Detection of Paramyxovirus, Reovirus and Adenovirus Infection in King Snakes (*Lampropeltis triangulum* spp.) by Transmission Electron Microscopy and Histopathology Techniques.

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***Abstract*— —** *Viruses diverse occur worldwide in reptilian. Paramyxoviruses that infect reptiles belong to Paramyxoviridae family and Ferlavirus genus and are one of the major agents responsible for causing pneumonia in snakes. Reptilian adenovirus has already been documented in various species of snakes, associated with liver, gastrointestinal, respiratory and central nervous system disease. Reptilian orthoreovirus has been demonstrated in several species of reptiles associated with sudden death, central nervous system disorders, skin lesions and pneumonia. In this study 3 kingsnakes (Lampropeltis triangulum spp), from a breeding in Rio de Janeiro, RJ, Brazil, presented a variable clinical picture and death. Feces and organ fragments were processed by the transmission electron microcopy (negative staining) and histopathology (H & E) techniques. By the negative staining, paramyxovirus-like particles, pleomorphic roughly spherical or filamentous, ranging in diameter from 100 to 500 nm, containing internal "herring-borne" nucleocapsid and an outer envelope covered by spikes, were visualized in all samples of the feces and fragments of organs examined. In samples of the small intestine, stomach, pancreas and spleen fragments, adenovirus-like particles, isometric, nonenveloped, containing icosahedral symmetry capsid, measuring 70-90 nm in diameter, were visualized. Reovirus-like particles, isometric, nonenveloped, spherical, characterized as "complete" or "empty", measuring between 65 and 70 nm in diameter, were also visualized in samples of the feces and small intestine. By the H & E, they were observed in the spleen numerous heterophiles, hypoplastic lymphoid follicles and hyperplastic red pulp. The lung presented hypertrophy and hyperplasia of the alveolar walls, alveoli with cellular debris and mucus; numerous heterophiles and monolymphocytic inflammatory cells. The liver had a marked macro and microgoticular steatosis, with a multifocal presence of nodules in the parenchyma. Monolymphocytic hepatitis and large nuclear basophilic inclusion bodies were also observed in hepatocytes, Kupffer cells and occasionally in endothelial cells. The large intestine presented monolymphocytic enteritis with hyperplasia of enteric lymph nodes and marked proliferation of eosinophils. Some areas showed flattened villi. The skin presented areas with hyperkeratosis, foci with balloonifrom degeneration and presence of eosinophilic inclusion corpuscles. In these areas a large number of eosinophils were observed. The kidneys presented monolymphocytic glomerulonephritis. The evaluation of the techniques employed allowed the rapid diagnosis of the viruses in the snakes.*

Keywords*— *Transmission electron microscopy, Histopathology, Snakes, Viruses.

I. INTRODUCTION

The breeding of captive snakes in Brazil has become an important activity, whose main segments of the market are directed to the commercialization of venom, export of non-poisonous snakes, slaughter of animals for sale of meat and leather and supply of snakes for specialized pet shops in pets animal (Tutzer, 2006).

Lampropeltis triangulum (false coral snake, also called milk snake), is one of the most widely distributed snakes in the Americas, occurring from southern Ontario and Quebec in Canada, to Colombia, Ecuador and Venezuela in South America. *Lampropeltis triangulum* is a mostly crepuscular or nocturnal and terrestrial snake that kills by constriction. Its diet consisting of a variety of prey items including insects, worms, spiders, birds, small snakes, frogs and small mammals (Aguilar-Lopes & Pineda, 2013). Because they are docile, non-venomous and easily manipulated, king snakes are kept in captivity, as pets (kingsnake Brazil, 2009). The milk snake is not listed by the IUCN (International Union for Conservation

of Nature), but in some areas, they may face significant pressure due to pet trade collection. Because of this species high value in the pet trade, many subspecies are now being bred in captivity for sale (Savitzky, 2004).

Reptilian paramyxoviruses belong to the *Paramixoviridae* family, genus *Ferlavirus*. They are negative sensed single stranded RNA viruses with a helical nucleocapsid packaged in a pleomorphic envelope (ICTV, 2016), and, among reptiles are found mainly in snakes of different families such as, Boidae, Elapidae, Colubridae, and Viperidae. Initially they were called ophidian paramyxovirus (OPMV) (Essbauer & Ahne, 2001), however, they were also isolated from lizards and tortoises (Marschang et al., 2009; Papp et al., 2010).

The genus *Ferlavirus* refers to a reptilian isolate, which consists of Fer-de-Lance virus (FDLV) found in the common lancehead snake (*Bothrops atrox*) (Clark et al., 1979).

Paramyxovirus is described as one of the major emerging agents that can threaten wildlife (Jacobson, 1993; Daszak et al., 2000) and is responsible for causing snake pneumonia (Marschang, 2011).

