Outbreak of paramyxovirus in Chestnut-bellied Seed-Finch (*Sporophila angolensis*). Transmission electron microscopy diagnosis

Surto de paramixovirose em curiós *Sporophila angolensis*. Diagnóstico por microscopia eletrônica de transmissão

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ABSTRACT
The Chestnut-bellied Seed-Finch (Sporophila angolensis) is a bird constantly sought after as a cage bird, which is the main threat and the cause of its disappearance from the most inhabited regions of the country. Paramyxovirus, present in most breeding sites and Ecological Parks, is the disease that most affects birds with a high mortality rate, causing significant losses, both economic and to nature. Avian paramyxoviruses belong to the family Paramyxoviridae, which includes 21 serotypes (APMVs-1-21). Serotypes 2 and 3 are the ones that most infect passerines. Type 2 causes respiratory disorders, while the disease caused by serotype 3 is characterized by pancreatitis, conjunctivitis, vomiting, diarrhea, dyspnea, dysphagia, pancreatitis, CNS symptoms, and death. Free-ranging birds can act as viral reservoirs and spread the disease when subjected to stress factors. During the period of 2009, during the illegal commercialization of Brazilian birds, 218 Chestnut-bellied Seed-Finch were apprehended and sent to the CRAS (Wild Animals Recovery Center) of the Tietê Ecological Park. Of these, 112 arrived dead and the rest died during the year. About 35 birds were sent to the Electron Microscopy Laboratory of the Biological institute, for research on viral agents. During the necropsy, it was observed that the intestines were dilated, containing yellowish and watery stools. Small intestine fragments were processed by negative staining (rapid preparation), antigen immunolabeling with colloidal gold particles and resin embedding. Under the transmission electron microscope, using the negative staining technique, pleomorphic, rounded or elongated, enveloped paramyxovirus particles containing helical herring-bone like nucleocapsid, measuring between 100 and 500 nm in diameter, were visualized in the samples of all birds. The antigen-antibody reaction was enhanced by colloidal gold particles. Ultrathin sections of the small intestine revealed the presence of nuclei with marginalized chromatin, containing intranuclear inclusions; amorphous intracytoplasmic inclusions, formed by helical nucleocapsids, complete particles measuring from 100 to 250 nm and incomplete ones, budding from the plasma membrane.

Keywords: Sporophila angolensis, Paramixovirus, Transmission Electron Microscopy.

RESUMO
O curió (Sporophila angolensis) é uma ave constantemente procurada como pássaro de gaiola, sendo esta a principal ameaça e causa de seu desaparecimento das regiões mais habitadas do país. A paramixovirose, presente na maioria dos criadouros e Parques Ecológicos, é a doença que mais acomete os pássaros com alta taxa de mortalidade, ocasionando relevantes perdas, tanto econômicas, quanto à natureza. Os paramixovírus aviais pertencem à família Paramyxoviridae, que inclui 21 sorotipos (APMVs-1-21). Os sorotipos 2 e 3 são os que mais infectam os passeriformes. O tipo 2 causa distúrbios respiratórios, enquanto a doença causada pelo sorotipo 3 é caracterizada por pancreatite, conjuntivite, vômito, diarreia, dispneia, disfagia, pancreatite, sintomas do SNC e morte. Aves de vida livre podem atuar como reservatórios virais e disseminar a doença quando submetidos a fatores de estresse. Durante o período de 2009, durante comercialização ilegal de pássaros brasileiros, 218 curiós foram apreendidos e encaminhados ao CRAS (Centro de Recuperação de Animais Silvestres) do Parque Ecológico do Tietê. Desses, 112 chegaram mortos e o restante morreu durante o ano. Cerca de 35 aves foram enviadas ao Laboratório de Microscopia Eletrônica do Instituto Biológico, para pesquisa de agentes virais. Durante a necropsia foi observado que os intestinos estavam dilatados, contendo fezes amareladas e aquosas. Fragmentos de intestino delgado foram processados pelas técnicas de contraste negativa (preparo rápido), de imunomarcação do antígeno com partículas de ouro coloidal e de inclusão em resina. Ao microscópio eletrônico de transmissão, pela técnica de contraste negativa, foram visualizadas partículas de paramixovírus, pleomórficas, arredondadas ou alongadas, envelopadas, contendo nucleocápside helicoidal com aspecto de “espinha de peixe”, medindo entre 100 e 500 nm de diâmetro, nas amostas de todas as aves. A reação antígeno-anticorpo foi realizada pelas partículas de ouro coloidal. As secções ultrassininas do intestino delgado revelaram a presença de núcleos com cromatina marginalizada, contendo inclusões intranucleares, inclusões intracitoplasmáticas amorfas, formadas por...
nucleocápsides helicoidais, partículas completas medindo de 100 a 250 nm e incompletas, brotando da membrana plasmática.

