Detection of Herpesvirus Infection (RaHV-1) in amphibians from commercial, experimental, and recreational breeding in São Paulo State, Brazil

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***Abstract*— *Frog farming represents an alternative animal production system with significant economic potential, especially in Brazil, recognized as one of the largest global producers of captive-bred frogs. However, inadequate management and environmental stressors may lead to outbreaks of infectious diseases, including those caused by herpesviruses (RaHV-1), posing severe health risks to animals and threatening production viability. This study aimed to detect the presence of herpesvirus in amphibians from commercial, experimental, and recreational facilities in São Paulo State, Brazil. Skin fragments and internal organs from 49 amphibians were analyzed using transmission electron microscopy and histopathological techniques. Findings revealed that 23 animals were positive for RaHV-1, showing pleomorphic viral particles, some enveloped, with diameters between 120 and 200 nm in both skin and organ fragments. Histopathological examinations also revealed intranuclear structures in various organs suggestive of RaHV-1. These findings indicate the circulation of RaHV-1 in Brazilian frog farms, highlighting the importance of further studies to better understand disease pathology and mitigate its impact on production.*

***Keywords*— Herpesvirus Infection (RaHV-1)., Amphibians, Transmission Electron Microscopy, Histology.**

I. INTRODUCTION

Frog farming has become a promising activity in Brazil, which ranks as one of the largest producers of captive-bred frogs. Although Brazil is among the world's leading producers of frog meat, regulation of frog farming remains highly deficient, and the industry faces cultural, economic, governmental, and health-related obstacles, hindering its expansion in the country. Additionally, this sector encounters challenges related to inadequate management, environmental stress, and outbreaks of infectious diseases. Among the diseases that can affect amphibians, herpesvirus infections, belonging to the Alloherpesviridae family, have drawn attention due to their ability to cause skin lesions, tumors, and adenocarcinomas, negatively impacting animal development and producer profitability.

Herpesviruses, in general, are known for establishing long-term latent infections in their hosts. These viruses have large genomes with double-stranded DNA and are classified into three main subfamilies based on target cell types and replication cycles:

1) *Alphaherpesvirinae*: typically cause acute infections and establish latency in neurons.
2) *Betaherpesvirinae*: characterized by longer lytic cycles and latency in immune system cells.
3) *Gammaherpesvirinae:* tend to associate with lymphatic system cells and may be linked to cancers in their hosts.

However, herpesviruses that infect amphibians, such as RaHV-1, belong to the *Alloherpesviridae* family, distinct from the three subfamilies of herpesviruses that affect mammals, birds, and other warm-blooded vertebrates.

Amphibians are ecologically and economically significant (VALENCIA-AGUIAR et al., 2013). Due to their thin skin, which facilitates gas exchange between their bodies and the environment, these animals are highly susceptible to ecosystem changes (JORGENSEN, 1994). Most species undergo a life cycle with two phases, a predominantly aquatic larval phase and a terrestrial adult phase. This characteristic makes amphibians excellent bioindicators of environmental quality (SEWELL and GRIFFITHS, 2009). However, this sensitivity to environmental changes, such as deforestation, pollution, and global warming, has led to significant population declines (GRANT et al., 2020; DIETRICH et al., 2020).

This class of vertebrates is divided into three main orders: Anura (including frogs, toads, and tree frogs), Caudata (salamanders and newts), and Gymnophiona (caecilians or blind snakes) (FROST et al., 2021). Some species of the Anura order are traded globally for human consumption. Among them, the North American bullfrog (*Aquarana catesbeiana*) stands out due to its easy adaptation to various management systems worldwide (OLIVEIRA, 2015). Frog farming represents a significant economic potential for producing countries since, in addition to meat, various by-products such as flour, pâté, hamburgers, and preserves can be marketed (OLIVEIRA, 2015). However, international trade in frogs has contributed to the spread of emerging pathogens, as the species appears resistant to high infection levels (JENKINSON et al., 2016; SCHLOEGEL et al., 2009; BRUNNER et al., 2019).

