Occurrence of Avian Pox Outbreaks in Wild and Canary Commercial Breedings. Diagnosis through Electron Transmission Microscopy and Histopathology Techniques

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Abstract— *Avian pox is one of the major viral diseases that affects canaries and due to its rapid spread, can decimate the entire breeding, causing great economic damages to breeders. Canaripoxvirus species belongs to the Poxviridae family and Avipoxvirus genus. Three main forms characterize the disease, the cutaneous, diphtheric and septicemic, but coryza and tumor forms can also occur. In the period from 2006 to 2021, outbreaks of avian pox occurred in canaries from commercial breeding and Ecological Park, in canaries victims of illegal trade in São Paulo, SP, Brazil. All birds had skin lesions in beaks and feets and sometimes in the eyes and tongue, in addition to anorexia, diarrhea, weight loss and death. Samples of skin lesion fragments, crusts and organs from all birds were processed by negative staining, immunoelectron microscopy and immunocytochemistry techniques. Skin lesions samples were also processed by the histopathology technique. Through the negative staining technique, a larger number of avipoxvirus particles was visualized in all samples of nodular lesions examined and in the sample of canary lung fragments from outbreak 7. Paramyxovirus particles were visualized in samples of liver, lung, heart, gizzard, intestine and proventriculus fragments of canaries 1 and 2 from outbreak 3. In the samples of lungs fragments, pleomorphic formations similar to mycoplasmas, were also visualized. In the samples of lung, heart, gizzard, liver, intestine and tongue fragments, from outbreak 5, the presence of mycoplasma particles was also observed. The presence of aggregates formed by the antigen-antibody interaction characterized the positive result for avipoxvirus obtained in the immunoelectron microscopy technique in all skin lesions samples. In the immunocytochemistry technique the antigen-antibody reaction was strongly enhanced by the dense particles of colloidal gold on avipoxviruses. In the histopathological examination many avipoxvirus inclusion bodies (Bollinger bodies), strongly eosinophilic ring-shaped, marked hyperkeratosis and epithelial hyperplasia were observed in feet skin of canaries. Areas with globular degeneration and monolymphocytic inflammatory infiltrate were also visualized.*

***Keywords*— *Avipoxvirus, Canary, Transmission electron microscopy, Histopathology*.**

I. INTRODUCTION

Canary breeding is a practice that has become increasingly profitable and widespread in Brazil and plays a fundamental role in preservation of species. The yellow color of the Belgian lineage is the most popular, but the search for new and different tonalities, size, genetic improvement, besides presentation in exhibitions and preservation, are the main objectives of the creators [1].

Avian pox is one of the major viral diseases that affects canaries and due to its rapid spread, can decimate the entire breeding, causing great economic damages to breeders [2]. *Canaripoxvirus* species belongs to the *Poxviridae* family and *Avipoxvirus* genus [3]. Transmission involves insects as vectors, direct contact with aerosols between infected and susceptible birds, ingestion of water, contaminated food and semen transmission [2, 4, 5]. Three main forms characterize the disease, the cutaneous, diphtheric and septicemic, but coryza and tumor forms can also occur [6]. Cutaneous or dry is the most common in passerines, being characterized by the formation of nodules or vesicles in regions devoid of feathers, such as around the eyes, paws, feet and beak and in some cases, papules in the peri and infra-orbital regions, nose, sinus and tongue causing dyspnea and dysphagia [6]. In this form, the lesions can lead to blindness when they occur around the eyes and obstruct the passage of food or air when they occur around the mouth and esophagus. On the paws and feet, they make perching difficult, often leading to the development of secondary bacterial infections [7]. In the diphtheric or wet form, most commonly seen in parrots, fibronecrotic lesions (white plaques) occur on the membranes of the oral cavity, tongue, pharynx and larynx. Birds may have dyspnea and asphyxia due to laryngeal obstruction. Parrots and macaws can be affected by diphtheric enteritis with myocardial necrosis [6]. Canaries are the species most affected by the septicemic form, evidenced by the absence of skin lesions and the presence of pulmonary lesions [5]. There is also loss of appetite, ruffled feathers, cyanosis and solitude. The disease progresses to desquamative pneumonia with capillary occlusion resulting in dyspnea and death within 3 days, with mortality ranging from 70 to 90% [8]. A fourth form, coryza, affects parrots, which begins with clear nasal discharge that progresses to fibrinous and mucous membranes, followed by conjunctivitis. The form of tumors, represented by skin nodules that evolve into tumors and adenomas, is more commonly observed in canaries [6]. An unusual form of poxvirus was described in a herd of canaries, affecting mainly young animals, whose signs were respiratory distress, loss of feathers and crusts on the head, neck and back, anorexia, weight loss, ruffled feathers and high mortality [9]. Avipoxvirus was also isolated from tongue for canaries, that showed severe localized proliferative glossitis [10]and an outbreak of systemic avian pox associated with B1 subgroup was related among canaries [11]. Other outbreak of canaripox was reported in breeder farms associated with co-infection by *Mycoplasma gallisepticum* [12]. In some birds, avipoxviruses may exist in a latent form [13,14] and may survive for months or even years in dry skin crusts [5]. More than one form can occur simultaneously in the same bird or in different birds from the same farm affected by the disease outbreak [6].

Secondary bacterial and fungal infections contribute to the worsening of the disease [15]. Nonspecific stressors may be associated with viral reactivation [6]. Birds with mild injuries can recover depending on their immunity, becoming carriers and spreading the virus [16].

