

Ultrastructural Identification of Circovirus in the Liver of Saffron Finch (*Sicalis flaveola spp.*)

Identificación Ultraestructural de circovirus en el
Hígado de Canarios Venezolanos (*Sicalis flaveola spp.*)

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SUMMARY: Circovirus are viral agents that cause disease in avian species. The main clinical symptoms of the disease are immunosuppression and, in young birds feather disorders. In neonates, the disease is known as "black spot" and characterized by abdominal enlargement, hepatobiliar congestion and failure to thrive. Also, it was observed in adult infected birds with other symptoms and clinical signs, such as enteritis, sinusitis, rhinitis, tracheitis, bronchopneumonia, myocarditis, nephritis, splenitis, dyspnea, anorexia, depression leading to high mortality. In April 2008, 317 saffron finch were apprehended during an illegal commercialization and were forwarded to the Wild Animals Recovery Center of the Tiete Ecological Park. Subsequently, 101 (31.66%) died and 20 of these were selected and sent to the Laboratory of Electron Microscopy, Biological Institute of São Paulo, Brazil to investigate possible etiological agents. After necropsy of these animals fragments of lung, liver and small intestine were processed using negative staining and resin embedding techniques. On the transmission electron microscopy, the negative staining technique allowed observation in 20 (100%) samples of liver suspension, a great number of particles with morphology similar to the circovirus, spherical, non-enveloped, isometric, characterized as "complete" and "empty", measuring between 17 and 20 nm in diameter. By the resin embedding technique, oval or rounded viral intracytoplasmatic inclusion bodies, containing viral particles disposed in paracrystalline arrays or loose arrangements were observed in ultrathin sections on the 20 (100%) samples of liver suspension. This is the first report on the presence of circovirus in saffron finch (*Sicalis flaveola spp.*).

KEY WORDS: Avian circovirus; Canaries; *Sicalis flaveola spp.*

INTRODUCTION

Circovirus are viral agents that cause disease in avian (Raue *et al.*, 2005) and swine species (Allan & Ellis, 2000). In avian species the main clinical symptoms are immunosuppression and, in young birds feather disorders (Raue *et al.*).

Several other symptoms, caused by immunosuppression and triggered by secondary bacterial, fungal or chlamydial infections such as enteritis, sinusitis, rhinitis, tracheitis, bronchopneumonia, myocarditis, nephritis, splenitis, dyspnea, anorexia, with a high mortality rate (Goldsmith, 1995; Rampin *et al.*, 2006) have been verified in canaries.

Circovirus in neonates has been long described in European industry of canaries, as the "black spot" and it is

characterized by abdominal enlargement, hepatobiliar congestion and failure to thrive (Goldsmith; Rampini *et al.*). Also, necrosis of epithelial tissue and oral mucosa (Phenix *et al.*, 2001) may occur. Most newborns die within seven days, and the percentage reaches 100% morbidity and 90% of mortality (Woods & Latimer, 2000).

The transmission of this disease is referred to as oral, intranasal and intra-cloacal routes (Ritchie *et al.*, 1991).

The Circoviridae family comprises two genera Gyrovirus and Circovirus. In the latter, the genome is organized in an ambisense direction. The replication-associated (Rep) protein is encoded from the virus-sense strand (ORF-V1) and the capsid protein from the

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complementary sense-strand (ORF-C1) of the genomes of all viruses of the genus Circovirus. Additional small open reading frames (ORFs) are present in some of the circoviruses (Niagro *et al.*, 1998; Johnne *et al.*, 2006). The DNA viral genome is circular single-stranded of 2 kb in size (Todd *et al.*, 2005).

When visualized in preparations on the electron microscope, circovirus present as small, non enveloped, round to icosahedral particles, with a diameter of 15-17 nm (Niagro *et al.*; Todd *et al.*, 1991).

To date, literature reports the existence of 11 species of circovirus, such as the *Beak and feather disease virus* of parrots (Bassami *et al.*, 1998; Sanada *et al.*, 1999; Kiatipattanasakul-Banlunara *et al.*, 2002), *Pigeon circovirus* (Todd *et al.*, 2001), *Canary circovirus* (Phenix *et al.*), *Goose circovirus* (Todd *et al.*, 2001), *Duck circovirus* (Soike *et al.*, 2004), *Finch circovirus* and *Gull circovirus* (Smyth *et al.* 2006, Todd *et al.*, 2007), *Starling circovirus* (Johnne *et al.*) and the *Swan circovirus* (Halami *et al.*, 2008). Also, there are two species in swine, the *Porcine circovirus-1*, not associated to any type of pathology and the *Porcine circovirus-2* that causes multisystemic wasting syndrome (Allan & Ellis).