Clinical signs associated with acute and chronic OPMV infection range from anorexia, occasional regurgitation, acute dyspnea, acute inspiration, pneumonia, emaciation, mucosal diarrhea, muscle weakness, head tremor, putrid odor and / or neurological disorders. The animals may also die without presenting any of the symptoms mentioned. Studies of OPMV isolated from snakes and other animals have shown that these are endogenous reptilian viruses (Ahne et al., 1987; Homer et al., 1995; Richter et al., 1996; Marschang et al., 2002; Sand et al., 2004; Jacobson and Samuelson, 2007; Abbas et al., 2011; Papp et al., 2013).

Recently a fatal systemic necrotizing infection associated with a novel paramyxovirus in *Eunectes murinus* juveniles was described (Woo et al., 2014).

Adenoviruses that infect reptiles are members of the family *Adenoviridae*, genus *Atadenovirus*. Virions are non-enveloped, 70–90 nm in diameter. The icosahedral capsid consists of 240 non-vertex capsomers (hexons), 8–10 nm in diameter, and 12 vertex capsomers (penton bases), each with a fiber protruding from the virion surface giving the characteristic morphology. The genome is a single, linear molecule of dsDNA and contains an inverted terminal repetition (ITR) (ICTV, 2016). Reptilian adenoviruses have already been documented in about 12 reptile species. Unlike mammalian and avian adenoviruses, reptilians were not well characterized in their pathogenic potential and the ability to induce a primary disease. Diagnosis by isolating the virus in fresh tissue is not always reliable and therefore confirmation of reptilian adenovirus infection depends on diagnosis by electron microscopy for the identification of virus particles associated with histopathological changes, such as the presence of nuclear inclusion corpuscles. Adenovirus infections were diagnosed in different species of snakes and associated with liver, gastrointestinal, respiratory and central nervous system disease (Heldstab & Bestett, 1984; Jacobson et al., 1985; Schumacher et al, 1994; Perkins et al, 2001; Kim et al., 2002; Raymond et al., 2003).

Reptilian orthoreovirus belongs to the *Reoviridae* family, *Spinareoviridae* subfamily and *Orthoreovirus* genus (ICTV, 2016). Orthoreoviruses are non-enveloped viruses with an icosahedral capsid 70–80 nm in diameter (Attoui et al., 2011). The double-stranded RNA genome of orthoreoviruses consists of 10 segments grouped into three categories based on their electrophoretic mobility, three larges (L1-L3), three mediums (M1-M3), and four small segments (S1-S4 (ORF) (Day, 2009). They can induce cell-to-cell fusion. It has been demonstrated in several species of reptiles associated with sudden death, central nervous system disorders, skin lesions and pneumonia (Ahne et al., 1987; Marschang et al., 2002; Ducan et al., 2004, Ugurtas et al, 2008).

The knowledge of the viral infections that affect the king snakes, both kept in captivity as in free life, becomes important to elucidate many of the diseases that infect these animals, which may also constitute important zoonoses. Thus, this study aimed to report the simultaneous presence of paramyxoviruses, adenovirus and reovirus in snakes breeding, using transmission electron microscopy and histopathology techniques.

II. MATERIAL AND METHOD

2.1 Animals

In this study, 3 kingsnakes (*Lampropeltis triangulum* spp) were sent from a breeding in Rio de Janeiro, RJ, Brazil, in 2009. They presented a variable clinical picture with leukocytosis, cutaneous abscesses, loss of motor coordination, skin retention, epidermal vesicles, emesis, diarrhea, dyspnea and death. Feces and various organ fragments (lung, liver, spleen, pancreas,

stomach and small intestine) were processed by transmission electron microscopy (negative staining) and histopathology (H-E routine) techniques.

2.2 Transmission Electron Microscopy

2.2.1 Negative staining technique (rapid preparation)

In this technique, the samples of organ fragments and feces of 3 snakes were suspended in 0.1 M phosphate buffer at pH 7.0. Drops of the obtained suspensions were placed in contact with metallic copper grids, previously covered with a film of 5% collodium amyl acetate and stabilized with carbon. Next, the grids were drained with filter paper and negatively stained with 2% ammonium molybdate at pH 5.0 (Brenner and Horne, 1959; Hayat and Miller, 1990; Madeley, 1997). The grids were observed in a Philips EM 208 transmission electron microscope, at 80 kV.

2.3 Histopathology

2.3.1 Routine Histological technique (&H&E)

All the samples of organ fragments of 3 snakes (lung, liver, spleen, kidneys, large intestine and skin) were fixed in 10% buffered formalin, dehydrated, diaphanized and embedded in paraffin. 5 µm thick sections were stained with hematoxylin and eosin technique and observed under the direct light optical microscope.