In Brazil, there are few reports on the occurrence of the disease in wild birds, in captivity or commercial breeding.

An outbreak of the disease occurred in 2003, was reported in an Ecological Park that affected several species, such as cowled-cardinal (*Paroaria dominicana*), white-throated seedeater (*Sporophila caerulescens*) and double-collared seedeater (*Sporophila albogularis*), with high mortality [17], and another outbreak that occurred in 2012 in the same Ecological Park, infected about 45 species of bay-winged-cowbird (*Gnorimopsar chopi*) [18]. Other occurrences signaled the presence of the virus in psittacines [19, 20], in a common barn owl (*Tyto alba*) [21], in penguins (*Spheniscus magellanicus)* [22] and in teal (*Dendrocygna autumnalis*) [23].

In commercial poultry, smallpox is a notifiable disease, being included in list B, among the communicable diseases considered important from a socio-economic and/or sanitary point of view at the national level and whose repercussions on the international trade of animals and products of animal origin are considerable [24].

This research aimed to detect the presence of avian poxvirus during outbreaks occurred in commercially farmed canaries and in an Ecological Park in the State of São Paulo, SP, Brazil, using transmission electron microscopy and histopathology techniques.

II. MATERIAL AND METHODS

2.1 Descriptions of the outbreaks

Outbreak 1 – In the period from the 2006 to 2009, during illegal commercialization of the Brazilian birds, 96 Saffron finches were apprehended and being forwarded to the Tietê Ecological Park, São Paulo, SP, Brazil. Twenty-nine birds presented cutaneous lesions around the beak and legs (fig. 1, arrow). After presenting weight loss, prostration and sudden death the birds were sent to Biological Institute of São Paulo, SP, Brazil, to investigate viral agents.

Outbreak 2 – In March and July 2013, 3 canaries from properties located in Itu, SP and São Paulo, SP, Brazil were sent. The birds had lesions on their feet. No other information was obtained.

Outbreak 3 – In April 2014, 2 canaries from São Paulo, SP, Brazil, were sent for examination. The birds presented diarrhea, weight loss, prostration, lesions on the legs and beaks. During the necropsy, we observed that the organs were hemorrhagic and one of the canaries had a dilated proventriculus.

Outbreak 4 – In March 2017, an outbreak occurred in an intensive sport-type canary farm in the State of São Paulo, SP, Brazil, with 200 animals. Initially 50 to 100 birds died and 50 became ill. The birds had symptoms and clinical signs of progressive emaciation and death within a few days of evolution. A total of 12 canaries, 8 months old, were sent to Biological Institute of São Paulo, SP, Brazil, for research on viral agents. Necropsy showed that the proventriculus, intestines and liver were enlarged, in addition to the presence of the nodular lesions with blood on the legs, feet, beaks and around the eyes (fig. 2, arrow).

FIGURE 1: Canaries with feet lesions (arrows).

FIGURE 2: Canary with bloody lesion on the feet (arrow).

Outbreak 5 – During the month of June 2018, an outbreak occurred in an intensive farm with 50 Belgian canaries, in Taubaté, SP, Brazil. Suddenly 7 animals became ill and one of them died. Posteriorly, all the animals died. During the necropsy of an animal, we could observe that the liver was friable, enlarged and yellowish, the intestines and lungs were hemorrhagic and the ventriculus had a dark content. It was also observed the presence of severe lesions in the beak, eyes and paws of the bird.

Outbreak 6 – In the period of December 2018, 2 canaries from intensive exploration and sport breeding in São Paulo, SP, Brazil were sent. The number of animals on the farm and the number of sick animals were not reported. At necropsy, it was observed that the intestines and lungs were hemorrhagic and the presence of lesions on the paws.

Outbreak 7 – In November 2021, an outbreak occurred in an intensive sport-type canary breeding, located in Santo André, SP, Brazil, with 70 animals. At the time, 7 birds became ill and 3 died. Two puppies were sent for necropsy, but they arrived very altered and it was not possible to perform the necropsy. The birds had lesions on their legs and beaks. A pool of organs from each bird was made. In February 2022, new animals were affected and a canary was sent for necropsy. The bird had lesions on the tongue, paws and beak. During the necropsy it was observed that the organs were hemorrhagic and the spleen and intestines were dilated.

III. METHODS

Samples of fragments of nodular lesions and dry crusts and organ fragments from all birds were processed by the negative staining (rapid preparation) technique.

3.1 Negative staining technique (rapid preparation)

In the negative staining process, the fragments of nodular lesions, dry crusts and organ fragments, were suspended in phosphate buffer 0.1 M and pH 7.0 and placed in contact with metallic grids. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate [25; 26; 27].

Poxvirus positive samples were processed by immunoelecton microscopy and immunocytochemistry techniques.

3.2 Immunoelectron Microscopy Technique (IEM)

The copper grids were prepared as described above, sensitized with a primary virus-specific antibody primary and were washed with phosphate buffer 0.1 M and pH 7.0. Upon incubation with the viral suspension, grids were washed successively with distilled water and negatively contrasted with 2% ammonium molybdate under the same conditions [26;28].

3.3 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 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 [29].

