USE OF POLYCLONAL ANTIBODIES, ELECTRON MICROSCOPY AND HISTOPATHOLOGY TO DETECT IRIDOVIRUS-LIKE PARTICLES IN BULLFROGS

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ABSTRACT

Iridoviruses of the *Ranavirus* genus have been implicated in the decline in amphibians worldwide, capable of affecting animals both in the wild and in captivity. This study aimed to detect iridovirus-like particles from three frog farms in southeastern Brazil using primary polyclonal antibodies, transmission electron microscopy (TEM) and histologic findings. The target organs were liver and kidneys. Sixty adults and sixty tadpoles of bullfrogs (*Lithobates catesbeianus*) were used in the study. TEM revealed the presence of iridovirus-like particles in hepatic tissue using the negative staining technique. Positive results were also observed by immunoelectron microscopy and immunocytochemistry (ICC). The histological analysis of the samples showed liver hemorrhage and corpuscles inclusion in hepatocytes as well as glomerulotubular degeneration and necrosis in the kidneys. The methods used in this study were highly efficient to detect the presence of iridovirus-like particles and possible infection of ranavirus.

Keywords: *Rana catesbeiana;* FV3; immunoelectron microscopy; ranavirus.

USO DE ANTICORPOS POLICLONAIS, MICROSCOPIA ELETRÔNICA E HISTOLOGIA PARA DETECÇÃO DE PARTÍCULAS SEMELHANTES AO IRIDOVÍRUS EM RÃS-TOURO

RESUMO

Os iridovirus do gênero *Ranavirus* têm sido implicados no declínio dos anfíbios em todo o mundo afetando animais de vida livre e aqueles em cativeiro. O objetivo deste estudo foi detectar a presença de particulas semelhantes ao iridovírus em três ranários na região sudeste do Brasil, utilizando anticorpos policlonais primários, microscopia eletrônica de transmissão (MET) e achados histológicos. Os órgãos alvo foram o figado e os rins. Sessenta rãs-touro adultas (*Lithobates catesbeianus*) e sessenta girinos da mesma espécie foram usados para o estudo. A MET revelou a presença de partículas semelhantes ao iridovírus em tecido hepático utilizando a técnica de contrastação negativa. Os resultados positivos foram também observados por imunomicroscopia eletrônica e imunocitoquímica. As análises histológicas nas mesmas amostras evidenciaram hemorragia no fígado e corpúsculos de inclusão em hepatócitos, e degeneração glomerulotubular e necrose nos rins. Os métodos usados neste estudo foram altamente eficientes na detecção de partículas semelhantes ao iridovírus e possivel infecção por ranavirus.

Palavras chave: *Rana catesbeiana*; FV3; imunoeletro microscopia; ranavírus

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INTRODUCTION

Frog breeding began in Brazil in the 1930s after bullfrog (*Lithobates catesbeianus*) breeding couples, native to North America, were introduced to the country. However, it was only during the 1970s when frog breeding became noticeably more common. With the decrease of empiricism regarding handling, new and more efficient fattening systems were developed to further intensify frog culture (FERREIRA *et al.*, 2002).

In southeastern Brazil, frog breeding is generally performed using two systems: a dry and a wet system. Both systems undergo weatherization using agricultural greenhouses, further improving the animals' thermal comfort. In 2006, Brazil produced 639 tons of frog meat, showing the importance of frog culture to the country (DIAS *et al*., 2010, FAO 2014).

High mortality and morbidity rates of amphibians have been reported in North and Latin America, Europe, Asia, and Australia, both in the wild and in breeding farms. The causes of these high rates were attributed mainly to a fungus known as *Batrachochytrium dendrobatidis* and a virus from the *Iridoviridae* family, considered a growing threat to the global biodiversity of these amphibians (MAZZONI *et al.,* 2009).

According to the OIE (2011), these pathogens cause important emerging diseases that require mandatory notification. The *Iridoviridae* family currently encompasses five genera: *Iridovirus, Chloriridovirus, Lymphocystivirus, Megalocytivirus* and *Ranavirus*. Of which, only the *Ranavirus* genus is capable of infecting ectothermic vertebrates such as amphibians, reptiles and fish, however, the *Megalocytivirus* genus also affects fish. Morphologically, iridoviruses are large icosahedral viruses (120-300 nm in diameter) with an internal lipid membrane located between the virus core and the outer capsid. The Major Capsid Protein (MCP) can be found within the capsid. The MCP is a common structural protein that is highly conserved in all iridoviruses. Members of this family have linear genomes with double-stranded DNA (CHINCHAR *et al.,* 2009; ICTV, 2011).