III. RESULTS AND DISCUSSION

3.1 Transmission Electron Microscopy

3.1.1 Negative staining (rapid preparation)

Under the transmission electron microscope, a great number of paramyxovirus-like particles (fig.1), pleomorphic roughly spherical or filamentous, ranging in diameter from 100 to 500 nm, containing internal "herring-borne" nucleocapsid (fig.1, big arrow) with 12 nm in diameter and an outer envelope covered by spikes (minor arrow) were visualized in all the samples of the feces and fragments of organs examined. In samples of the small intestine, stomach, pancreas and spleen fragments, adenovirus-like particles (fig.2), isometric, nonenveloped, containing icosahedral symmetry capsid, measuring 70-90 nm in diameter, were visualized. Reovirus-like particles (fig.3), isometric, nonenveloped, spherical, characterized as "complete" (fig.3, big arrow) or "empty" (fig.3, minor arrow), measuring between 65 and 70 nm in diameter, were also visualized in samples of the feces and small intestine.

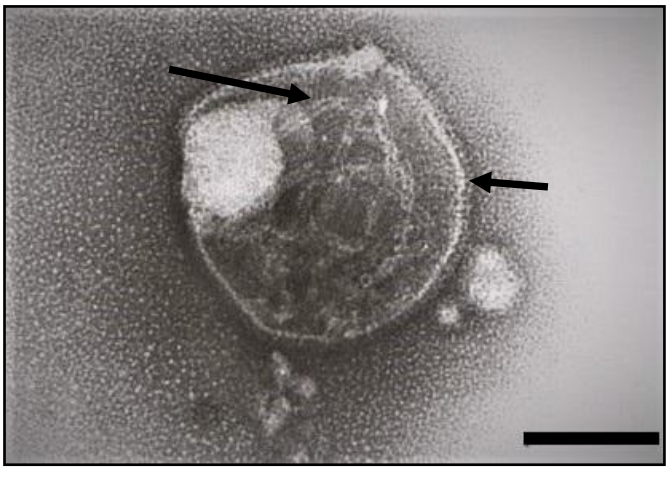

FIGURE 1: Negatively stained paramyxovirus particles, pleomorphic, roughly spherical, containing internal "herring-borne" nucleocapsid (big arrow) and an outer envelope covered by spikes (minor arrow). Bar: 120 nm.

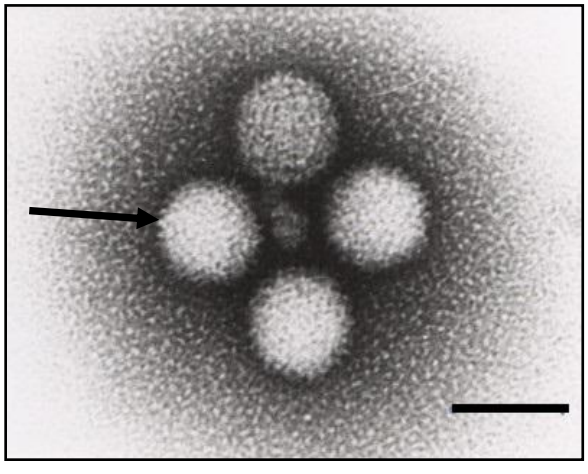

FIGURE 2: Negatively stained adenovirus particles, in small intestine suspension, showing a hexagonal shape with distinct closely packed capsomers (arrow). Bar: 80 nm.

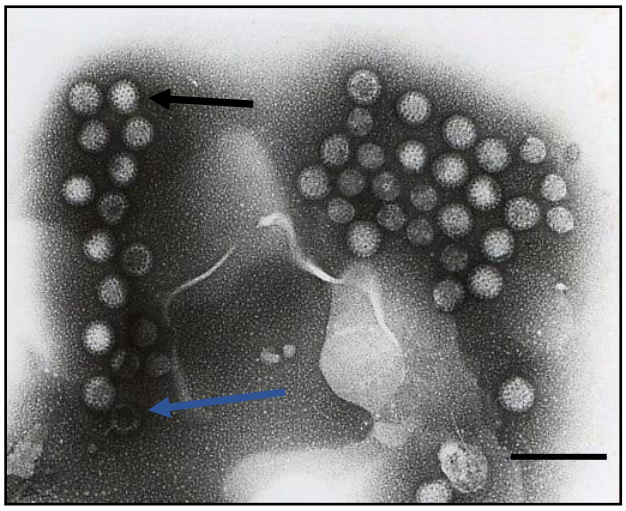

FIGURE 3: Negatively stained reovirus particles, in feces suspension by negative staining, showing "stain-penetrated" (black arrow) and "non-penetrated" (blue arrow) particles. Bar: 190 nm.