IV. RESULTS

4.1 Negative Staining Technique (Rapid Preparation)

Through the negative staining technique (rapid preparation) a larger number of avipoxvirus particles was visualized in all samples of nodular lesions examined and in the sample of canary lung fragments from outbreak 7. The particles showed irregular distribution of the tubules on the outer membrane (fig. 3, arrow), some enveloped (fig. 4, arrow), measuring on average 350 nm in lenght x 250 nm in diameter. Enveloped, pleomorphic, characteristic paramyxovirus particles (fig. 8, arrow), measuring between 100 and 300 nm in diameter, were visualized in samples of liver, lung, heart, gizzard, intestine and proventriculus fragments of canaries 1 and 2 from outbreak 3. In the samples of lungs fragments, pleomorphic formations similar to mycoplasmas, measuring between 100 and 800 nm, were also visualized. In the samples of lung, heart, gizzard, liver, intestine and tongue fragments, from outbreak 5, the presence of mycoplasma particles was also observed.

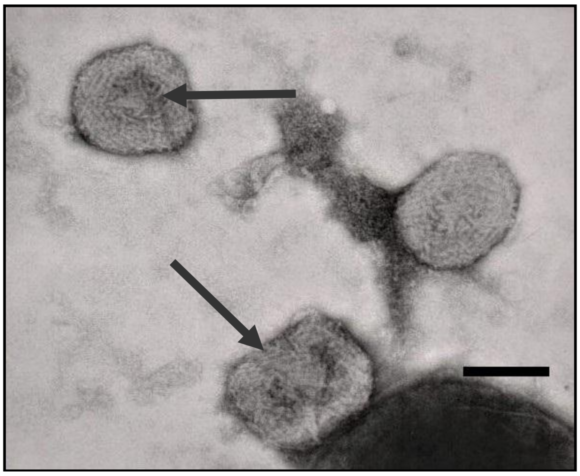


FIGURE 3: Negatively stained of avipoxvirus particles, ovoide, showing irregular distribution of the tubules on the outer membrane (arrows). Bar: 190 nm.

FIGURE 4: Negatively stained of avipoxvirus particle showing outer envelope (arrow). Bar: 270 nm.

4.2 Immunoelectron microscopy

The presence of aggregates formed by the antigen-antibody interaction characterized the positive result for avipoxvirus obtained in the immunoelectron microscopy technique in all skin lesions samples (fig. 5, arrow).

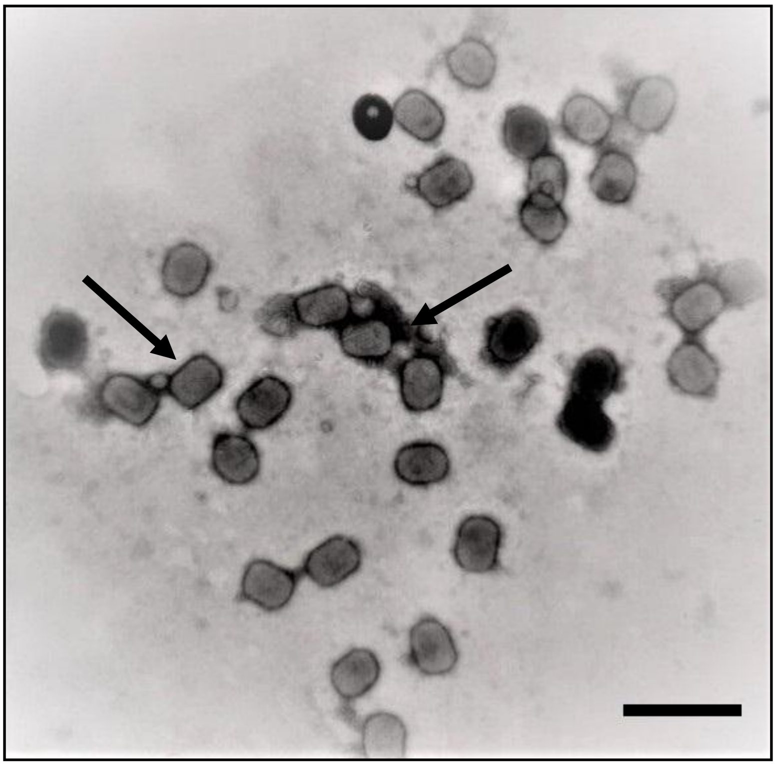
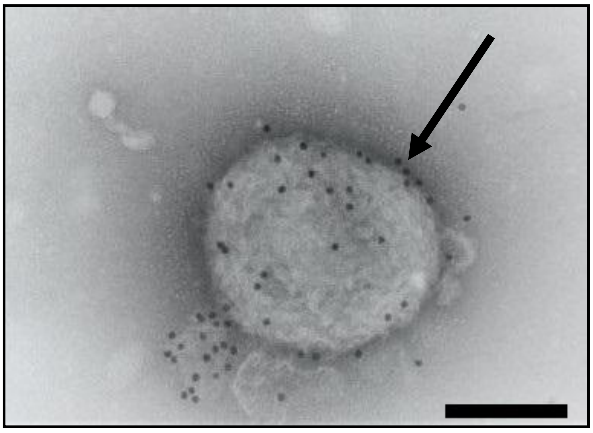
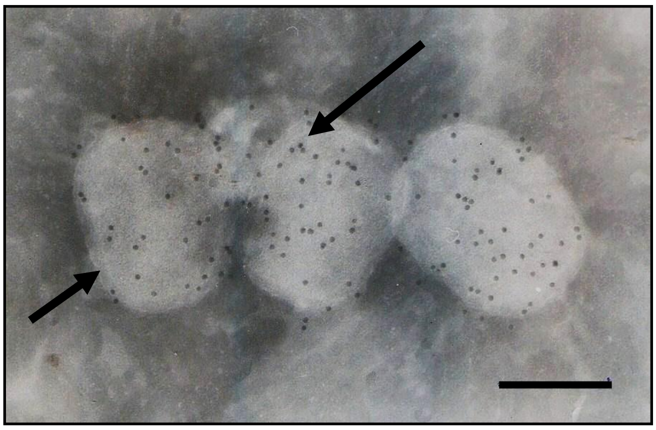


FIGURE 5: In the immunoelectron microscopy technique the avipoxvirus particles were aggregated by antigen-antibody interaction (arrows). Bar: 690 nm.