Ranaviruses are highly virulent, with a high death incidence in most cases. Amphibian larvae are the most vulnerable to ranaviruses, however, adults of some species are also susceptible to infections (GALLI *et al.,* 2006). According to ROBERT *et al.* (2011), the use of *Xenopus laevis* in laboratory trials showed that the infection might occur through water, physical contact, biting, predation and cannibalism, which is the most common form of spreading ranaviruses to other individuals. The clinical signs of ranavirus infections are not always apparent. Immunocompromised animals develop systemic infections with ulcers in the distal parts of their members as well as an increase in ventral volume (*e.g*. fluid) or loss of body weight, hemorrhaging, lethargy and death. There can also occur severe internal lesions in the kidney, liver, spleen and in the gastrointestinal tract (HOVERMAN; GRAY and MILLER, 2010).

In Brazil, MAZZONI *et al.* (2009) detected ranavirus in sick tadpoles and bullfrogs using the PCR technique and transmission electron microscopy (TEM), associating ranavirus with the high number of casualties in three frog farms in midwestern Brazil. In southeastern Brazil, the presence of viral particles similar to ranavirus has already been registered through TEM using negative staining technique (HIPOLITO *et al.,* 2002). Apparently, this emerging disease is established in Brazil resulting in occasional fatalities, but its etiology lacks further explanations. We need to study the presence and the development of ranavirus concomitantly with other infectious agents in the host because the presence of this disease could facilitate infection of a second pathogen (CONVERSE and GREEN 2005; ROMANSIC et al.; 2007).

This study aimed to detect irodovirus-like particles in bullfrogs from three frog farms in southeastern Brazil using primary polyclonal antibodies, TEM and histologic findings in liver and kidney samples of bullfrogs.

MATERIALS AND METHODS

Sample collection

Animal populations from three different frog farms were used as samples. These farms use two

different breeding systems: the flooding system (farm A) and the semi-dry system (farms B and C). The flooding system is characterized by completely filling the frog farm with water about 5 cm deep, thus eliminating any shelters and troughs, which are used in other breeding systems to minimize stress in animals, and to facilitate for the frogs to reach the food (FAO, 2014). The feed was given by hand casting and it remained floating in tank water due to extrusion. The density used by frog farmers in Brazil in the flooding system is 100 bullfrogs per square meter (FERREIRA *et al.*, 2002). The semi-dry system consisted of an area of variable dimensions (from 10 to 1600 m^2), made of cinder block or agricultural shade cloth. Some pens also had a top mesh to prevent predation from birds. Between 75 and 90% of the total floor surface area of the pen was covered by grass, while the rest had shallow (<10 cm) concrete-lined puddles (FAO, 2014). The density used by frog farmers in Brazil in the semi-dry system is 50 bullfrogs per square meter (FERREIRA *et al.*, 2002).

The animals were chosen randomly. Twenty adult animals were collected from each farm with an average weight of 150 grams. We also collected twenty tadpoles with an average weight of 7 g, totaling 120 animals in the study sample.

The first collection began in September 2011 on farm A (23º31'S and 47º08'W), the second in February 2012 on farm B (21º15'S and 48º19'W) and the third in March 2012 on farm C (22º45'S and 47º24'W), during the spring/summer seasons in the southern hemisphere.

The animals were transported alive in thermal boxes. The adult specimens were kept in expanded polystyrene boxes, with a layer of wet foam at the bottom. The tadpoles were kept alive with portable aeration systems connected to a 16L box.

Upon receipt by São Paulo Fisheries and Biology Institutes (*Instituto de Pesca e Instituto Biológico/SP/Brazil*) Interinstitutional Aquaculture Health Lab (*Laboratório Interinstitucional de Sanidade em Aquicultura*, LISA), the adult animals were stowed away for 24 h in a 100 L (26 gallons) polyethylene water box, with approximately 5 cm column of dechlorinated water. The animals were anesthetized in the following order (ice and benzocaine hydrochloride 4:1) and then euthanized (tadpoles and adults) by performing an incision on the cervical vertebrae, located under the head. Then, the animals were weighed.