Genetic studies attribute the co-evolution of this viral group with their hosts, where latent infections occur without causing severe diseases with high mortality, thereby favoring the spread of herpesviruses (FRANCO & ROECHE, 2007; CATROXO et al., 2003). Latency can be defined as viral persistence in which the virus remains within cells in a non-pathogenic form, with intermittent periods of reactivation and shedding (ADAMEK et al., 1996). Any latent carrier animal is a potential source of infection, as the virus can resume multiplication and be shed through secretions under low resistance conditions, promoting disease spread. During latency, the viral episomal genome becomes circular, and gene expression is limited. Reactivation is associated with various stressors, such as transport, adverse weather conditions, overcrowding, and concurrent infections (QUINN et al., 2005).

Herpesviruses spread primarily through three routes: cell-to-cell, bloodstream, and nervous system. Cell-to-cell transmission, common in these viruses, occurs through intercellular bridges that prevent antibody action. The virus can also reach the nervous system, being transported to specific ganglia neurons, such as the trigeminal or sacral, depending on the infection site. Blood circulation (in lymphocytes) also facilitates its dissemination, allowing the virus to reach reproductive organs, though viremia is brief. In animals with low immunity, infection can range from asymptomatic to mild but may worsen with high viral doses. As a result, some animals may be asymptomatic carriers of viruses and, when exposed to a drop in immunity, may trigger a viral process, even if the viral presence cannot be linked to the animal's cause of death, and it is unknown whether the viral particle quantity found contributes to worsening the condition (MEYER, 2001; LOPEZ & SILVA, 2019).

Studies on the presence of herpesvirus in bullfrogs (*Lithobates catesbeianus*) are limited in Brazil, motivating this research to detect the occurrence of this pathogen in commercial and experimental facilities in São Paulo State, Brazil.

II. OBJECTIVES

The primary objective of this study was to detect herpesvirus infection (Ra HV-1) in bullfrogs from commercial, experimental, and recreational fa

Specific objectives included:

1) Analyzing skin and internal organ samples using transmission electron microscopy (TEM) and light microscopy.
2) Identifying the presence of herpesvirus particles with characteristic morphology in the analyzed samples.
3) Assessing the infection frequency relative to the total number of animals and samples collected.

III. MATERIALS AND METHODS

3.1 Sample Collection:

Skin fragments and internal organs, such as liver, kidney, spleen, intestine, heart, lung, and brain lesions, were collected from 49 amphibians: 42 captive bullfrogs (*Lithobates catesbeianus*) raised at different locations in São Paulo State, as well as one horned frog (*Proceratophrys boiei*), two river frogs (*Hylodes asper*), two small frogs (*Leptodactylus podicipinus*), and two dart frogs (*Adelphobates galactonotus*) from the wild in various locations within São Paulo State. In total, 211 samples from the 49 individuals were processed and sent to the Biological Institute for microorganism analysis in organ and skin fragments.

3.2 Sample Processing:

Samples were processed at the Aquaculture Health Laboratory using routine H&E histological techniques and at the Electron Microscopy Laboratory with negative staining for rapid preparation.

3.3 Light Microscopy Analysis or Photomete:

H&E staining: Tissues from 49 amphibians were collected and fixed in 10% formalin for 36–48 hours, dehydrated in a graded series of alcohols (70°, 80°, 95°, and absolute), cleared in xylene, and embedded in paraffin at 58°C overnight. Sections 4 µm thick were placed on slides and stored at room temperature. For analysis, slides were deparaffinized with xylene, rehydrated in a descending alcohol series (absolute, 95°, 80°, 70°), and water, and stained with hematoxylin and eosin.

3.4 Transmission Electron Microscopy (TEM):

The fragments were suspended in 0.1M phosphate buffer at pH 7.0. Drops of this suspension were applied to metal grids covered with collodion and carbon, drained with filter paper, and contrasted with 2% ammonium molybdate at pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997). The samples from 49 amphibians were examined using a Philips EM 208 transmission electron microscope.