**Palavras-chave:** Sporophila angolensis, Paramixovírus, Microscopia Eletrônica de Transmissão.

1 INTRODUCTION

The Chestnut-bellied Seed-Finch (*Sporophila angolensis*) is a bird constantly sought after as a cage bird (Sick, 1997). In Brazil, it has the highest seizure rates recorded by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) (Júnior et al., 2014; Parreira de Freitas et al., 2015), showing a drastic reduction in its natural population in the states of Minas Gerais, Rio Grande do Sul, São Paulo and Paraná (Nunes, 2010).

Paramyxovirus, present in most breeding sites and Ecological Parks, is the disease that most affects birds with a high mortality rate, causing significant losses, both economic and in nature (Macwhirter, 1994; Joseph, 2003).

Wild birds may act as natural reservoir hosts of paramyxoviruses and assume an important role in the spread of the virus in the environment (Rahman et al., 2018).

The risk of spillover of enzootic paramyxoviruses in the susceptibility of human and domestic animal is defined by the interaction of several ecological and molecular factors that are not yet established (Thibault et al., 2017).

Paramyxovirus particles are pleomorphic, elongated or filamentous with a lipid envelope surrounded a nucleocapsid and measured 150-500 nm of diameter (Rima et al., 2019). Negatively stained virus rupture easily, releasing the internal “herring-bone” nucleocapsid, which may be rigid ou flexible (Lamb & Parks, 2007).

Avian paramyxoviruses belongs to the *Paramixoviridae* Family, *Avulavirinae* subfamily, whose genus were recently classified in, *Metaavulavirus*, *Orthoavulavirus* and *Paraavulavirus*, which include 21 serotypes (APMVs). Genus *Metaavulaviruses* include APMVs 2,5,6,7,8,9,10,11,14,15 and 20; *Orthoavulavirus* genus includes APMVs 1,8,12,13,16,17,19,21 and *Avian Orthoavulavirus* 21. *Paraavulavirus* genus includes only APMV-3 and APMV-4 (Rima et al., 2019).

The genome of APMVs contain a non-segmented negative sense single-stranded RNA with approximate by 15 to 17 kb in lenght and encodes six structural proteins which include the nucleoprotein (NP), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) (Samal, 2011; Karamendin et al., 2020).

Newcastle disease (APMV-1) is a highly contagious disease of poultry and constitute major problem in existing or developing poultry industries. There are diferente pathotypes of NDV,
velogenic (high virulence), mesogenic (moderate virulence), and lentogenic (low virulence) (Maqbool et al., 2017).

The feral birds including aquatic and migratory birds and other wild birds may act as natural reservoir hosts of Newcastle disease and may play a remarkable role in the spread of the virus in environment (Rahman et al., 2018).

The most commonly observed clinical signs and symptoms in birds affected by APMV-1 are represented by anorexia, weight loss, depression, diarrhea, nasal and ocular discharges, conjunctivitis, pseudomembrane formation in the larynx, dyspnoea, ataxia, torticollis, opisthotonus, dilation of the pupils, tremor and paralysis of the limbs, sagging wings and death, in weaver finches (Ritchie et al., 1994), cockatoos, budgerigars, maxaw, lory, parrot, love birds, conure, yellow headed, Amazon parrots and yellow-maped Amazon Parrots (Samanta & Bandyopadyay, 2017) and in pigeons (He et al., 2020). APMV-1 has also been reported in Common starlings found dead (Dodovski et al., 2015) and in House sparrows, apparently healthy (Silva et al., 2006).