4.3 Immunocytochemistry technique

In the immunocytochemistry technique the antigen-antibody reaction was strongly enhanced by the dense particles of colloidal gold on avipoxviruses (figs. 6, 7, arrow).





FIGURES 6, 7: Antigen-antibody interaction strongly enhanced by the dense gold particles over the avipoxvirus (big arrow). Observe enveloped particles in fig. 7 (minor arrow). Bar. fig. 6: 160 nm; Bar. fig. 7: 200 nm.

4.4 Histopathology technique

In the histopathological examination many avipoxvirus inclusion bodies (Bollinger bodies), strongly eosinophilic ring-shaped (fig. 9), marked hyperkeratosis and epithelial hyperplasia (fig. 10) were observed in feet skin of canaries. Areas with globular degeneration and monolymphocytic inflammatory infiltrate were also visualized (fig. 11).

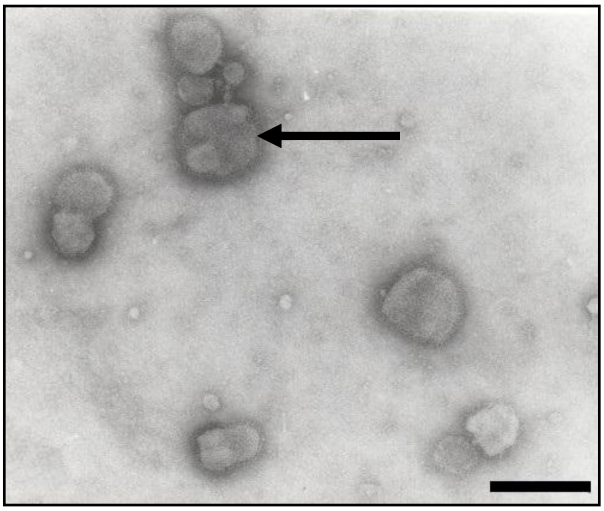


FIGURE 8: Negatively stained of enveloped, pleomorphic, characteristic paramyxovirus particles, measuring between 100 and 300 nm in diameter (arrow). Bar: 430 nm.

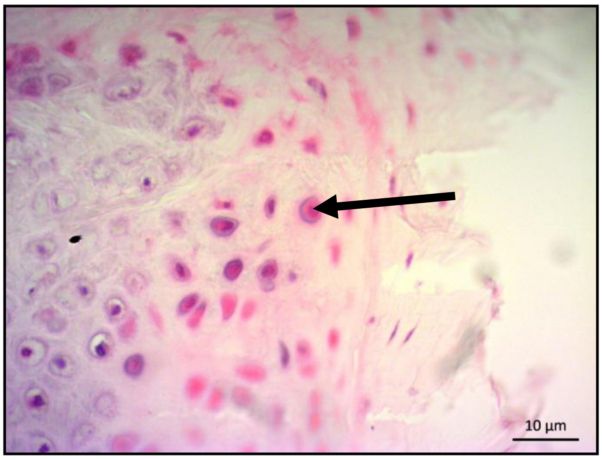


FIGURE 9: Photomicrograph of canary feet skin, showing many inclusion bodies (Bollinger bodies), ring-shaped and strongly eosinophilic (arrow). X630.

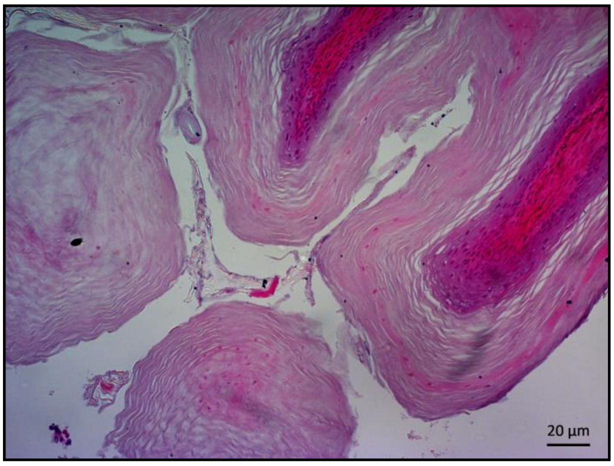


FIGURE 10: Photomicrograph of canary feet skin, showing marked hyperkeratosis and epithelial hyperplasia. X200

