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SIMULTANEOUS INFECTION BY PARAMYXOVIRUS AND MYCOPLASMA IN RED SISKIN (*SPINUS CUCULLATA*), THREATENED SPECIES

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Abstract: The red-siskin (*Spinus cucullata)* is an increasingly rare species, due to its illegal hunting, which classifies it in the status of endangered. Paramyxoviruses have been isolated from many avian species around the world, causing high morbidity and mortality in breeding grounds, commercial farms and Ecological Parks. Paramyxoviruses that infect birds belong to the *Paramyxoviridae* family with 21 serotypes (APMV-1-21). Serotype 2 affects Passeriformes and Psittaciformes, being more frequent in Passeriformes, causing weakness, weight loss, tracheitis, diarrhea, pneumonia and death. Passerines and Psittaciformes infected by serotype 3 may presented conjunctivitis, pancreatitis, dysphagia, dyspnea, vomiting, diarrhea, steatorrhea, in addition to neurological symptoms. Mycoplasmas are small prokaryotes belonging to the *Mycoplasmatacea* family. In passerines, the disease is characterized by coughing, sneezing, rales, nasal and ocular discharge and conjunctivitis. In April 2017, 2 red-siskin (*Spinus cucullata*) were sent to the Electron Microscopy Laboratory of the Biological Institute of São Paulo, SP, Brazil, for research of viral agents. After necropsy, samples of organ fragments (lung, heart, ventricle, liver, intestine) and feces were processed for transmission electron microscopy using the negative staining technique (rapid preparation). In the transmission electron microscope, paramyxovirus particles, pleomorphic, enveloped, containing nucleocapsid in the form of a "fishbone", measuring between 100 and 300 nm in diameter, were observed in all examined samples. In the lung fragments of the 2 birds, the presence of mycoplasma-like pleomorphic formations was also visualized, measuring between 100 and 800 nm. This report constitutes the first occurrence of these agents in *Spinus cucullata*.

Keywords: *Spinus cucullata,* Paramyxovirus, Mycoplasma, Transmission electron microscopy.

INTRODUCTION

The red-siskin is an increasingly rare avian species, and its capture has been illegal since 1940. Its conservation status is considered threatened by the IUCN (2012), due to illegal capture, which promotes the reduction of its natural habitat for agriculture, constituting a serious threat to its complete disappearance. Captive breeding programs for their subsequent reintroduction into the wild are being instituted to prevent their extinction (BirdLife International, 2012).

Avian paramyxoviruses belongs to the *Paramixoviridae* family, *Avulavirinae* sub-family, whose genus were recently classified in, *Metaavulavirus, Orthoavulavirus* and *Paraavulavirus*, which include 21 sero-types (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. Paramyxoviruses (PMVs) are enveloped particles, com nucleocápside helicoidal and negative-sense, single-stranded RNA viruses with genes coding for at least six major proteins, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion glycoprotein (F), receptor binding protein (RBP, formerly designated variously as HN, H, or G), and the large protein (L) that possesses RNA-dependent RNA polymerase (RdRp) activity (Rima et al., 2019). Several of the most devastating diseases of animals, such as rinderpest, Newcastle disease, and canine distemper, are caused by paramyxoviruses (Samal, 2008). Paramyxoviruses are at risk of spillover, originating from wild hosts and may pose an ongoing threat to global human and animal health (Clayton, 2017; Thibault et al., 2017).

Paramyxoviruses have been isolated from many avian species around the world, causing high morbidity and mortality in bird breeding, chicken farming and Ecological Parks (Macwhirse, 1994; Joseph, 2003). Wild bird carriers may shed the virus for up to six weeks and may potentially spread endemic APMV-1 to susceptible poultry flocks (Animal Health Australia 2013) and disease susceptibility and severity varies between affected species (Alexander, 2000). Passerines, psittacines, columbiformes and raptors infected by Newcastle disease may show variable clinical signs and symptoms, such as anorexia, weight loss, depression, diarrhea, ruffled feathers, ocular and nasal secretion, coughing, conjunctivitis, dyspnoea, ataxia, torticollis, opisthotonus, tremor and paralysis of the limbs, sagging wings and death (Ritchie et al., 1994; Tarello et al., 2004; Norod et al., 2017; Samanta & Badyopadyay, 2017; He et al., 2020). The presence of convulsions and accelerated movements are signs that precede death (Ritchie et al., 1994).

