perform qPCR to quantify viral load, did not measure stress biomarkers, and the observation period was limited to 14 days. Future research should address these limitations by incorporating qPCR, stress biomarker measurement, longer observation periods, and viral dose quantification.

Future Research Directions: Future studies should investigate the effects of different viral doses, longer observation periods, and other stress factors on ISKNV infection dynamics. The development of qPCR assays for viral load quantification and the measurement of stress biomarkers (cortisol, glucose) would provide more comprehensive data on the mechanisms underlying stress-induced susceptibility to ISKNV.

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DECLARATIONS

Conflict of Interest: The authors declare no conflicts of interest regarding the publication of this manuscript.

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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