3.2 Histopathology

3.2.1 Routine Histological technique (H&E)

In the optic microscope we observed in the spleen numerous eosinophils, hypoplastic lymphoid follicles and hyperplastic red pulp (figs. 4,5). The lung presented hypertrophy and hyperplasia of the alveolar walls, alveoli with cellular debris and mucus; numerous eosinophils and monolymphocytic inflammatory cells (fig. 6). The liver had a marked macro and microgoticular steatosis, with a multifocal presence of nodules in the parenchyma. When enlarged, these nodules had a necrotic center surrounded by monolymphocytic cells, macrophages and innumerable eosinophils. The nodules were surrounded by connective tissue. Foci of monolymphocytic hepatitis and large nuclear basophilic inclusion bodies were also observed in hepatocytes, Kupffer cells and occasionally in endothelial cells. The capsule was thickened. Presence of granulomas (figs. 7-12). The large intestine presented monolymphocytic enteritis with hyperplasia of enteric lymph nodes and marked proliferation of eosinophils. Some areas showed flattened villi (fig. 13). The skin presented areas with hyperkeratosis, foci with balloniform degeneration and presence of eosinophilic inclusion corpuscles. In these areas (dermis and epidermis) a large number of eosinophils were observed (fig. 14). The kidneys presented monolymphocytic glomerulonephritis and melanomacrophages fig. 15).

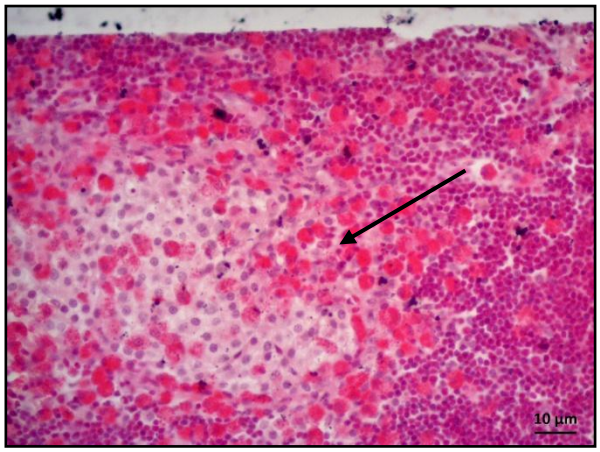

FIGURE 4: Photomicrograph of the spleen of snake. Observe numerous eosinophils (arrow), hypoplastic lymphoid follicles and hyperplastic red pulp. X400.

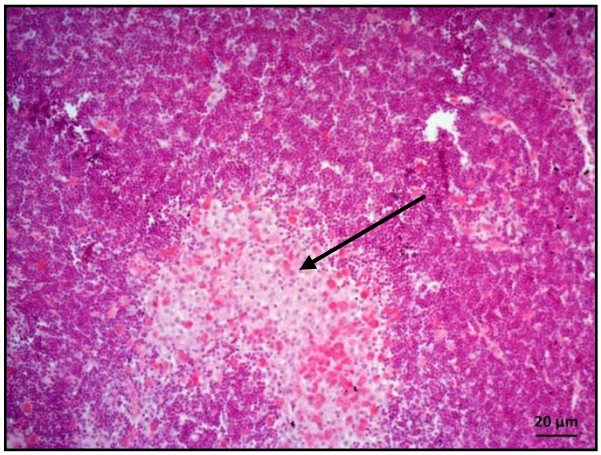

FIGURE 5: Photomicrograph of the spleen of snake. Observe lymphoid follicles (arrow). X200.

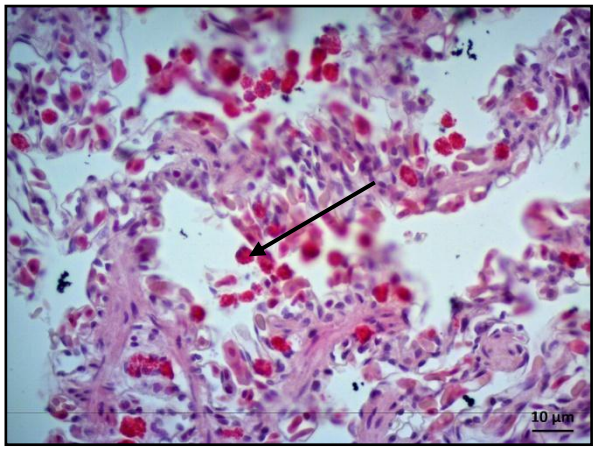

FIGURE 6: Photomicrograph of the lung of snake. Note numerous eosinophils (arrow).