Serotype 2 affects passerines and psittacines, being more frequent in passerines, causing ematiation, pneumonia and diarrhea, in addition to weakness, weight loss, tracheitis, and death in psittacines (Collins et al., 1975; Goodman & Hanson, 1988; Ritichie et al., 1994; Ritchie et al., 1995; Ritchie & Carter, 1995; Zhang et al., 2006).

Parrots, parakeets and finches infected by serotype 3 may show clinical signs of conjunctivitis, pancreatitis, dyspaghia, dyspnea, vomiting, diarrhea, and, steatorrhoea, and neurological signs such as torticollis, circling and opisthotonus (Schemera et al., 1987; Ritchie et al., 1994; Shivaprasad, 1998; Shihmanter et al., 1998; Kaleta, 1999; Beck et al., 2003; Jung et al., 2009).

Doves, pigeons, eagles and crows have already been infected by serotype 4 (Kydirmanov et al., 2018). Caged budgerigars are the species most affected by serotype 5, and, new borns may show depression, dyspnoea, diarrhea, torticollis, acute enteritis with high mortality (Nerome et al., 1978; Gouch et al., 1993). Regarding serotypes 6, 7, 8 and 9, these have already been described in house sparrow and the 6, 8, 13, 16 and 20 have already been found in eagles, doves and crows (Kyrdimanov et al., 2018).

Mycoplasmas are the smallest prokaryotes that belong to the class *Mollicute*, order *Mycoplasmatales*, *Mycoplasmatacea* family and *Mycoplasma* genus (Sirand-Pusnet et al., 2007). Due to the absence of a rigid cell wall they are pleomorphic and measure 0.1-0.15 μm in lenght. The pathogenic strain of *Mycoplasma gallisepticum* presents an external formation with the appearance of a bubble, called a "bleb", which has the function of mobility and adherence to host cells (Balen et al., 1991; Nakane & Miyata, 2009). They has a worldwide distribution, cause acute or chronic diseases and are transmitted vertically via infectious aerossol and through contamination of feed, water, and, the enviromment (Razin et al., 1998). The incubation period for finches is 4 to 14 days (OIE, 2018). *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are considered the most pathogenic with economic impact on commercial breeding. Mycoplasmas have been detected in different species of passerines (Ley et al., 1996, 1997, 2012, Mikaelian et al., 2001; Hawley et al., 2011; Dhont et al., 2014; Sawicka-Durkalec et al., 2021; Fischer et al., 1997; Frasca et al., 1997; OIE, 2018), psittacines (Bozeman et al., 1984; Lierz et al., 2008; Gomes et al., 2012; Carvalho et al., 2017) , columbids (Poveda et al., 1990; Guimarães, 2014) and falconids (Poveda et al., 1990). The main clinical signs and symptoms observed in these species are characterized by cough, sneezing, rales, eye and nasal discharges, conjunctivitis,

epiphora, hyperaemia of palpebrae and nictitans (Nascimento et al., 2005b; Ley et al., 2012).

Considering the effectiveness and speed of transmission electron microscopy, this work aimed to report the simultaneous presence of paramyxovirus and mycoplasma particles in samples of organ fragments and feces of red-siskin, using the negative staining technique.

MATERIAL AND METHOD

DESCRIPTION OF THE CASE

In April 2017, 2 red-siskin (*Spinus cucullata*), from a breeding facility located in Barueri, São Paulo, SP, Brazil, were sent to the Electron Microscopy Laboratory of the Biological Institute of São Paulo, SP, Brazil, for research. of viral agents. The property had about 40 animals, 8 of which fell ill and 3 died. The birds showed clinical signs of weight loss, lack of appetite, conjunctivitis, diarrhea, respiratory disorders, pneumonia and sudden death.

NEGATIVE STAINING TECHNIQUE (RAPID PREPARATION)

The two birds were submitted to necropsy and samples of organ fragments (lung, heart, ventricle, liver, intestine) and feces were collected and processed by the negative staining technique (rapid preparation), being suspended in 0.1M phosphate buffer and pH 7.0, placed in contact with metallic grids, covered with collodion and carbon film, drained with filter paper and negatively contrasted with 2% ammonium molybdate (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997).

RESULTS

During necropsy it was observed that the intestines were dilated, containing yellowish and watery stools. The lungs had small whitish spots along their entire length. Under the transmission electron microscope, particles with morphology similar to paramyxovirus were observed using the negative staining technique (rapid preparation), in samples of fragments from the lung, heart, ventricle, liver, intestine and feces of the 2 birds (Fig. 1), pleomorphic, enveloped, containing a "fishbone" shaped nucleocapsid, measuring between 100 and 300 nm in diameter. In samples of lung fragments from the 2 birds, the presence of pleomorphic formations similar to mycoplasmas was also visualized (fig. 2), measuring between 100 and 800 nm.