IV. RESULTS

At necropsy, hemorrhagic and friable internal organs (Figure 1) were observed in a few amphibians, and some bullfrogs also exhibited skin lesions on the body and digits (Figure 2). Electron microscopy results revealed that out of the 49 amphibians, 23 (46.9%) tested positive for herpesvirus (Figures 12 and 13), including 16 captive bullfrogs (*Lithobates catesbeianus*), one horned frog (*Proceratophrys boiei*), two river frogs (*Hylodes asper*), two small frogs (*Leptodactylus podicipinus*), and two dart frogs (*Adelphobates galactonotus*) from the wild. Herpesvirus particles were pleomorphic, icosahedral, some of which were enveloped and measured between 120 and 200 nm in diameter. A total of 26 (53.1%) animals tested negative for herpesvirus. Histological examination showed various histopathological changes, such as hemorrhage, mononuclear and polymorphonuclear inflammatory reactions, edema, several melanomacrophage centers, necrosis, and the presence of inclusion bodies, primarily intranuclear, in various organs, including the skin, liver, kidney, and spleen, in 14 bullfrogs (Figures 3, 4, 5, and 6; Graphs 1, 2, 3, and 4).

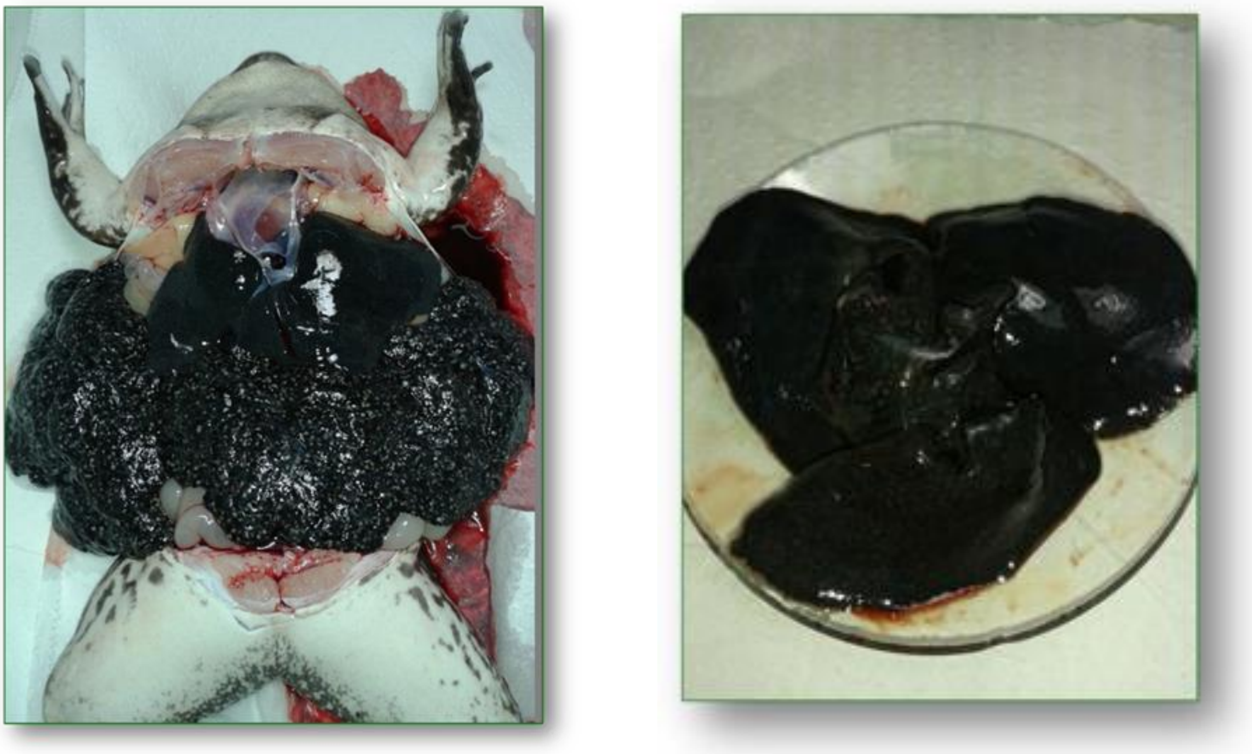
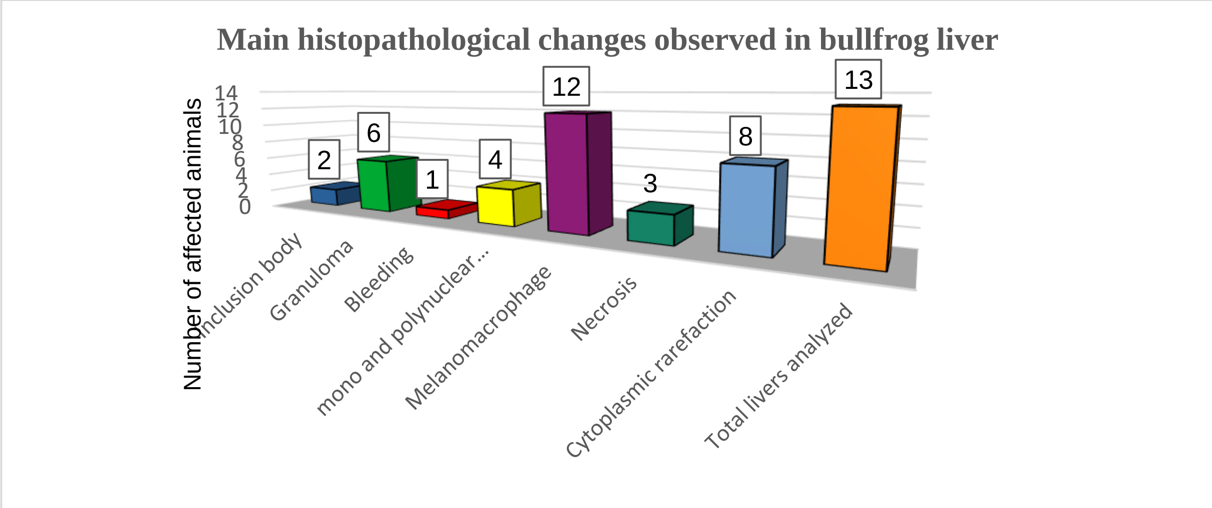
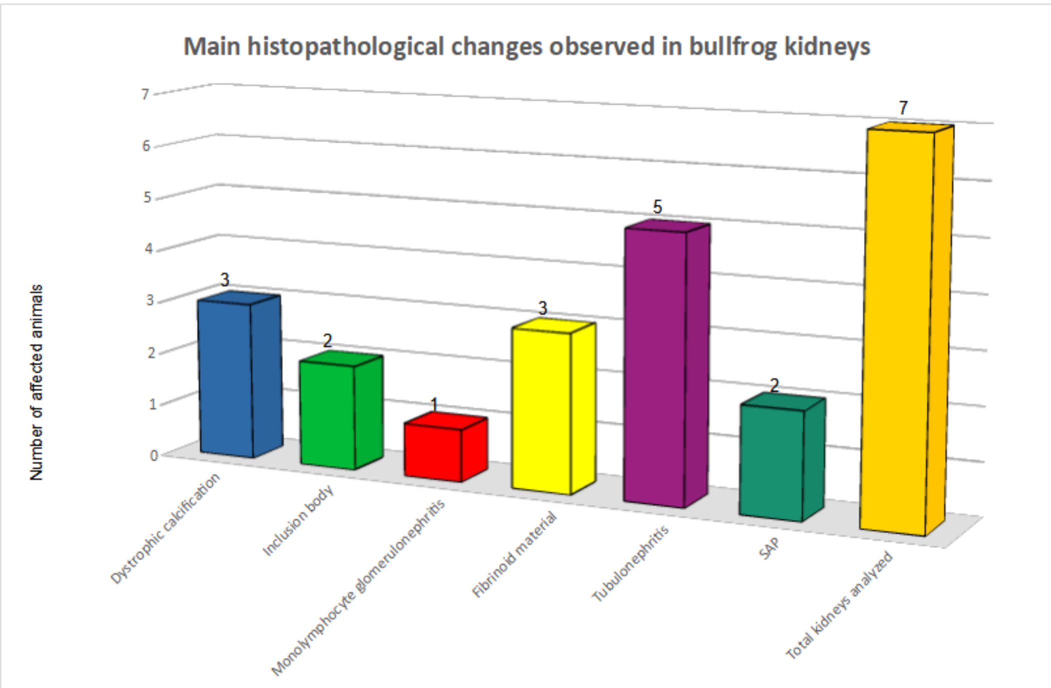


FIGURE 1: Bullfrog positive for herpesvirus showing altered organs (left). Macroscopic aspects of organs at necropsy (right)



GRAPH 1: Main histopathological changes observed in bullfrog liver and number of affected animals