The middle liver lobe and one of the kidneys were collected. The material extracted from each animal was fragmented into 0.1 g portions, and placed in 1.5 mL microtubes, which were kept frozen until samples for TEM started to be prepared. The rest of the collected material was preserved in 80 mL universal containers partially filled with 10% buffered formalin at pH 7.4. The histological cuts were made from the fixed tissue.

Sample processing for Transmission Electron Microscopy (TEM)

Three TEM techniques were performed: 1) negative staining (rapid preparation) to identify viral particles; 2) immunoelectron microscopy; and 3) immunocytochemistry (immunolabelling with colloidal gold particles). These last two techniques were used to confirm the presence of the iridovirus-like particles.

For negative staining, after centrifugation (GOLDSMITH and MILLER, 2009) the material was suspended in PBS 0.1M pH 7.0 and an automated pipetting system was used to collect a 40 µL drop from the suspended material. The drop was placed on a piece of parafilm glued to a glass slide. A copper grid covered with colloidal film and stabilized with carbon was placed under each drop and incubated for 10 min. The screens were drained using filter paper and underwent negative staining with ammonium molybdate at 2% pH 5.0 (BRENNER and HORNE, 1959).

For the immunoelectron microscopy during TEM, the copper screens were sensitized with a primary polyclonal antibody (Abcam™) obtained from the iridovirus Major Capsid Protein (MCP), diluted in 1:200, washed with 40 PBS buffer drops, incubated for 10 min with sample suspension, washed with distilled water and subjected to negative staining with ammonium molybdate on 2% pH 5.0. In order to control the technique, for every 10 incubated samples, one was treated as

described previously, replacing the antibody with distilled water (HAYAT and MILLER, 1990).

In preliminary positive samples made with immunoelectron microscopy, the immunolabelling with colloidal gold technique (immunocytochemistry) was applied. The copper screens, also prepared beforehand, were incubated for 15 min with 40 µL viral suspension drops, which were sensitized with the same antibody diluted at 1:80 during 30 min. Afterwards, the screens were washed with PBS. Afterward, the screens were incubated again for 30 min with a second antibody (protein A conjugated with colloidal gold particles of 10nm in diameter – Electron Microscopy Sciences) diluted in 1:20 in PBS at 0.5%. The screens also underwent negative staining with the use of ammonium molybdate (KNUTTON, 1995). The samples were examined using a Philips EM 208 electron microscope from the Electron Microscopy Laboratory of Instituto Biológico, São Paulo, Brazil.

Preparing histological samples

The samples were dehydrated in 70^{o}, 80^{o}, 95^{o} alcohol as well as in absolute alcohol. They were diaphanized in xylene and were included in paraffin blocks for microtome cuts. The cuts were 5 µm thick and the samples underwent H&E staining. The samples were observed with light transmitted optical microscope and the results were quantified through occurrence percentages of each different type of lesions observed.

RESULTS

TEM allowed visualizing iridovirus-like particles, enveloped icosahedral particles with 120 to 300nm in diameter, which underwent negative staining with ammonium molybdate (Figure 1). The morphological characteristics described in this study were similar to those described by other researchers regarding bullfrogs and other amphibians (MAZZONI *et al*., 2009). The electron microscopy confirmed the presence of agglutination further stimulated by antigen-antibody reactions (Figure 2) (Table 1). In the ICC technique (immunolabelling with colloidal gold), the antigen-antibody reaction was greatly highlighted by the colloidal gold particles (Figure 3).

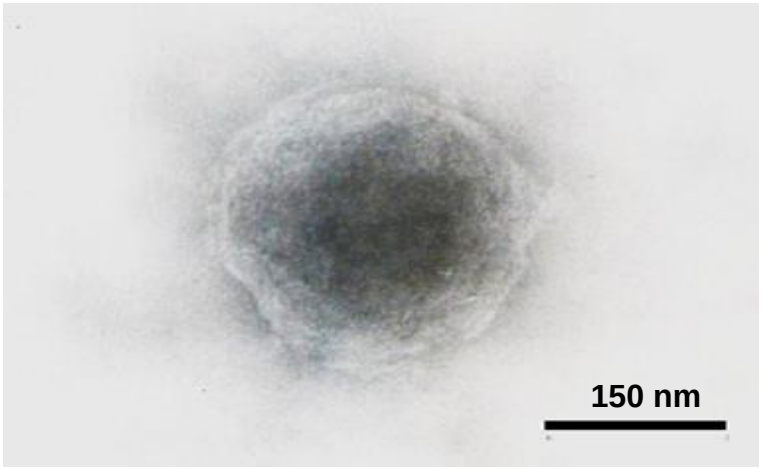

Figure 1. Photoelectromicrograph of viral suspension obtained from *Lithobates catesbeianus* liver, showing an icosahedral viral particle after negative staining. Zoom: 120.000x