Serotype 2 occurs most frequently in passerines causing ematiation, severe pneumonia, and diarrhea (Goodman & Hanson, 1988; Ritchie et al., 1995; Ritchie & Carter, 1995; Zhang et al., 2006). Psittacines may present with pneumonia, weakness, weight loss, tracheitis, diarrhea, and death (Ritchie et al., 1994; Collins et al., 1975).

Serotype 3 affects psittacines more than passerines (Beck et al., 2003), and the clinical signs can be symbolized by conjuntivitis, acute pancreatitis, dysphagia, dyspnea, torticollis, circling, opisthotonus, ataxia, steatorrhea, vomiting, diarrhea and death in parrots, parakeet and finches (Schemera et al., 1987; Ritchie et al., 1994; Shivaprasad, 1998; Shihtmanter et al., 1998; Kaleta, 1999; Beck et al., 2003; Jung et al., 2009).

The presence of serotype 4 has been reported in eagles, doves and pigeons and in crows (Kydirmanov et al., 2018), while serotype 5 is responsible for causing disease in budgerigars (*Melopsittacus undulatus*), with symptoms of depression, dyspnoea, diarrhea, torticollis and acute fatal enteritis in newborns, leaving to high mortality (Nerome et al., 1978; Gough et al., 1993).

The serotypes 6, 7, 8 and 9 have been described in house sparrow (Maldonado et al., 1995) and the APMV-6, 8, 13, 16 and 20 in eagles, doves and crows (Kydirmanov et al., 2018).

Transmission electron microscopy is an important tool with several technological advantages, due to its speed and diagnostic accuracy, in addition to the direct visualization of the various viral agents in the samples, being used in emergency situations to identify unknown or unsuspected viruses (Zhang et al., 2013).

This work aimed to detect the presence of viral agents in feces and small intestine fragments of the Chestnut-bellied Seed-Finch, through transmission electron microscopy techniques.
2 MATERIAL AND METHOD

Description of the case

In 2009, during the illegal sale of Brazilian birds, 218 Chestnut bellied Seed-Finch, were apprehended and sent to the CRAS (Wild Animal Recovery Center) of the Tietê Ecological Park. Of those, 112 arrived dead and the rest died during the year. About 35 birds were sent to the Electron Microscopy Laboratory of the Biological Institute, to research viral agents. During necropsy it was observed that the intestines were dilated, containing yellowish and watery stools. Feces and small intestine fragments were processed by negative staining (rapid preparation), immunocytochemistry and resin embedding techniques.

Negative staining technique (rapid preparation). In this technique, stool and small intestine fragments samples were suspended in phosphate buffer 0.1 M, pH 7.0. Drops of the obtained suspensions were placed in contact with metallic copper grids with carbon stabilized supporting film of 0.5% collodion in amyl acetate. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate, pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997).

Immunocytochemistry technique. At the immunolabeling technique with colloidal gold particles for negative staining, the copper grids were placed in contact with viral suspension of the samples of stool and small intestine fragments and, after removing the excess with filter paper, the same were put on specific primary antibody drops. After further washing in PBS drops, the grids were incubated in protein A drops, in association with 10 nm colloidal gold particles (secondary antibody). Grids were then contrasted with 2% ammonium molybdate at pH 5.0 (Knutton, 1995).

Resin embedding technique. Fragments of small intestine were fixed in 2.5% glutaraldehyde in 0.1 M, pH7.0 phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in cetonic series, the fragments were embedded in Spurr resin (González- Santander 1969; Luft, 1961). Ultrathin sections were cut on the LKB ultratome and mounted on copper grids. The sections were contrasted with uranyl acetate-lead citrate (Watson, 1958; Reynolds, 1963).