DISCUSSION

In this study samples of organ fragments and feces of 2 red-siskin (Spinus cucullata) were investigated by the negative staining technique for transmission electron microscopy. Pleomorphic rounded or elongated paramyxovirus particles, with a diameter of 100-500 nm, containing a envelope with a single fringe of surface projections approximately 17 nm in lenght, and helical herring-bone-like nucleocapsid, were visualized in the samples of organ fragments and feces of red-siskin (*Spinus cucullata*). In other avian paramyxovirus research, particles with these basic features were described by the negative staining technique in rusty collared seedeater and red cowled cardinal (Catroxo et al., 2000), *Chloebia gouldiae* (Zhang et al., 2006), owl (Catroxo et al., 2010), dove (Catroxo et al., 2011), helmeted manakin, common waxbill, double collared seedeater, 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, greatbilled, seed-finch, red-crested finch, ultramarine grosbeak, lined seedeater, variable oriole,

seven colored tanager, hooded siskin, white-naped jay, brassy breasted tanager, swallow tanager, buffy-fronted seedeater, gilt-edgerd tanager (Catroxo et al., 2012) and chestnut-bellied seed-finch (Catroxo et al., 2022).

The clinical signs represented by lack of appetite, emaciation, conjunctivitis, diarrhea, pneumonia and sudden death, presented by the two specimens of red siskin that we examined, were also reported in other species of passerines infected by serotype 1, such as house sparrow (Khalaffala et al., 1990 a, b), minahs (Korbel & Kosters, 1998) and weaver finches (Ritchie et al., 1994), pelo serotype 2, gouldian finch (Zhang et al., 2006) and serotype 3, house sparrow (Stillknecht et al., 1991), finch (Ritichie & Carter, 1995; Beck et al., 2003), and, canary (Schemera et al., 1987). In contrast, some serotypes have already been detected in healthy birds and found dead. During paramyxovirus research, serotype 1 has already been detected in healthy canaries and sparrows (Ritchie et al., 1994; Silva et al., 2006) and in common starlings found dead (Dodovsky et al., 2015). Serotypes 2, 6, 7, and, 8 have been found in apparently healthy house sparrows (Maldonado et al., 1994). Species of passerines such as great kiskadee, although asymptomatic, presented sudden death (Catroxo et al., 2012).

The passerines in our work were co-infected with Mycoplasma. Mycoplasma particles, pleomorphic, filamentous or elongated, measuring between 100 and 800 nm, were visualized in lung fragments. Similar ultrastructural features have also been reported in strains of *Mycoplasma gallisepticum* (Balen et al., 1991; Nakane & Miyata, 2009) and in canary feces (Queiroz et al., 2016).

The *Spinus cucullata* in our study showed conjunctivitis, in addition to the other clinical signs already mentioned, this being the main one observed among passerines infected by Mycoplasma. In other surveys, the presence of Mycoplasma has been reported in *Coccothraustes vespertinus* and *Pinicola enucleator* (Mikaelian et al., 2001), in *Petrochelidon pyrrhonota* (Ley et al., 2012), *Sturnus vulgaris* (Frasca et al., 1997), *Haemorhous mexicanus, Spinus tristes Haemorhous purpureus, Poecile atricapillus* (Dhondt et al., 2014), *Haemorhous mexicanus* and *Spinus tristes* (Fischer et al., 1997; Hartup et al., 2000), *Haemorhous purpureus* (Hartup et al., 2000), and, *Haemorhous mexicanus* and *Aphelocoma californica* (Rogers et al., 2019). Other clinical signs such as erythema, discharge, rhinitis, sinusitis, emaciation, lethargy, blindness and bilateral blepharitis were also mentioned (Frasca et al., 1997; Hartup et al., 2000; Dhont et al., 2014). Sawicka-Durcale et al. (2021), however, reported the presence of Mycoplasma in passerines with no clinical signs.

The birds in our study also presented respiratory disorders and pneumonia, which was not observed in other species of passerines. Nascimento et al. (2005), however, reported that the muscovy-ducks they studied exhibited cough, sneezing, rales, eye and nasal discharges, while Lierz et al. (2008) stated that birds of prey, denoted respiratory dysfunction, air sacculitis, pneumonia and tracheitis.

