Detection of avipoxvirus in a cutaneous lesion of a swallow tanager (*Tersina viridis*) by transmission electron microscopy

Detecção de avipoxvírus em lesões cutâneas de saí-andorinha (*Tersina viridis*) por microscopia eletrônica de transmissão

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Marcia Helena Braga Catroxo
Doutora em Infectologia pela Universidade Federal de São Paulo (UNIFESP)
Instituição: Laboratório de Microscopia Eletrônica do Centro de Pesquisa de Sanidade Animal do Instituto Biológico de São Paulo, SP, Brasil
Endereço: Avenida Conselheiro Rodrigues Alves, 1252, Vila Mariana, São Paulo, SP, Brasil
E-mail: marcia.braga@sp.gov.br

Ana Maria Cristina Rebello Pinto da Fonseca Martins
Doutora e Pós Doutora em Patologia Especial e Comparada (FMVZ/USP)
Instituição: Laboratório Institucional de Sanidade em Aquicultura do Centro de Pesquisa de Sanidade Animal do Instituto Biológico de São Paulo, SP, Brasil
Endereço: Avenida Conselheiro Rodrigues Alves, 1252, Vila Mariana, São Paulo, SP, Brasil
E-mail: ana.martins@sp.gov.br

Liliane Milanelo
Mestre em Ciências pela Escola Paulista de Medicina - UNIFESP / Médica Veterinária pela Universidade Estadual Paulista
Instituição: Centro de Recuperação de Animais Silvestres do Parque Ecológico do Tietê
Endereço: Rodovia Parque, 8054 - Vila Santo Henrique, São Paulo – SP, Brasil
E-mail: lmilanelo@yahoo.com.br

Lilian Sayuri Fitorra
Bióloga pela Universidade Cruzeiro do Sul
Instituição: Centro de Recuperação de Animais Silvestres do Parque Ecológico do Tietê
Endereço: Rodovia Parque, 8054 - Vila Santo Henrique, São Paulo – SP, Brasil
E-mail: l.sayurifitorra@yahoo.com.br

Bruno Simões Sergio Petri
Médico Veterinário pela Universidade Metodista de São Paulo
Instituição: Centro de Recuperação de Animais Silvestres do Parque Ecológico do Tietê
Endereço: Rodovia Parque, 8054 - Vila Santo Henrique, São Paulo – SP, Brasil
E-mail: brunopetri_mv@yahoo.com.br

Edson Mora dos Santos
Acadêmico de Ciências Biológicas pela Universidade Anhembi Morumbi
Instituição: Laboratório de Microscopia Eletrônica do Centro de Pesquisa de Sanidade Animal do Instituto Biológico de São Paulo, SP, Brasil
Endereço: Avenida Conselheiro Rodrigues Alves, 1252, Vila Mariana, São Paulo, SP, Brasil
E-mail: edson_moura01@outlook.com
ABSTRACT
Avianpox is a highly contagious disease that affects worldwide both commercial and wild birds. The etiologic agent is a poxvirus, belonging to the Poxviridae family and Avipoxvirus genus. The disease manifests itself in three ways, cutaneous, diphtheric and septicemic. In June 2007, during the illegal commercialization of birds, a Swallow tanager (Tersina viridis) was apprehended by the Forestry Police, among other birds, which was sent to the Tietê Ecological Park, São Paulo, SP, Brazil. After one month, the bird presented skin lesions on its legs, anorexia, emaciation, mobility difficulties, diarrhea, dehydration and death, and was sent to the Electron Microscopy Laboratory of the Biological Institute, São Paulo, SP, Brazil, to search for viral agents. Fragments of skin lesions and crusts were collected and processed for transmission electron microscopy using the techniques of negative staining (rapid preparation) and immunocytochemistry (immunolabelling with colloidal gold particles). Under the transmission electron microscope using the negative staining technique, a large number of typical poxvirus particles ovoid in shape, showing irregular arrangement of the tubules on the outer membrane, some enveloped, measuring an average of 240 nm in length x 200 nm in diameter, were seen in suspension of skin lesions or crusts. In the immunocytochemistry technique the antigen-antibody reaction was strongly enhanced by the dense particles of colloidal gold on poxviruses.