FIGURE 2: External macroscopic lesions observed in bullfrogs. Body skin lesions (A) and digit lesions (B) in bullfrogs



GRAPH 2: Main histopathological changes observed in bullfrog kidneys and number of affected animals

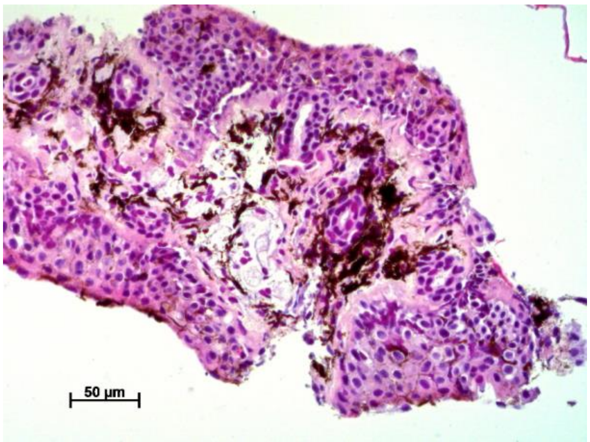


FIGURE 3: Panoramic photomicrograph of skin lesions in bullfrogs. H&E staining, 200x magnification

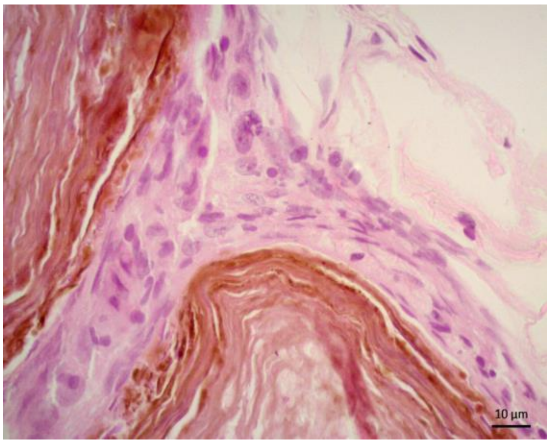


FIGURE 4: Photomicrograph of hyperplastic epidermal lesion and keratosis in bullfrog, showing neoplastic cells and inclusion bodies. H&E staining, 630x magnification

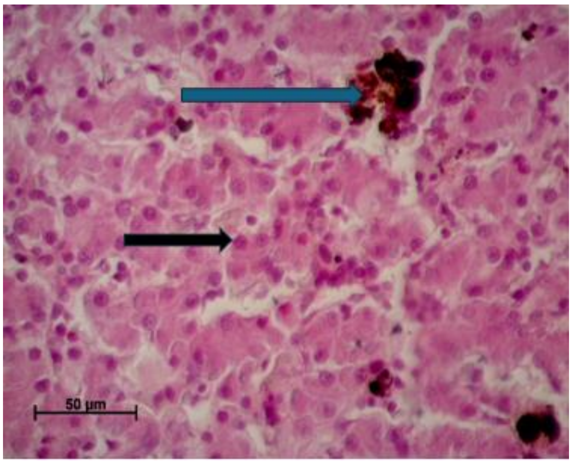


FIGURE 5: Photomicrograph of bullfrog liver histological section indicating basophilic nuclear inclusion bodies suggestive of herpesvirus (black arrow) and multiple melanomacrophage centers (blue arrow). H&E staining, 400x magnification