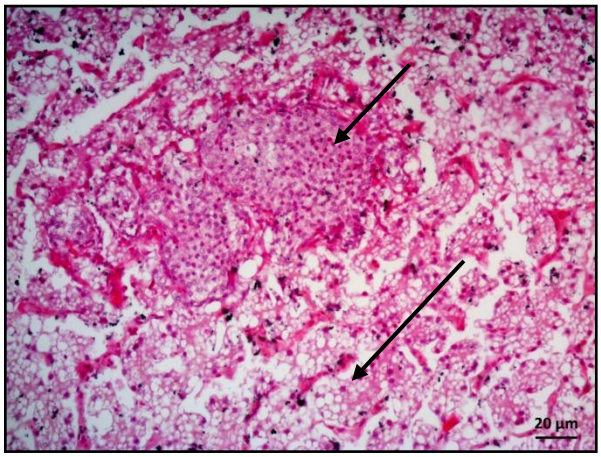

FIGURE 7: Photomicrograph of the liver of snake showing microgoticular steatosis (big arrow), with a multifocal presence of nodules in the parenchyma (minor arrow). X200.

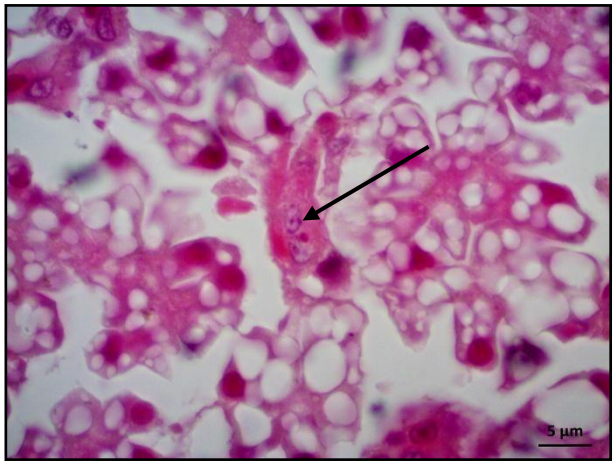

FIGURE 8: Photomicrograph of the liver of snake showing large nuclear basophilic inclusion bodies (arrow). X1000.

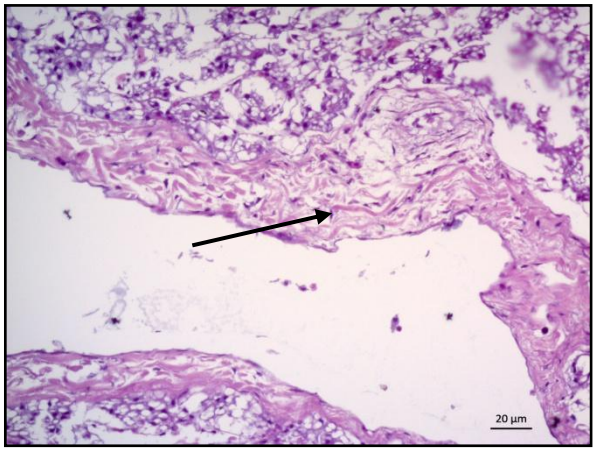

FIGURE 9: Photomicrograph of the liver of snake. Observe thickened capsule (arrow). X20.

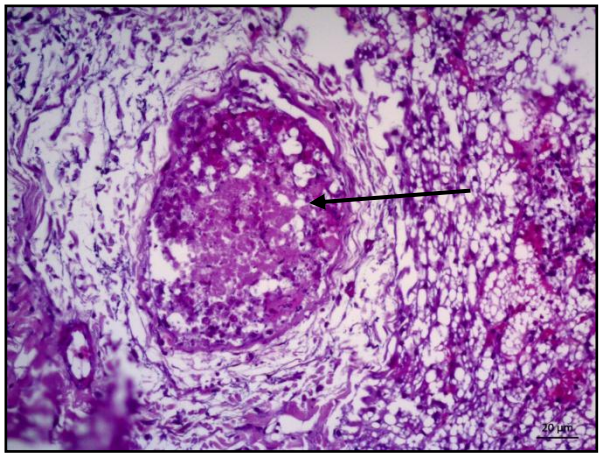

FIGURE 10: Photomicrograph of the liver of snake displaying granuloma (arrow). X20.

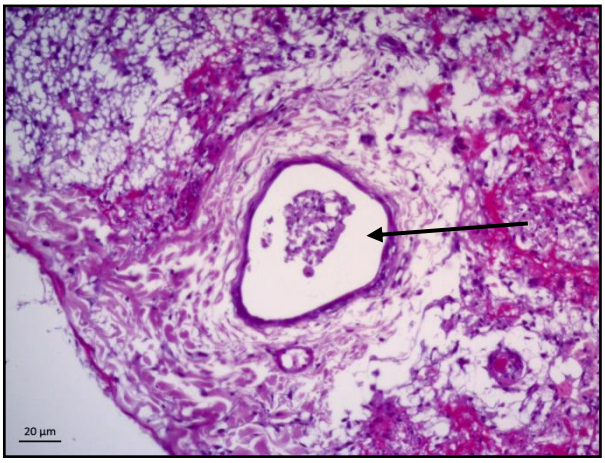

FIGURE 11: Photomicrograph of the liver of snake exhibiting granuloma (arrow). X20.