All grids submitted to the above reactions were observed in a Philips EM 208 electron microscope, at 80 kV.
3 RESULTS

Negative staining (rapid preparation) technique. Under the transmission electron microscope, using the negative staining technique, paramyxovirus particles, pleomorphic (fig. 1, big arrow), rounded or elongated, measuring 100 to 500 nm in diameter, containing an envelope covered by spikes (fig. 1, minor arrow) and helical herring-bone-like nucleocapsid (fig. 2, arrow), were visualized in the samples of all birds.

Immunocytochemistry Technique. In the immunocytochemistry technique, the antigen-antibody interaction was strongly enhanced by the dense colloidal gold particles over the parapoxvirus (fig. 3, arrow) in all samples of stool and small intestine fragments (figure 2, arrow), confirming the results of negative staining technique.

Resin embedding technique. The ultrathin sections of small intestine fragments revealed the presence of nuclei with compact marginalized chromatin (fig. 4, white arrow; fig. 5, minor arrow), containing intranuclear inclusions (figs. 4,5 big arrow). Intracytoplasmic granular amorphous inclusions surrounded by membrane (figs. 4,5 (minor arrow), formed by viral nucleocapsids were also observed. Complete particles measuring 100 to 250 nm in diameter (fig. 6, big arrow) and incomplete particles, measuring on average 70 nm in diameter, budding from the plasma membrane could also be identified (fig. 6, minor arrow).

4 DISCUSSION

This paper describes the identification of paramyxovirus particles in approximately 35 stool and small intestine samples from Chestnut-bellied Seed-Finch (Sporophila angolensis) using transmission electron microscopy techniques.

Paramyxovirus particles, pleomorphic, rounded or elongated, measuring 100 to 500 nm in diameter, containing an envelope covered by spikes and helical herring-bone-like nucleocapsid, were visualized in the samples of all the 35 birds, using the negative staining technique, under transmission electron microscope.

Particles with these ultrastructural characteristics have also been described in other studies of paramyxoviruses in several other avian species as, rusty collared seedeater, red cowled cardinal, curl crested and (Catroxo et al., 2000); helmeted manakin, waxbill, double-collared seedeater, Turdus sp., Thraupis sp, rufous-bellied thrush, great kiskadee, bananaquit, bay-winged cowbird grey monjita, surucua trogon, green-winged saltator, common canary, wild canary, saffron finch, brazilian tanager, campo troupial, great-billed seed-finch, red-crested finch, ultramarine grosbeak,
lined seedeater, variable oriole, seven-colored tanager, hooded siskin, red siskin, white-naped jay, brassy breasted tanager, Carduelis sp., swallow tanager, buffy-fronted seedeater, gilt-edgerd tanager (Catroxo et al., 2012); gouldian finch (Zhang et al., 2006); parrot and parakeet (Steffens, 1998, Grund et al., 2002), owl (Catroxo et al., 2010); dove (Catroxo et al., 2011); eurasian teal (Sobolev et al., 2016); geese (Yamamoto et al., 2015), duck (Lee et al., 2017) and, in gull (Karamendin et al., 2017).

The immunocytochemistry technique we applied highlighted sharply the paramyxovirus particles with colloidal gold, allowing the visibility of the antigen-antibody interaction, also used by other researchers to confirm the paramyxovirus viral strain (Martins et al., 2013, 2016; Catroxo et al., 2009 a,b, 2017, 2020).

Through the resin embedding technique, we could observe in ultrathin sections, nuclei with compact marginalized chromatin, containing intranuclear inclusions, in addition to granular intracytoplasmic amorphous inclusions surrounded by membrane, formed by viral nucleocapsids, aspects which were also observed by other authors (Mannl et al., 1987; Leach et al., 1988; Granzow et al., 1999; Jacobson et al., 2001; Catroxo et al., 2012).