Keywords: Tersina viridis, Avianpox, Transmission electron microscopy

RESUMO
A bouba aviária é uma doença altamente contagiosa que afeta aves comerciais e de vida livre em todo o mundo. O agente etiológico é um poxvírus, pertencente à família Poxviridae e ao gênero Avipoxvirus. A doença se manifesta de três formas, cutânea, diftérica e septicêmica. Em junho de 2007, durante comercialização ilegal de aves, foi apreendida pela Polícia Florestal, entre outras aves, uma Sai-andorinha (Tersina viridis), que foi encaminhada ao Parque Ecológico do Tietê, São Paulo, SP, Brasil. Após um mês, a ave apresentou lesões cutâneas nas patas, anorexia, emaciação, dificuldade de locomoção, diarreia, desidratação e morte, e foi encaminhada ao Laboratório de Microscopia Eletrônica do Instituto Biológico, São Paulo, SP, Brasil, para pesquisa de agentes virais. Fragmentos de lesões de pele e crostas foram coletados e processados para microscopia eletrônica de transmissão utilizando as técnicas de contrastação negativa (preparação rápida) e de imunocitoquímica (imunomarcação com partículas de ouro coloidal). Ao microscópio eletrônico de transmissão utilizando a técnica de contrastação negativa, um grande número de partículas típicas de poxvírus, de forma ovóide, apresentando disposição irregular dos túbulos sobre a membrana externa, algumas envelopadas, medindo em média 240 nm de comprimento x 200 nm de diâmetro, foi observado na suspensão de lesões de pele ou de crostas. Na técnica de imunocitoquímica, a reação antígeno-anticorpo foi fortemente marcada pelas densas partículas de ouro coloidal, sobre os poxvírus.

Palavras-chave: Tersina viridis, Bouba aviária, Microscopia Eletrônica de Transmissão.
1 INTRODUCTION

Birds are among the animals, the most threatened by deforestation and habitat fragmentation resulting from human activities, especially those related to agricultural activities and urban expansion. Other relevant threats are fires and the capture of animals, whether for consumption or for illegal trade to serve as pets (Ibama 2008; Red Book of the Endangered Brazilian Fauna of Extinction volume III - birds 2018). Brazil ranks second in the world ranking of endangered bird species (Renctas 2008).

Due to the natural beauty of their song and plumage, they are undoubtedly the most sought after in this market, generating great profit for traffickers (Pereira & Brito 2005). This type of trade has already contributed to the extinction of some Brazilian avian species (Renctas 2001). The Swallow tanager (*Tersina viridis*) is a passerine bird of the family Traupidea, measures approximately 14 cm in length and weighs, on average, 30 grams. It has a marked sexual dimorphism, the male is bright light blue, with a black mask and throat and a white belly; the female and the immature are greenish, with a gray mask and a more yellowish tone in the lower parts. The plumage of the adult male is acquired only after 3 or 4 years. The iris of adult individuals, in both sexes, is red (Hoffling & Camargo 2002).

It is a migratory bird, preferably frugivorous that occurs in open areas, edges of forests and even cities in Central and South American countries (Sibley & Monroe 1990), frequently feeding on magnolia seeds (Sick 1997). According to a study by Borges et al. (2006) the Swallow tanager was listed among the species most apprehended by the Military Police of the Environment of Juiz de Fora, MG, Brazil.

Avianpox or smallpox (also known as contagious epithelioma, bladder, popcorn, avian diphtheria) is a highly contagious disease that affects worldwide both commercial and wild birds (Yoshikkawa & Alam 2002).

The etiologic agent is a poxvirus, double-stranded DNA, belonging to the *Chordopoxviridae* subfamily, *Poxviridae* family and *Avipoxvirus* genus (ICTV, 2020). They do not have a nuclecapsid and the outer membrane contains a central zone in the form of alteres and two lateral bodies. The irregular arrangement of the tubules on the external lipoprotein bilayer gives it a textured appearance (Fenner et al. 1992).

The disease manifests itself in three ways. The cutaneous is the most common and is characterized by proliferative lesions on the skin and regions devoid of feathers, such as legs, beaks and commissures of the eyes. In diphtheric, fibronecrotic lesions occur in the membranes of the upper respiratory tract and esophagus. Ruffled plumage, somnolence and anorexia characterize the septicemic form. In the diphtheric and septicemic form, a high mortality is observed and birds of all
ages are susceptible. Transmission occurs through sucking insect bites or through contact with contaminated food, water, secretions and fomites. Infection is most common during the summer and autumn months when mosquitoes are most prevalent (Ritchie et al. 1994).

Avianpox have been reported worldwide in several avian species, but in Brazilian birds it is rarely mentioned (Catroxo et al., 2009). Due to the absence of literary data on diseases that affect this avian species, the purpose of this study was to identify the presence of avipoxvirus in skin lesions of the Swallow tanager, using transmission electron microscopy techniques.

Figs. 1 and 2. Male and female Swallow tanager photograph. (https://pt.wikipedia.org/wiki/Tersina_viridis)

2 MATERIAL AND METHODS

In June 2007, during the illegal commercialization of birds, a Swallow tanager (*Tersina viridis*) was apprehended by the Forestry Police, among other birds, which was sent to the Tietê Ecological Park, São Paulo, SP, Brazil. After one month, the bird presented skin lesions on its legs, anorexia, emaciation, mobility difficulties, diarrhea, dehydration and death, and was sent to the Electron Microscopy Laboratory of the Biological Institute, São Paulo, SP, Brazil, to search for viral agents. Fragments of skin lesions and crusts were collected and processed for transmission electron microscopy using the techniques of negative staining (rapid preparation) and immunocytochemistry (immunolabelling with colloidal gold particles).