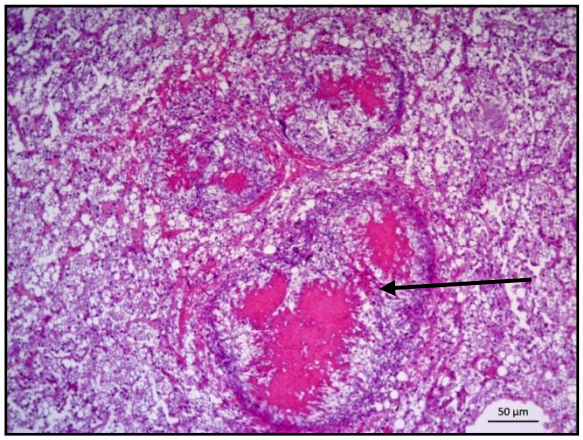

FIGURE 12: Photomicrograph of the liver of snake. Note presence of the nodules in the parenchyma (arrow) with a necrotic center surrounded by monolymphocytic cells, macrophages and innumerable eosinophils. X20.

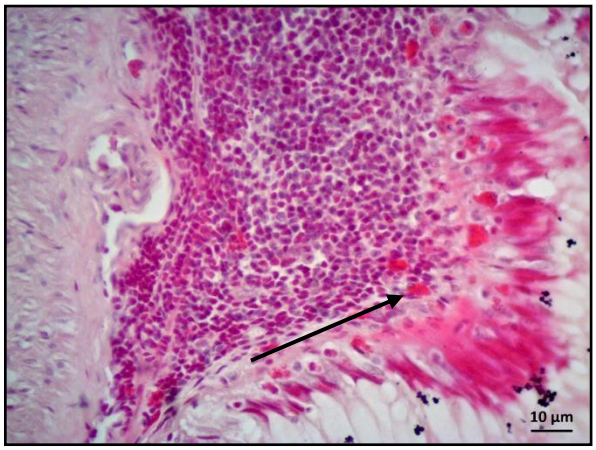

FIGURE 13: Photomicrograph of the small intestine of snake showing monolymphocytic enteritis with hyperplasia of enteric lymph nodes and marked proliferation of eosinophils (arrow). X400.

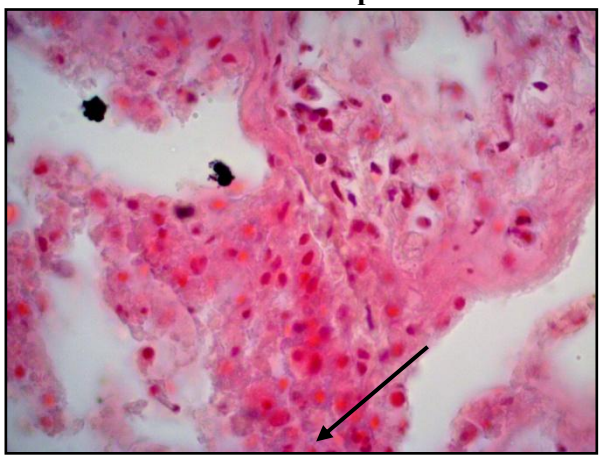

FIGURE 14: Photomicrograph of the skin of snake. Observe: balloniform degeneration and presence of eosinophilic inclusion corpuscles (arrow). X1000.

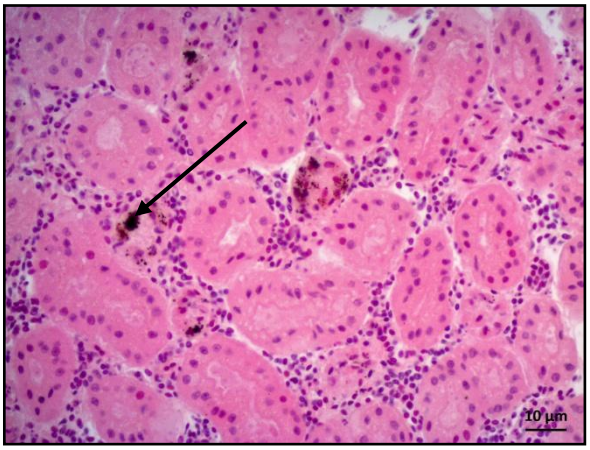

FIGURE 15: Photomicrograph of the kidney of snake showing monolymphocytic glomerulonephritis and melanomacrophages (arrow). X400.