The presence of complete particles measuring 100 to 250 nm in diameter and incomplete, measuring on average 70 nm in diameter, budding from the plasma membrane that we identified, were consistent with previous findings in other researches (Mannl et al., 1987; Leach et al., 1988; Granzow et al., 1999; Jacobson et al., 2001; Catroxo et al., 2012).

The passerines of the our study initially showed clinical signs of diarrhea and then died suddenly.

Most birds affected by Newcastle disease have diarrhea such as, minahs (Korbel & Kosters, 1998); weaver finches (Ritchie et al., 1994), pigeons (He et al., 2020) and psittacine birds (Clavijo et al., 2000; Samanta & Bandyopadhay, 2017).

Serotype 2 is the one that most infects passerines causing various symptoms, including profuse diarrhea, reported in weaver finches (Ritchie et al., 1994; Ritchie & Carter, 1995; Goodman & Hanson, 1988), gouldian finch (Zhang et al., 2006) and also observed in psittacines, such, parrots (Collins, 1975). Serotype 3, although it occurs more frequently in psittaciformes, such as parakeet and parrot (Shivaprasad, 1998; Shihtmanter et al., 1998; Kaleta, 1999; Beck et al., 2003; Jung et al., 2009), can also affect passerines, such as house sparrow (Stallknecht et al., 1991), finch and canary (Schemera et al., 1987), causing yellowish diarrhea. Immature budgerigars infected with serotype 5 had acute fatal enteritis with high mortality (Nerome et al., 1978; Gough et al., 1993). On the other hand, serotype 1 has already been detected in common starlings, found dead (Dodovski et al., 2015) while serotype 1 infected canaries rarely develop clinical signs (Ritchie et al., 1994). In apparently
healthy birds, serotype 1 has already been detected in sparrows (Silva et al., 2006), serotypes 2 and 3 in woodchat shrike, house sparrow and wood pigeon (Maldonado et al., 1994), the serotypes 4, 6, 8, 13, 16 and 20 in eagles, doves and crows (Kydirmanov et al., 2018) and 6, 7 and 8 in house sparrows (Maldonado et al., 1995).

According to OIE (2013), the susceptibility of passerines is highly variable, ranging from no signs of disease with excretion of the virus to severe clinical signs and may be carriers for months before the onset of clinical signs (Shivaprasad, 1998). Stress factors, including nutritional, husbandry, breeding, and the introduction of new birds in flock aviaries, may play a significant role in disease outbreaks (Joseph, 2003).

This report constitutes the first occurrence of paramyxoviruses in Chestnut-bellied Seed-Finch (Sporophila angolensis).

Very little is known about the molecular biology of APMVs in wild bird populations, but an understanding of the molecular and pathological characteristics of APMVs is of general epidemiological interest, and is important for developing vaccines in the case of emergence of novel pathogenic strains (Karamendin et al., 2017).

The application of transmission electron microscopy techniques was essential for the rapid detection of paramyxoviruses in the samples, promoting the immediate adoption of control measures in the creation, avoiding losses due to the occurrence of new outbreaks.

Figure 1: Negative staining of paramyxovirus particles in feces suspension, showing pleomorphic particles (big arrow) and an envelope covered by spikes (minor arrow). Bar: 140 nm.
Figure 2: Negative staining of paramyxovirus particle in small intestine suspension, showing helical herring-bone-like nucleocapsid (arrow). Bar: 220 nm.

Figure 3: Antigen-antibody interaction strongly enhanced by the dense gold particles over the paramixovirus (arrow) in the immunocytochemistry technique (arrow). Bar: 100 nm.

Figure 4: Ultrathin section of the small intestine showing a nucleus with marginalized chromatin (white arrow); intranuclear (big arrow) and intracytoplasmic inclusions (minor arrow). Bar: 560 nm.
Figure 5: Ultrathin section of the small intestine, showing intranuclear inclusion (big arrow) and aspect of marginalized chromatin (minor arrow). Bar: 1000 nm.

Figure 6: Ultrathin section of small intestine, revealing complete (big arrow) and incomplete (minor arrow) paramyxovirus particles budding from cell membranes. Bar: 800 nm.
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