Considering that Mycoplasma is an opportunistic agent, the association with other agents is understandable. Ley et al. (2012) reported the occurrence of the association of *Mycoplasma sturni* and cryptosporidiosis in cliff swallows. Queiroz et al. (2016) reported co-infection of circovirus, retrovirus and mycoplasma in domestic canary.

Studies that demonstrate the molecular characterization of APMV isolates should be conducted in order to better evaluate and understand their genetic origin and relationship with the occurrence of sudden

outbreaks (Peroulis-Koutis et al., 2002). Considering that wild birds are important reservoirs of pathogenic agents with the possibility of mutation and recombination in order to produce new pathogens that can spread over long distances and cause new outbreaks in animals and humans, they constitute a danger to human health (Elmberg et al., 2017; Yin et al., 2017). The monitoring of viruses and bacteria in wild and captive birds should be used as a fin system for the incursion of zoonotic agents (Shan et al., 2022).

The technique employed was efficient for the rapid diagnosis and taking of prophylactic and control measures at the breeding site, in addition to enabling the protection of this endangered species. This report constitutes the first occurrence of these agents in *Spinus cucullata*.

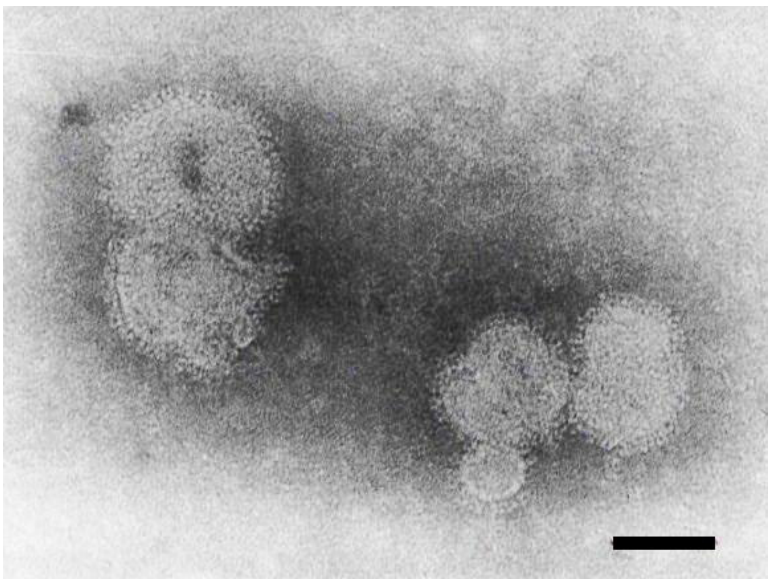

Fig. 1 – Negative staining of paramyxovirus particles in a stool sample, showing envelope covered by spicules (arrows). Bar: 100 nm.

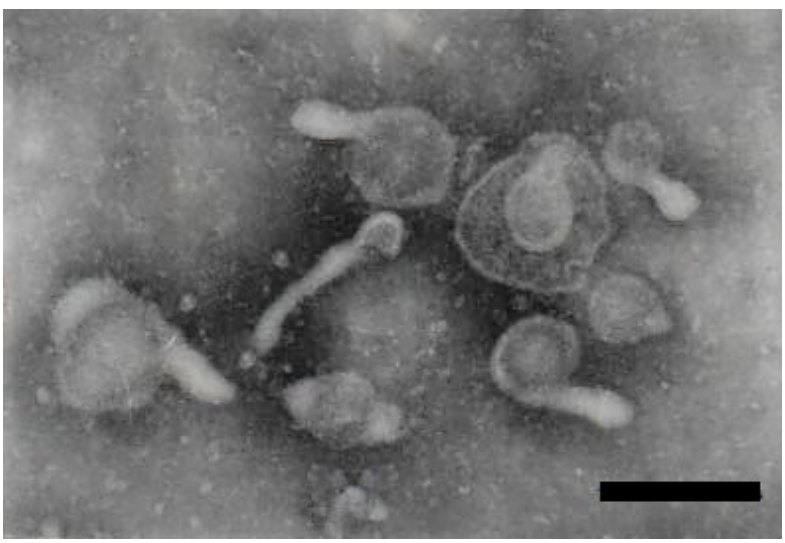

Fig. 2 – Mycoplasma particles, pleomorphic, filamentous (big arrow) or elongated (minor arrow) negatively contrasted in red-siskin lung suspension. Bar: 330 nm.

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