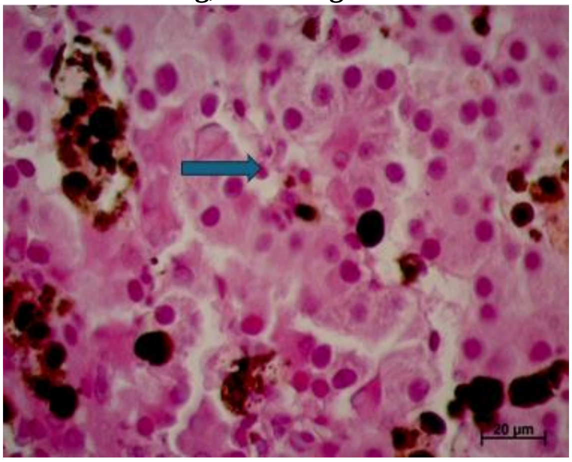
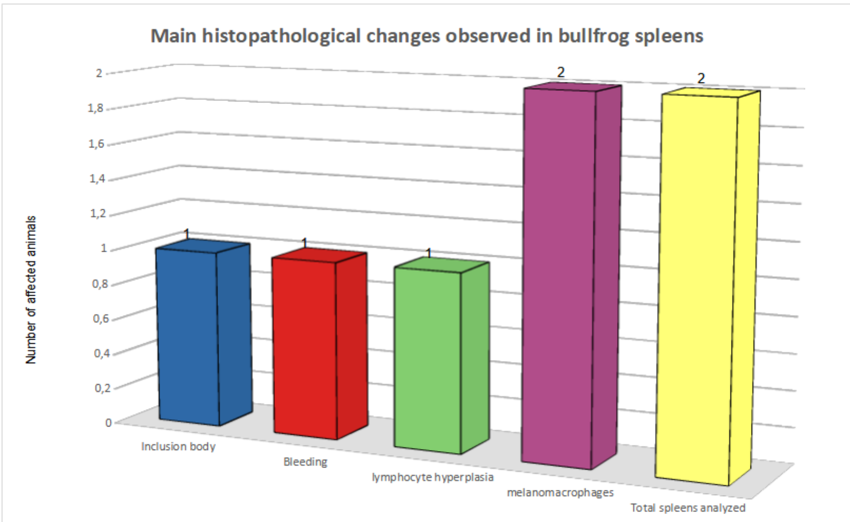
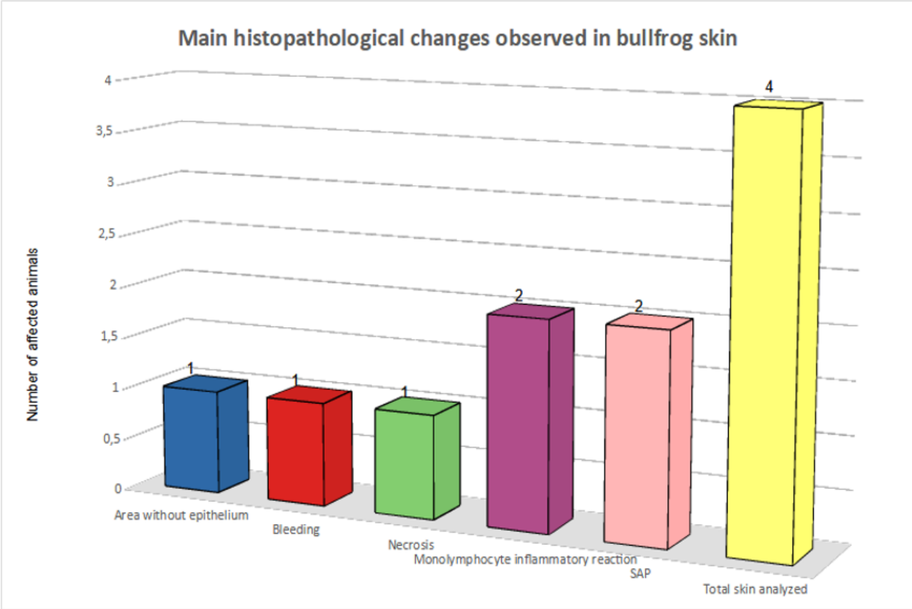


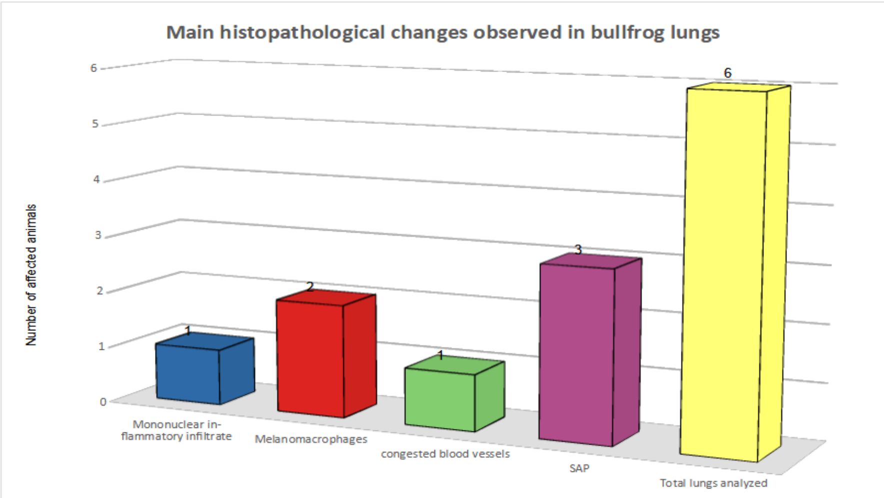
FIGURE 6: Photomicrograph of bullfrog liver histological section, arrow indicating basophilic nuclear inclusion body. H&E staining, 630x magnification