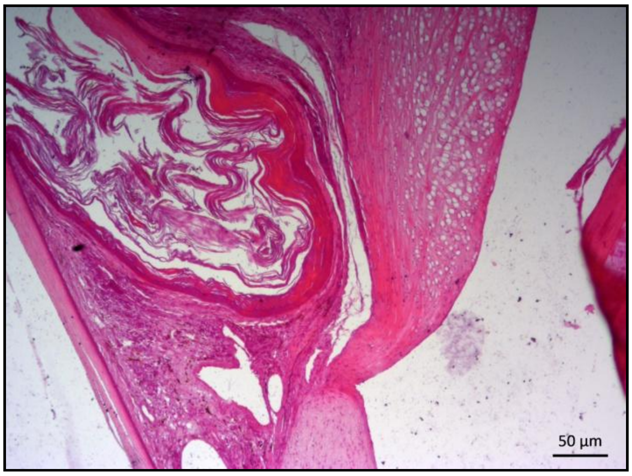


FIGURE 11: Photomicrograph of canary feet skin, where epithelial hyperplasia with areas of globular degeneration and monolymphocytic inflammatory infiltrate is observed. X100

V. DISCUSSION

In this work we report the occurrence of outbreaks of avian poxvirus in commercial canary farms and in an Ecological Park in the State of São Paulo, SP, Brazil. Similar outbreaks have been reported worldwide, causing unfortunate animal losses with consequent damage to breeders and nature.

Several forms of the disease affect canaries, the septicemic form with the absence of skin lesions and the presence of lung lesions [5], tumors [6; 7; 30] and an uncommon form, characterized by respiratory problems, associated with loss of feathers and crusts on the head, neck and back, with high mortality [9]. The cutaneous or nodular form, however, is the most prevalent among the creations in Brazil, leading to the death of most flock canaries.

All the canaries in our study had cutaneous lesions on the feet, beak and sometimes in the eyes, in agreement with the report of other authors, in canaries [9; 12; 30; 31; 32; 33] and in other avian species, such as, *Gnorimopsar chopi* [18], *Paroaria dominicana*, *Sporophila caerulescens* and *Sporophila albogularis* [17]; *Tersina viridis* [34]; *Tyto alba* [21]; hummingbirds [35]; *Parus major* [36]; *Gymnorhina tibicen* [37]; house sparrow [38]; pigeons [39]; *Bubo virginianus* [40]; *Dendrocygna autumnalis* [23]; *Corvus macrorhynchos* [41] and in Psittacines [19].

Two canaries in our study, from outbreaks 5 and 7, had pink to reddish lesions on the tongue. Catania et al. [10] also verified the presence of similar nodular formations on the tongue of the canaries in their study, however, they did not find any other type of lesion or sign of pneumonia. The canaries from outbreak 4 had lesions with blood in the feet. Sheron-Bochler et al. [42] described hemorrhagic lesions in the head of a Southern giant petrel infected by avipoxvirus, while lesions in the lungs and liver were described in a great horned owl by Echenique et al. [40]. Flamingos with nodular lesions on their beaks not showed other clinical signs [43].

Anorexia, diarrhea, weight loss and death were the clinical signs most commonly observed in the canaries in our study, also observed in canaries by other authors [9; 11; 12; 33]. The presence of diarrhea has been described in bay-winged cowbird, cowled -cardinal, white-throated seedeater, double-collared seedeater and swallow tanager [17; 18; 34]. Most of the canaries in our research had bleeding organs and dilated intestines; one animal showed an enlarged spleen. Reza et al. [30] reported the presence of an enlarged spleen and liver in the canaries of their study.

Mycoplasma particles were seen in the lungs of 2 canaries from outbreak 3 and in organ fragments from a canary from outbreak 5. Concurrent infection of Avipoxvirus with *Mycoplasma gallisepticum* was also reported in the air sacs of two canaries by Shaib & Barbour [12]. Co-infections or secondary infections in diseases initiated by viral agents may provide a more serious condition, leading to increases in the mortality rate [44]. In mixed infections, mycoplasma can affect the physical barriers of the upper and lower airways, causing changes in tracheal cilia and mucosal destruction, aggravating respiratory disease [12].

Paramyxovirus particles were also observed in the canaries organ fragments from outbreak 3. Avipox and PMV-1 infections were responsible for high mortality of houbara bustards from a rehabilitation center in Pakistan. The association of PMV-1 and diphtheric and septicemic avipox was observed mainly in houbara bustards victims of illegal trade [45]. Serotype 2, however, is the most common in passerines, causing weight loss, severe pneumonia and diarrhea [5,6]. Stress conditions in birds due to transport, temperature changes, and poor nutrition can favor higher mortality [11] making birds more susceptible to PMV-1 infection, in addition to activating latent avipoxvirus infections [5; 46].

The viral presence was confirmed by the application of transmission electron microscopy techniques. A large number of poxvirus particles showing irregular distribution of the tubules on the outer membrane measuring 350 x 250 nm and enveloped particles, were visualized in all lesions samples examined by negative staining technique. These viral morphological aspects were also observed by this technique in skin lesions of canaries [10; 32], Australian magpie [37], penguin [42], partridge [47], bay-winged cowbird, cowled-cardinal, white-throated seedeater, double-collared seedeater and swallow tanager [17; 18; 34].

In the immunoelectron microscopy technique, the poxvirus particles were aggregated forming an immune complex, result also confirmed by other authors who used this technique to agglutinate poxvirus particles in swinepox cases [48]; herpesvirus in non-human primates [49]; swine coronavirus [50]; paramyxovirus, retrovirus and coronavirus in owls [51].