In this study, organ and feces fragments of 3 king snakes (*Lampropeltis triangulum* spp) were examined by transmission electron microscopy and histopathology techniques.

Paramyxovirus particles were visualized in all fragments of organs, skin, faeces, lavage and lung mucus of all 3 snakes studied.

The variable clinical signs presented by the snakes, such as subcutaneous abscesses, skin retention, vomiting, mouth opening, wheezing, pneumonia, dyspnea, motor incoordination and leukocytosis, were also mentioned by other authors in several other species of snakes, infected by paramyxovirus, such as, *Bothrops atrox* (Clark et al., 1979), *Crotalus unicolor* (Jacobson et al., 1997), *Vipera xanthena xanthena* (Potgieter et al., 1987), *Phyton sp.* (Manvell et al., 2000), *Homalopsis buccata* (Papp et al., 2013), (Abbas et al., 2011; Starck et al., 2017), *Bothrops alternatus* (Kolesnikovas et al., 2011), *Bothrops jararaca* (Junqueira de Azevedo et al., 2011) in several species of snakes (Prpic et al., 2017) and in *Draecena guianensis* (Jacobson et al., 2001).

Histopathological examination showed the presence of interstitial multifocal pneumonia.

The main lesions caused by paramyxovirus in snakes are observed in the lungs, the main target organ and occasionally in the brain (Homer et al., 1995).

These findings were confirmed in other studies, in the species, *Crotalus terificus* (Homer et al., 1995), *Python regius* (Ucellini et al., 2014), *Crotalus unicolor* (Jacobson et al., 1997), *Homalopsis buccata* (Papp et al., 2013), *Pantherophis*

guttatus (Starck et al., 2017) and in several species of snakes (Jacobson et al., 1992).

Severe nephritis or necrotizing inflammation of multiple organs was related in *Eunectes murinus* (Woo et al., 2014).

Although proliferative pneumonia is highly suggestive of paramyxovirus infection, the definitive diagnosis requires additional tests such as isolation and viral identification or immunohistochemistry (Holmer et al., 1995).

The transmission electron microscopy examination revealed that the size and morphology of the typical paramyxovirus particles are similar of those other paramyxoviruses observed in other snakes species, as in *Eunectes murinus* (Woo et al., 2014), in *Bothrops atrox* (Lunger & Clark, 1979), *Crotalus durissus terrificus, Crotalus unicolor* and *Atheris* sp (Richter et al., 1996) in a lizard, *Draecena guianensis* (Jacobson et al., 2001) and in cell culture supernatant from isolates of various species (Ahne et al., 1999).

Adenovirus has been described causing severe diseases in reptiles. In birds and mammals adenovirus is mainly dependent on immunosuppression. In reptiles, however, adenovirus is not completely characterized by the fact that there are reports of infection without other recurrent diseases (Jacobson et al., 1992; Ogawa et al., 1992; Schumacher et al, 1999).

The major clinical signs reported in snakes and infected lizards, many of them also presented by the snakes of our study, were represented by vomiting and anorexia, also found in *Boa contrictor* and *Elaphe g. guttata* (Jacobson et al., 1985, Ramis et al., 2000; Mahapatra et al., 2013), pneumonia in *Elaphe guttata*, (Juhasz & Ahne, 1992), opisthotonus in *Pogona vitticeps* (Kim et al., 2002), stomatitis in *Elaphe quatuorlineata* (Heldstab & Bestetti, 1984), dermatitis in *Boa contrictor imperator* (Perkins et al., 2001), cycling in *Lampropeltis zonatta* (Raymond et al., 2003) and head tilt in *Boa constrictor* (Jacobson et al., 1985).

Histopathological diagnosis in the case of adenovirus infection is based on the presence of nuclear basophilic inclusion corpuscles in Kupffer cells and sinusoid endothelial cells, according to our findings. According to Marschang (2011) the most common histological change described in infected animals is hepatic necrosis. Eosinophilic intranuclear inclusion bodies were related in *Phyton regius* (Ogawa et al., 1992), in *Pogona vitticeps* (Wellehan et al., 2004), in *Elaphe g. Guttata* (Mahapatra et al., 2013), in *Boa constrictor* (Jacobson et al., 1985; Ramis et al., 2000; Perkins et al., 2001) in *Heloderma horridum* and *Heloderma suspectum* (Papp et al., 2009) and in *Lichanura trivirgata* (Schumacher et al., 1994).

We verified that the large intestine presented monolymphocyte enteritis with hyperplasia of enteric lymph nodes.

The presence of adenovirus was also associated with the cause of gastroenteritis in Chinese vipers (Jacobson, 1985).