GRAPH 3: Main histopathological changes observed in bullfrog spleens and number of affected animals



GRAPH 4: Main histopathological changes observed in bullfrog skin and number of affected animals



GRAPH 5: Main histopathological changes observed in bullfrog lungs and number of affected animals

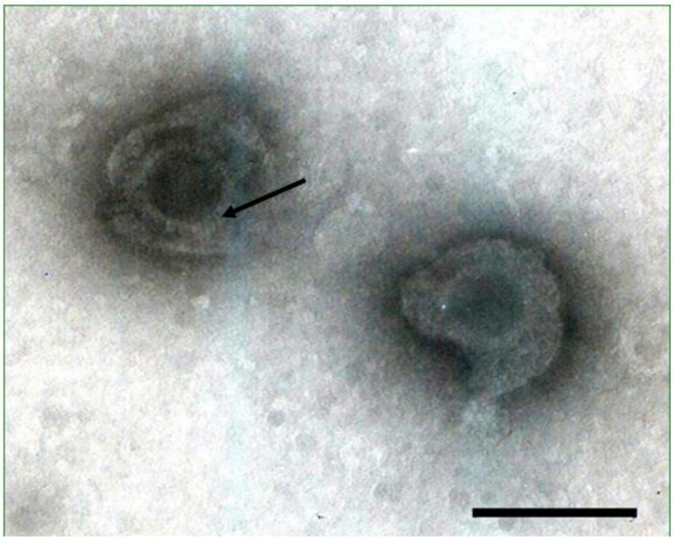


FIGURE 7: Enveloped herpesvirus particles in a bullfrog liver suspension, showing individual capsomeres (arrow). Bar: 140 nm

FIGURE 8: Herpesvirus particles, both enveloped (larger arrow) and non-enveloped (smaller arrow), in a bullfrog liver suspension. Bar: 150 nm

These transmission electron microscopy images provide detailed views of the herpesvirus particles found in infected bullfrog tissues, highlighting their pleomorphic nature and structural characteristics, such as the presence or absence of an envelope.

V. DISCUSSION

Frog farming holds significant potential for expansion within Brazilian agribusiness. However, several obstacles have hindered its progress nationally. In addition to previously mentioned economic challenges, the lack of governmental and private incentives, absence of specific regulations, and insufficient communication between research institutions and producers represent substantial barriers to advancing this activity within aquaculture. Improving access to knowledge for producers, especially regarding research on diseases affecting amphibians and appropriate prevention and treatment methods, would also be necessary.

Transmission electron microscopy revealed the presence of pleomorphic, icosahedral particles with herpesvirus-like morphology in the skin and organ fragments of 23 amphibians, some of which were enveloped and measured between 120 and 200 nm in diameter. Morphologically identical particles were visualized in other studies with bullfrogs from farms in São Paulo State, Brazil (HIPÓLITO et al., 2003; ANTONUCCI et al., 2014). The detection of the virus in multiple organs, including skin and liver, supports the hypothesis that this pathogen may affect multiple systems within the host. In total, 211 samples from 49 animals were processed using the negative staining technique (rapid preparation), with 23 (48.9%) animals testing positive for herpesvirus. A similar study by Antonucci et al. (2014) found herpesvirus in the small intestine of 7 (15.5%) out of 45 bullfrogs.

The most common clinical symptoms and signs observed in the amphibians in this study were sudden death and the presence of skin and digit lesions. HIPÓLITO et al. (2003) used the negative staining technique to detect herpesvirus particles in three animals with liver lesions, five animals with nodules in the leg muscles and back, and the liver and ascitic fluid of six animals. The occurrence of Lucké tumor, characterized by renal adenocarcinoma caused by herpesvirus, has been described in leopard frogs (*Rana pipiens*) (McKINNELL & CUNNINGHAM, 1982; CARLSON et al., 1994), with metastasis potentially mediated by low temperatures (TWEEDELL, 1989).