In the negative staining the scabs and fragments of skin lesions were suspended in phosphate buffer 0.1 M, pH 7.0. Drops of the obtained suspension were placed in contact with metallic copper grids with carbon stabilized supporting film of 0.5% collodium in amyl acetate. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate, pH 5.0 (Brenner & Horne, 1959).
At the immunolabeling technique with colloidal gold particles for negative staining, the copper grids were placed in contact with viral suspension and, after removing excess with filter paper, the same were put on specific primary antibody drops. After successive washings in PBS drops, the grids were incubated in protein A drops in association with 10 nm gold particles (secondary antibody). Grids were then contrasted at 2% ammonium molybdate, pH 5.0 (Knutton, 1995). Observations were made in a Philips EM 208 electron microscope, at 80 kV.

3 RESULTS AND DISCUSSION

In the present work, we verified that the animal presented proliferative and crusted lesions, located in the legs, characteristic of the nodular form of the avianpox, also observed by other authors, in other species (Smits et al. 2005, Catroxo et al., 2009, 2012; Ha et al., 2013). Others, however, report in different species, this type of lesion in the eyes, eyelids, dewlap, beak, ceroma, head, nostrils, neck, legs, wings and abdomen (Docherty et al. 1991; Reed & Schrader 1989; Raidal et al 1996, Deem et al. 1997; Kreuder et al. 1999; Saito et al., 2019; Gang et al. 2020; Le Net et al., 2020; Baek et al., 2020; Kurihara et al., 2020; Saker et al., 2020; 2021). Lesions on the tongue, characteristic of the diphtheric form, have been reported in canaries (Catania et al., 2017), falcons (Krone et al., 2004) and in pigeons (Sharma et al., 2019).

In our research, we observed that the clinical signs commonly observed by bird were lack of appetite, emaciation, locomotion difficulties, diarrhea, dehydration and death, also observed by other authors in cowled cardinal, white-throated seedeater, double-collared and bay-winged cowbird (Catroxo et al., 2009, 2012). Other clinical signs such as blindness, blepharitis, conjunctivitis, rhinitis, depression, anorexia, ruffling of the feathers, epistaxis, dyspnea, lethargy, hypothermia and pneumonia, have also been described (Nuttal et al. 1985, Raidal et al. 1996, Kreuder et al 1999; Krone et al. 2004, Medina et al. 2004, Greenacre 2005, Gulbahar et al. 2005, Pledger 2005; Catania et al., 2017).

Under the transmission electron microscope using the negative staining technique, a large number of typical poxvirus particles (fig. 3), ovoid in shape, showing irregular arrangement of the tubules on the outer membrane (fig. 3, big arrow), some enveloped (fig.3, minor arrow), measuring an average of 240 nm in length x 200 nm in diameter, were seen in suspension of skin lesions or crusts.

These morphological characteristics were also observed by this technique in fragments of skin lesions from Cowled cardinal, White-throated seedeater, Double-collared and Bay-winged cowbird (Catroxo et al., 2009, 2012), Canaries (Docherty et al. 1986; Catania et al., 2017),
Australian magpie (Sarker et al., 2020); Red-Flanked Blue Robin (Song et al., 2020), Nudlark (Sarker et al., 2021), sparrow and pigeon (Weli et al. 2004) and falcons (Krone et al., 2004).

In the immunocytochemistry technique in negative staining applied to the samples, the antigen-antibody reaction was clearly marked by the colloidal gold particles (fig. 4, arrow), strongly showing those of the poxvirus, confirming the findings of the negative staining technique.

Other researchers have also used this technique to highlight poxvirus particles in skin lesions and crusts of cowled cardinal, white-throated seedeater, double-collared and bay-winged cowbird (Catroxo et al., 2009, 2012).

The constant presence of mosquitoes observed during the summer and autumn months at the outbreak site probably contributed to the onset of the disease. Mites and other sucking insects have also been implicated in the spread (Ritchie 1995). In addition, the animal was subjected to various stress factors since it was victimized by the illegal trade. According to Greenacre (2005), stress can activate a latent infection.

Transmission electron microscopy techniques were essential for the detection of the viral etiologic agent. According to Fenner et al. (1992) transmission electron microscopy is the method of choice for accomplish the laboratory diagnosis of poxviruses, where viruses can be easily identified through lesion or crust samples (Nistche et al. 2006). The infection definitive diagnosis is made through morphological characterization of the virus (Silva et al., 2010).

Krone et al. (2004) reported that the application of the Elisa and propagation in cell culture techniques that they used, did not identify the avipoxviruses, however, virus particles were detected by electron microscopy in skin and tongue lesions.

Studies on this important disease must have real continuity aiming to protect both free-living birds and those that are on the list of IBAMA (Brazilian Institute of the Environment and Renewable Natural Resources) of species threatened with extinction, contributing to their effective preservation, but also to prevent the spread to other species of breeding and commercial farms, also avoiding unnecessary economic losses.
Fig. 3. Negative staining of poxvirus particles in skin suspension of the Swallow tanager, showing irregular tubules arrangement on the outer membrane (big arrow) and outer envelope (minor arrow). Bar: 140 nm.

Fig. 4. Immunocytochemistry technique. The antigen-antibody reaction was strongly enhanced by the colloidal gold particles on the virus (arrow). Bar: 160 nm.
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