In samples of the small intestine, stomach, pancreas and spleen, adenoviurs-like particles, isometric, nonenveloped, containing icosahedral symmetry capsid, measuring 70-90 nm in diameter, were visualized.

Due to the ease of visualization of viral particles within the clinical specimens, most of the studies on adenoviruses in reptilians have been realized utilizing transmission electron microscopy, both through the negative staining technique and the resin embedding technique followed by ultrafine sections. The morphological characteristics that we described were also described by other authors (Pénzes et al., 2014; Wellehan et al., 2004; Mahapatra et al., 2013; Juhasz & Ahne, 1992; Ahne & Juhasz, 1995; Jacobson et al., 1985; Ramis et al., 2000; Perkins e al., 2001; Papp et al., 2009; Raymond et al., 2003; Ogawa et al., 1992; Schmacher et al., 1994). Starck et al. (2017) utilized electron microscopy to study quantitative changes in the respiratory epithelial surface in the lung of infected snakes with paramyxovirus.

With reference to the reovirus, the significance of the infection caused by this viral agent in snakes is not completely elucidated.

The major clinical signs reported in reovirus infected reptiles have been, hepatic necrosis in *Opheodrys aestivus* (Landolfi et al., 2010), pneumonia in *Elaphe* sp (Lamirande et al., 1999), neurological signs in *Crotalus viridis* (Vieler et al., 1994) and dry lesions in *Lacerta viridis* (Raynaud & Adrian, 1976). The reovirus was also found in asymptomatic Chinese vipers (Blahak & Gobel, 1991).

The reovirus was experimentally inoculated into snakes species, producing pneumonia, indicating that reovirus can be a primary agent (Lamirande et al., 1999).

In birds, reovirus is a facultative pathogen that can trigger severe disease when associated with stress factors. This behavior can also occur among reptiles (Lamirande et al., 1999; O'Rourke & Lertpiriyapong, 2015). The presence of the reovirus was demonstrated in cutaneous lesions of lizards, corroborating with our findings in lesions of the king snakes of our study

(Ugurtas et al, 2008).

The clinical picture in a reptile with reoviral disease typically presents as pneumonia and neurologic signs, and is very similar to the clinical picture seen with paramyxovirus infection. The histologic lesions also resemble paramyxoviral disease (Wellehan et al., 2009).

Electron microscopy, however, allows a rapid and low cost diagnosis where viral particles can be easily visualized directly on specimens of tissue or faeces and has been used by many authors, associated with histopathology, PCR and cell culture or other techniques diagnosis (Latney & Wellehan, 2013). The negative staining technique also allows the detection of different viral particles in the same sample (Gentile & Gelderblom, 2014; Catroxo & Martins, 2015).

Typical reovirus particles were visualized in samples of the *Elaphe moellendorffi* and *Elaphe taenuris* (Lamirande et al., 1999), *Trimeresurus stejnegeri* (Jacobson, 1986), *Corallus Caninus*, *Elaphe longissima* and *Iguana iguana* (Blahak et al., 1995), *Phyton regius* (Ahne et al., 1987; Duncan et al., 2004), *Uromasty hardwickii* (Drury et al., 2002); *Opheodrys aestivus* (Landolfi et al., 2010), *Savannah monitor* (Jacobson et al., 1986) and in *Lacerta viridis* and *Varanus exanthematicus* (Ugurtas et al., 2008).

Simultaneous detection of the three types of viral agents (paramyxovirus, adenovirus and reovirus) in the three snakes of our study was also reported in three corn snakes (*Pantherophis guttatus*) by Abbas et al. (2011).

Concurrent infections with multiple infections agents, including multiple viruses, have been shown to occur and the interactions and effects of these concurrent infections are most yet understood (Marschang, 2011).

Coinfections with paramyxovirus and reovirus (Marschang et al., 2002) and with reovirus and adenovirus (Papp et al., 2009) have been documented in lizard. Simultaneous infection with herpesvirus and mycoplasma has been reported in tortoises (Salinas et al., 2011).

The occurrence of this co-infection is probably associated with the introduction of animals of uncertain origin to creation or inappropriate disinfection of the terrariums, after occurrence of diseased animals, consisting of source of infection to other animals (Abbas et al., 2011).

To prevent the introduction of infectious diseases into livestock, biosecurity measures such as correct disinfection of the environment, quarantine procedures, physical examinations of animals and rapid laboratory diagnostic tests should be instituted in the creation, avoiding losses of animals and losses economic (Prpic et al., 2017).

IV. CONCLUSION

Considering that viruses that affect reptiles are caused by emerging agents that can threaten wildlife, efficient techniques that provide rapid diagnosis are of paramount importance to assist in the immediate adoption of prophylactic and disease control measures, during outbreaks, avoiding important economic losses in the creations.

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