Regarding bullfrog histopathology, the most significant alteration observed in all bullfrogs (*L. castebeianus*) was the presence of inclusion bodies in the liver, kidney, spleen, and ovary. These findings align with those of other authors studying the presence of Lucké tumor in leopard frogs (*Rana pipiens*) (GRANOFF, 1983; CARLSON et al., 1994).

We also observed hemorrhage in the liver, spleen, and skin, as well as lymphocytic inflammation in the kidney, spleen, and hepatic necrosis in some muscle areas and skin lesions. These findings were also reported by CARLSON et al. (1994) in the kidney of leopard frogs (*Rana pipiens*). However, other alterations we found, such as the presence of melanomacrophages in the liver, spleen, and lung, tubulonephritis in the kidneys, and monolymphocytic inflammatory reaction in the skin, were not reported in the literature. The variety of positive species affected suggests that multiple herpesvirus types may occur in Brazil.

The study by ORIGGI et al. (2021) investigated the pathophysiology and molecular mechanisms of herpesvirus in frogs and toads. Common and agile frogs (*Rana temporaria and R. dalmatina) infected with RaHV3 and common toads (Bufo bufo)* infected with BfHV1 were analyzed. At different developmental stages, six frogs and 14 toads exhibited transient skin lesions associated with and necessary for the viral replication cycle. Transcriptomic analysis in infected and non-infected animals (two frogs and two toads) revealed altered expression of signaling and cellular remodeling genes in infected individuals. Finally, viral transcriptomics indicated high expression of a viral immunomodulatory gene encoding a receptor for the tumor necrosis factor in the skin of infected hosts, suggesting a coordinated cycle between epidermis and virus involving host signaling, remodeling genes, and viral immunomodulatory genes.

Experimental transmission studies on ranid herpesvirus 1 (RaHV1, *Batravirus ranidallo*), the first characterized amphibian herpesvirus, indicate that viral-associated renal adenocarcinoma likely occurs when amphibians are infected during the early embryonic stage (pre-metamorphosis) but not in adult or juvenile stages (post-metamorphosis). We investigated the potential occurrence of RaHV3 infection in free-living, pre-metamorphic common frogs (ORIGGI et al., 2023).

According to TWEEDELL (1989), electron microscopy and histopathology are the primary initial tools for diagnosing Lucké tumor. When histological analysis suggests a viral disease, transmission electron microscopy is a highly useful method for identifying the etiological agent. Numerous herpesviruses causing diseases in fish and frogs that could not be successfully isolated in cell cultures were detected via electron microscopy (HANSON et al., 2011).

In the animals testing positive via the negative staining technique, no organ preference was observed, as viral particles were present in all examined organs. In routine hematoxylin-eosin histology, the liver was the most affected organ. The liver can be chosen due to its easy access and relatively large size compared to the animal.

Although 26 animals tested negative for herpesvirus, the detected infection rate raises concerns about the potential impact of this infection on Brazilian frog farms, particularly in terms of economic losses. The correlation between inadequate management, environmental stress, and herpesvirus outbreaks in other animal species suggests that prophylactic measures, such as improved management practices and sanitary control, are essential for mitigating outbreak risks.

VI. CONCLUSION

The presence of pleomorphic particles, some enveloped, measuring between 120 and 200 nm, confirms herpesvirus-1 infection, consistent with previous studies in other amphibians. The detection of the virus in multiple organs, including the skin and liver, supports the hypothesis that this pathogen may affect multiple systems within the host. This study identified the presence of herpesvirus in captive bullfrogs in São Paulo State, Brazil, with a significant infection rate. These findings suggest that herpesvirus (RaHV-1) is circulating in Brazilian frog farms, potentially harming animal health and the economic viability of frog farming. Therefore, it is crucial to continue studies in this area to gain a better understanding of viral pathology and develop management and prevention practices that reduce losses caused by these infections.

In this regard, we emphasize the importance of a coordinated effort among various sectors within the country to ensure better prospects for the development of frog farming in Brazil.

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