The detection of the viral antibody was also performed by the application of the immunocytochemistry technique, which enhancedly marked the avipoxvirus particles by protein-A-gold. Other researchers have reported similar results in *Gnorimopsar chopi, Paroaria dominicana*, *Sporophila caerulescens*, *Sporophila albogularis* and *Tersina viridis* [17;18;34].

In the histopathological examination of the skin of canaries, the most relevant results, such as inclusion bodies (Bollinger bodies), hyperkeratosis, epithelial hyperplasia and monolymphocytic infiltrate, are in agreement with the findings of other authors in canaries [9; 11; 12; 30; 32; 33], in barn owl [21]; great horned owl [40]; duck [23]; penguin [22; 42], [47], jungle crow [41] and in turkeys [52].

Considering that there is no specific treatment available for the disease, in infected captive birds, biosecurity standards should be established for flocks, such as the practice of quarantine before the introduction of new birds into the aviary, isolation of birds when infected, elimination of vector mosquitoes by installing screens on windows and application of low toxicity insecticides [6]. Adequate vaccination of all birds helps to contain the spread of the disease [10]. The use of caustic pencil to cauterize the lesions, associated with an optimized diet with vitamin and mineral supplements and probiotics can be useful in the recovery process [53].

In the presence of outbreaks of viral diseases, transmission electron microscopy has been extremely useful, acting in the rapid detection of multiple agents [54; 55]. The application of the techniques was paramount for the diagnosis of avian pox and the immediate adoption of prophylactic measures, prevention and control of the disease in the canary breedings.

REFERENCES

[1] Hosken, F.M. & Silveira, A.C. Criação de Canários de Cor, Ed. Aprenda Fácil, Viçosa, MG, vol 1, 2001. 237p.

[2] van Riper III, C., van Riper, S.G. & Hansen, W.R. (2002). Epizootiology and effect of avian pox on Hawaiian forest birds. The Auk., 119(4):929-942.

[3] ICTV, Virus Taxonomy (2021). Available in: https://talk.ictvonline.org/taxonomy/

[4] Metz, A.L., Hatcher, L., Newman, A.J. & Halvorson, D.A. (1985). Venereal pox in breeder turkeys in Minnesota. Avian Diseases, 29(3):850-853.

[5] Ritchie, B.W. & Carter, K. Avian viruses: Function and control. Lake Worth. Florida, Ed. Publishing Incorporated, 1995. pp.285-311.

[6] Ritchie, B.W., Harrison, G.J. & Harrison, L R. Avian Medicine: Principles and application. Florida, Ed. Wingers Publishing Inc., 1994. pp.865-74.

[7] van Riper, C., III, & Forrester, D. J. Avian Pox. In: Infectious and parasitic diseases of wild birds. Eds. N. Thomas, B. Hunter & Atkinson, C. A. Ames, Iowa, Blackwell Publishing Professional, 2007. pp. 131-76.

[8] Tripathy, D.N., Schnitzlein, W.M., Morris, P.J., Janssen, D.L., Zuba, J.K., Massey, G. & Atkinson, C.T. (2000). Characterization of poxvirus from forest birds in Hawaii. Journal of Wildlife Disease, 36:225–230.

[9] H. L. Shivaprasad, T. Kim, D. Tripathy, Woolcock, P.R. & Uzal, F. (2009). Unusual pathology of canary poxvirus infection associated with high mortality in young and adult breeder canaries (*Serinus canaria*). Avian Pathology, 38(4):311-316.

[10] Catania, S., Carnaccini, S., Mainenti, M., Moronato, M.L., Gobbo, F. & Calogero, T. (2017). Isolation of Avipoxvirus from tongue of canaries (*s*) show severe localized proliferative glossitis. Avian Diseases, 61(4):531-535.

[11] Ávila-Reyes, V. A., Díaz-Morales, V., Chávez-Maya, F., García-Espinosa, G., & Sánchez-Godoy, F. D. (2019). Outbreak of systemic avian pox in canaries (*Serinus canaria domestica*) associated with the B1 subgroup of avian pox viruses. Avian Diseases, 63(3):525-530.

[12] Shaib, H. & Barbour, E. (2018). Characterization of a canarypox virus from an outbreak among canaries (*Serinus canaria domesticus)* in Lebanon. J. Appl. Anim. Res., 46(1):932-937.

[13] Deern, S.L., Heard, D.J. & Fox, J.H. Avian pox in eastern screech owls and barred owls from Florida. Journal of Wildlife Diseases, 33(2):323-327, 1997.

[14] Ciganovich, E. A. In Field Manual of Wildlife Diseases: General Field Procedures and Diseases of Birds. United States Geological Survey, Washington, DC. 1999, pp. 163-170.

[15] McDonald, S. E., Lowenstine, L. J. & Ardans, A. A. (1981). Avian pox in blue-fronted Amazon parrots. Journal of American. Veterinary Medical Association, 179(11):1218-1222.

[16] Cubas, Z.S. & Godoy, S.N. Algumas doenças de aves ornamentais. 2004. Disponible in:
http://canarilalmada.com/download/download/Dossierdedoencas.pdf Acesso em: 04/04/2022.

[17] Catroxo M.H.B., Pongiluppi T., Melo N.A., Milanelo L., Petrella S., Martins A.M.C.P.F. & Rebouças M.M. (2009). Identification of poxvirus under transmission electron microscopy during outbreak period in wild birds, in São Paulo, Brazil. International Journal of Morphology 27(2):577-585.