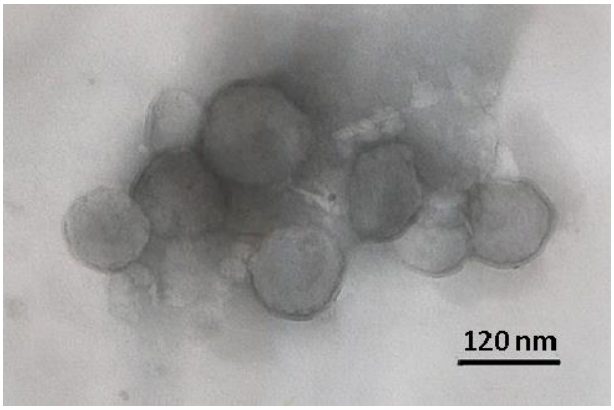

Figure 2. Photoelectronmicrograph of viral suspension obtained from *Lithobates catesbeianus* liver, showing agglutinated iridovirus particles due to the antigen-antibody interaction with the immunoelectron microscopy technique. Zoom: 100.000x

Table 1. Immunoelectron microscopy positive percentage (%) in liver samples detected in bullfrogs from southeastern Brazil, frog farms A, B, and C (n=120)

Samples	A	B	C
Adult bullfrogs	90%	65%	15%
Tadpoles	90%	0%	0%

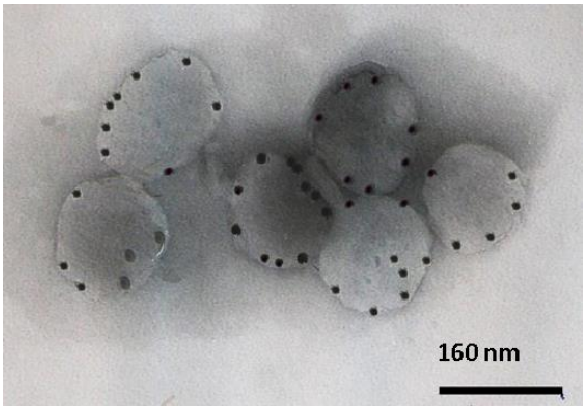

Figure 3. Photoelectronmicrograph of viral suspension obtained from *Lithobates catesbeianus* liver, showing agglutinated iridovirus-like particles due to the antigen-antibody interaction in the immunoelectron microscopy technique. The particles were highlighted by colloidal gold particles. Zoom: 100.000x

In this study, 43.33% of the sampled animals had iridovirus-like particles associated to signs of ranavirus infection (*i.e.* ulcers in distal parts of members, increase in abdominal volume, body weight loss, hemorrhage and lethargy) (MAZZONI *et al.* 2009; HOVERMAN; GRAY and MILLER, 2010). In adult bullfrogs of farms A, B, and C, the iridovirus-like particles were present in 90%, 65% and 15% of the animals, respectively. The iridovirus-like particles were present in 90%

of the sampled tadpoles on farm A; however, tadpoles on the other two farms did not show signs of ranavirus infection. According to field observations, infected animals showed similar behavior to non-infected animals and production of these animals is currently not inflicting any losses to farmers.

Necropsy findings revealed that frogs from farm A had a higher number (40%) of external clinical signs *(i.e.* epidermal lesions). These lesions were characterized as deep skin ulcers on the head and distal limbs. The internal lesions most frequently observed were friable organs, brown to dark yellow livers, swollen spleens and pigmented areas in the external regions of the kidneys.

The light microscopic analysis revealed lesions with varied degrees of severity in the kidneys and liver. In general, liver tissue with cytoplasmic rarefaction (mineral protein disorder) showed an increased number of melanomacrophage centers, which are monolymphocytary hepatic clusters. Tubular nephrosis and interstitial hemorrhage were also observed. Inclusion bodies were observed in areas of necrosis and hemorrhage. Melano-macrophage centers were increased in the kidney and interstitial hemorrhage and tubular nephrosis were also observed.