[18] Catroxo, M.H.B., Martins, A.M.C.FR.P.F., Petrella, S. & Milanelo L. Detection of poxvirus using transmission electron microscopy techniques during outbreak in Bay-Winged Cowbird (*Gnorimopsar chopi*). In: Perez-Marin, C.C. A Bird's-Eye View of Veterinary Medicine. Rijeka, Croatia, InTechOpen, chapter 25. 2012, pp. 557-566.

[19] Esteves, F.C.B., Marín, S.Y., Resende, M., Silva, A.S.G., Coelho, H.L.G., Barbosa, M.B., D'Aparecida, N.S., Resende, J.S., Torres, A.C.D., Martins, N.R.S. (2017). Avian pox in native captive psittacines, Brazil, 2015. Emerging Infectious Diseases, 23(1):154-156.

[20] Murer, L., Westenhofen, M., Kommers, G.D., Furian, T.Q., Borges, K.A., Kunert-Filho, H.C., Streck, A.F. & Lovato, M. (2018). Identification and phylogenetic analysis of clade C *Avipoxvirus* in a fowlpox outbreak in exotic psittacines in southern Brazil. Journal of Veterinary Diagnostic Investigation, 30(6):946-950.

[21] Vargas, G.D., Albano, A.P., Fischer, G., Hübner, S., Sallis, S.E., Nunes, C.F., Raffi, M.B. & Soares, M.P. (2011). Avian pox virus infection in a common barn owl (*Tyto alba*) in southern Brazil. Pesquisa Veerinária Brasileira, 31(7):620-622.

[22] Niemeyer, C., Favero, C.M., Kolesnikovas, C.K.M., Bhering, R.C.C., Brandão, P. & Catão-Dias, J.L. (2013). Two different avipoxviruses associated with pox disease in Magellanic penguins (*Spheniscus magellanicus*) along the Brazilian coast. Avian Pathology, 42:6, 546-551.

[23] Pereira, W.L.A., Gabriel, A.L.M., Monger, S.G.B., Moraes, L.A., Queiroz, D.K.S. & Souza, A.J.S. (2014). Lesões cutâneas tipo tumorais associadas à infeção por avipoxvirus em uma marreca-cabocla (*Dendrocygna autumnalis*). Ciência Animal Brasileira, 15(2):234-238.

[24] OIE, Terrestrial Manual – Fowlpox, chapter 3.3.10, Paris, France. p.906-914, 2018.

[25] Brenner, S. & Horne, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. Biochimica et Biophysica Acta, 34:103-10.

[26] Hayat, M. A. & Miller, S. E. Negative Staining. McGraw-Hill Publ. Company, 1990. p.235.

[27] Madeley, C. R. (1997). Origins of electron microscopy and virus diagnosis. Journal of Clinical Pathology, 50(6):454-456.

[28] Katz, D. & Kohn, A. (1984). Immunosorbent electron microscopy for detection of viruses. Advances in virus Research, 29:169-194.

[29] Knutton, S. (1995). Electron microscopical methods in adhesion. Methods in Enzymology, 253:145-158.

[30] Reza, K., Nasrin, A. & Mahmoud, S. (2013). Clinical and pathological findings of concurrent poxvirus lesions and aspergillosis infection in canaries. Asian Pacific Journal of Tropical Biomedicine, 3(3):182-185.

[31] Donnelly, T.M. & Crane, L.A. (1984). An Epornitic of Avian Pox in a Research. Avian Diseases, 28(2):517-525.

[32] Johnson, B.J. & Castro, A.E. (1986). Canary pox causing high mortality in an aviary. Journal of AmericanVeteterinary Medicine Association,189:1345–1347.

[33] Zarifi, F., Nakhaei, P., Nourani, H., Mirshokraei, P. & Razmyar, J. (2019). Characterization of Iranian canarypox and pigeonpox virus strains. Archives of Virology, 164:2049–2059.

[34] Catroxo, M.H.B., Martins, A.M.C.R.P.F., Milanelo, L., Sayuri Fitorra, L.S., Petri, B.S.S. & Santos, E.M. (2022). Detection of avipoxvirus in a cutaneous lesion of a swallow tanager (*Tersina viridis*) by transmission electron microscopy. Brazilian Journal of Animal and Environment Research, 5(1):1311-1320, 2022.

[35] Baek, H.E., Bandivadeka, R.R., Pandit, P., Mah, M.; Sehgal, R.N.M. & Tell, L.A. (2020). TaqMan quantitative real-time PCR for detecting *Avipoxvirus* DNA in various samples types from hummingbirds. PLoS ONE, 15(6): e0230701., 1-17.

[36] Kozdruń, W., Styś-Fijoł, N., Czekaj, H., Indykiewicz, P., Sandecki, R. & Niczyporuk, J.S. (2018). Avian poxvirus infection in Polish great tits (*Parus major*). Journal of Veterinary and Research, 62, 427-430.

[37] Sarker, S.; Batinovic, S.; Talukder, S.; Das, S.; Park, F.; Petrovski, S.; Forwood, J.K.; Helbig, K.J. & Raidal, S.R. (2020). Molecular characterisation of a novel pathogenic avipoxvirus from the Australian magpie (*Gymnorhina tibicen*). Virology, 540:1-16.

[38] Ruiz-Martinez, J., Ferraguti, M., Figuerola, J., Martinez-de la Puente, J., Williams, R.A.J., Herrera-Dueñas, A., Aguirre, J.A., Soriguer, R., Escudero, C., Moens, M.A.J., Perez-Tris, J. & Benítez, L. (2016). Prevalence and genetic diversity of Avipoxvirus in house sparrows in Spain. PLoS ONE, 11(12): e0168690. 1-13.

[39] Abd El Hafez, M.S., Shosha, E.A.E-M. & Ibrahim, S.M. (2021). Isolation and molecular detection of pigeon pox virus in Assiut and New Valley governorates. Journal of Virological Methods, 293:114142, 1-9.

[40] Echenique, J.V.Z., Bandarra, P.M., Brauner, R.K., Soares, M.P., Coimbra, M.A.A. & Schild, A.L. (2016). Infecção por poxvirus e *Aspergillus fumigatus* em *Bubo virginianus* (Coruja jacurutu). Pesquisa Veterinária Brasileira, 36(7):630-633.

[41] Joshi, S., Mudasir, M., Sharma, D. & Singh, R. (2012). Histopathological study of cutaneous form of Avipoxvirus infection in Jungle crow (*Corvus macrorhynchos*). Veterinary World, 5(10):628-630.

[42] Shearn-Bochsler, V., Green, D.E., Converse, K.A., Docherty, D.E., Thiel, T., Geisz, H.N., Fraser, W.R. & Patterson-Fraser, D.L. (2008). Cutaneous and diphtheritic avian poxvirus infection in a nestling Southern Giant Petrel (*Macronectes giganteus*) from Antarctica. Polar Biology, 31:569–573.

[43] Terasaki, T, Kaneko, M. & Mase, M. (2010). Avian poxvirus in flamingos (*Phoenicopterus roseus*) in a Zoo in Japan. Avian Diseases, 54:955-957.

[44] Couto, R.M., Braga, J.F.V., Gomes, S.Y.M., Resende, M., Martins, N.R.S. & Ecco, R. (2016). Natural concurrent infections associated with infectious laryngotracheitis in layer chickens. Journal of Applied Poultry Research, 25:113–128.

[45] Bailey, T.A., Silvanose, C., Manvell, R., Gough, R.E., Kinne, J., Combreau, O. & Launay, F. (2002). Medical dilemmas associated with rehabilitating confiscated houbara bustards *(Chlamydotis undulata macqueenii)* after avian pox and paramyxovirus type 1 infection. Journal of Wildlife Disease, 38(3):518-32.

[46] Mohamed, M.A., & Hanson, R.P. (1980). Effect of social stress on Newcastle disease virus infection. Avian Diseases, 24: 908–915.

[47] Brower, A.I., Cigel, F., Radi, C. & Toohey-Kurth, K. (2010). Beak necrosis in Hungarian partridges (*Perdix perdix*) associated with beak-bits and avian poxvirus infection. Avian Pathology, 39(3):223-225.

[48] Bersano, J.G., Catroxo, M.H.B., Villalobos, E.M.C., Leme, M.C.M., Martins, A.M.C.R.P.F., Peixoto, Z.M.P., Portugal, M.A.S.C., Monteiro, R.M., Ogata, R.A. & Curi, N.A. (2003). Swine variola: study on the occurrence of the outbreaks in São Paulo and Tocantins States, Brazil. Arquivos do Instituto Biológico, 70(3):269-278.

[49] Catroxo, M.H.B., Martins, A.M.C.R.P.F., Lara, M.C.C.S.H., Villalobos, E.M.C. & Milanelo, L. (2020). Outbreak of encephalitis caused by human herpesvirus type 1 (HSV-1) in non-human captive primates in São Paulo, SP, Brazil. Clinical Microbiology and Research, 3(1):2-7.

[50] Martins, A.M.C.R.P.F., Bersano, J.G., Ogata, R., Amante, G., Nastari, B.D.B. & Catroxo, M.H.B. (2013). Diagnosis to detect porcine transmissible gastroenteritis virus (TGEV) by optical and transmission electron microscopy techniques. International Journal of Morphology, 31(2):706-715.

[51] Catroxo, M.H.B., Taniguchi, D.L., Melo, N.A., Milanelo, L., Alves, M., Martins, A.M.C.R.P.F. & Rebouças, M.M. (2010). Viral research in Brazilian owls (*Tyto alba* and *Rhinoptynx clamator*) by transmission electron microscopy. Journal of Morphology, 28(2):627-636.

[52] Ferreira, B.C., Ecco, R., Couto, R.M., Coelho, H.E., Rossi, D.A., Beletti, M.E. & Silva, P.L. (2018). Outbreak of cutaneous form of avian poxvirus disease in previously pox-vaccinated commercial turkeys. Pesquisa Veterinária Brasileira, 38(3):417-424.

[53] Les Stocker. (2013). Treatment of Avian Pox. Corvid Isle. Meeting place for people interested in corvids. https://corvid-isle.co.uk/treatment-of-avian-pox

[54] Richert-Pöggeler, K.R., Franzke, K., Hipp, K. & Kleespies, R.G. (2019). Electron Microscopy methods for virus diagnosis and high resolution analysis of viruses. Frontiers in Microbiology, 9(3255):1-8.

[55] Roingeard, P., Raynal, P-I., Eymieux, S. & Blanchard, E. (2019). Virus detection by transmission electron microscopy: Still useful for diagnosis and a plus for biosafety. Reviews in Medical Virology, 29:e2019., 1-9.