DISCUSSION

The results show that the highest incidence of iridovirus-like particles were found on farm A. Structurally, farm A uses a flooding system to breed and fatten the animals. This system is widely used in southeastern Asia and adopted by a number of frog breeders in Latin America. This system ensures that all individuals are constantly in direct contact with water from metamorphosis until harvest. This system, combined with the high density of individuals (resulting in excessive contact between animals) and the natural occurrence of cannibalism among members of the species, provides a supportive environment for ranavirus propagation, as reported by ROBERT *et al.* (2011). The other animals sampled from farms B and C did not show signs of lesions or other external clinical signs. These farms use a semi-dry breeding system (FAO, 2014). We assume that conditions at farms that use the flooding system may help spreading the pathogen and increase iridovirus-like particles.

The results of negative staining through TEM indicated the presence of the iridovirus-like particles, confirmed by the immunoelectron microscopy and ICC techniques. Negative staining is important when an emerging viral infection is suspected, when it is necessary to perform a quick diagnosis or when the alternative standard methods do not produce satisfactory results. The same technique was used by HIPOLITO *et al*. (2012) on *Litopenaeus vannamei* suspected of contamination with viral particles of the *Whispovirus* genus (*Nimaviridae* family), responsible for the white spot syndrome (WSS) in shrimp.

The situation of the sampled individuals in this study suggests that, aside from the viral agent, their bodies host various pathologies. Although there were no external clinical signs in the tadpoles and adult frogs from farms B and C, many individuals showed problems with hepatic and renal functions due to a high degree of tissue degeneration. The situation of the sampled individuals in this study suggests that, aside from the viral agent, their bodies host various pathologies, with many individuals showing problems with hepatic and renal functions due to a high degree of tissue degeneration which could be related to nutritional disorders, water quality and secondary pathogenic agents. Although ranavirus infections are common in formation of inclusion bodies in leukocytes and erythrocytes (CONVERSE and GREEN 2005; MILLER *et al.*, 2007), we cannot guarantee that the inclusion bodies observed in the sampled tissue was a specific clinical sign of ranaviruses, requiring more histological studies.

Some studies have reported higher mortality and morbidity rates in animals cauased by ranavirus. In Canada, the works of GREER; BERRIL and WILSON (2005) described events of mortality in free animals infected with the ranavirus in central southern Ontario. DUFFUS *et al.* (2008) collected anurans and salamanders from natural populations in small lakes in the southeastern Ontario that had been infected with

Frog Virus-3 (FV3), species of the *Ranavirus* genus. During experiments in South America, ZUPANOVIC *et al.* (1998), using electron microscopy and restriction enzymes, detected ranavirus for the first time in healthy *Rhinella marina* living in the wild, collected from various locations in Venezuela in the early 1990s. GALLI *et al.* (2006) also detected ranavirus by using molecular techniques in tadpoles originated from frog farms in midwestern Brazil and Uruguay. The decline in the population of the *Atelognathus patagonicus* species of anuran was related to ranavirus associated with ecological changes in its habitat. FOX et al. (2006) reported this fact in a study on population fluctuations in lakes around Laguna Blanca National Park in the Neuquén province in the Patagonia region of Argentina. These reports prove that ranavirus occurs in specific locations in the Americas; however, there is still no information regarding surges in natural populations in Brazil and other nearby regions.

MAJJI *et al.* (2006) identified various degrees of pathogenicity in two different *Ranavirus* species: FV3 and RCV-Z. The bullfrog registered the strongest resistance to the FV3, because it is capable of hosting the virus without manifesting any clinical signs. For ROBERT *et al.* (2011), pathogenicity of ranavirus in *Xenopus laevis* is related to the compromised immune system. In this study, it was not possible to determine the degree of ranavirus pathogenicity. Studies on this issue in commercial frog farms are required.

Bullfrog bred in captivity in Brazil has known so far a small number of *L. catesbeianus* individuals that may escape from captivity, invading natural environments close to rural properties. On the other hand, wild anurans are reported to have accidentally penetrated frog farms. In this bidirectional flow, where individuals enter and leave frog farms, an exchange of pathogens between farmed bullfrog populations and other wild amphibian populations may occur. Albeit exploratory, investigating the presence of viral particles that identify ranavirus for the tracking process and eradication of this pathology, lethal to amphibians and amphibians bred for commercial purposes, is essential. In this context, the TEM techniques used in this study were efficient to detect viral particles of the *Iridoviridae* family and diagnose diseases caused by the ranavirus in liver tissue of bullfrogs.

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