The circovirus was also identified in Australian raves (Stewart *et al.*, 2006) and in ostrich (Els & Josling, 1998).

In canaries from aviaries with a history of high mortality, the circovirus was detected by transmission electron microscopy in organs from poultry farms (Guercio *et al.*, 2000, Todd *et al.*, 2001; Rampin *et al.*).

Cytoplasmic inclusion bodies can be observed in ultrathin sections of small intestine, skin and bursa of Fabricius (Rampin *et al.*; Kiatipattanasakul-Banlunara *et al.*; Todd *et al.*, 2007).

The present study describes the occurrence of circovirus in liver fragments of Saffron finch (*Sicalis flaveola* spp.), using transmission electron microscopy techniques.

MATERIAL AND METHOD

Samples. In April 2008, during an illegal commercialization of wild birds 317 Saffron finch (*Sicalis flaveola* spp) were apprehended and sent to the Center of Recovery of Wild Animals of the Tietê Ecological Park. Of these, 101 (31.66%) had sudden apathy, ruffled feathers and died. Twenty of these animals were selected and sent to the Laboratory of Electron

Microscopy, Biological Institute of São Paulo, where the necropsy was performed by collecting fragments of lung, liver and small intestine which were processed for transmission electron microscopy, using negative staining (rapid preparation) and resin embedding techniques to search for the etiologic agent.

Negative Staining Technique. In this technique, samples were suspended in 0.1 M phosphate buffer, pH 7.0, placed in contact with metallic copper grids, previously covered with carbon and collodion film, drained with filter paper and negatively stained at 2% with ammonium molybdate, pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997).

Resin Embedding Technique. The samples were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, post fixed in 1% osmium tetroxide in the same buffer, dehydrated in cetic series and embedded in Spurr resin (González-Santander, 1969; Luft, 1961). Ultrathin sections 70 nm thick were positive stained by the combination of 2% uranyl acetate (Watson, 1958) and 0.5% lead citrate (Reynolds, 1963).

All samples were examined and photographed on a transmission electron microscope Philips EM 208, voltage 80 kV.

RESULTS

On the transmission electron microscopy, by the negative staining technique, a large number of particles with morphology similar to the circovirus spherical, non-enveloped, isometric, characterized as "complete" and "empty", measuring between 17 and 20 nm in diameter, were visualized in suspensions of liver fragments of the 20 (100%) examined birds (Fig. 1).

Using the resin embedding technique (positive staining) electron-dense viral intracytoplasmic inclusions bodies, rounded or oval, were observed mostly located close to or associated with cell nucleus, measuring 200 to 700 nm in diameter (fig. 2). They had viral particles from 10 to 14 nm in diameter (Fig. 3, big arrow) in loose arrangements or in paracrystalline arrays (Fig. 3, minor arrow).

In suspensions of liver fragments, no other pathogen besides circovirus was observed.

When fragments of lungs and intestines were analyzed, the presence of circovirus or other agents was not observed.

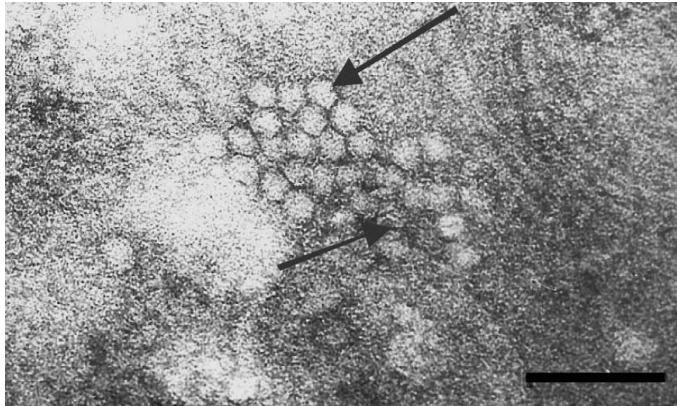


Fig. 1. Negatively stained circovirus particles, non-enveloped, spherical, isometric, characterized as “complete” (big arrow) and “empty”. (minor arrow). Bar: 110nm.

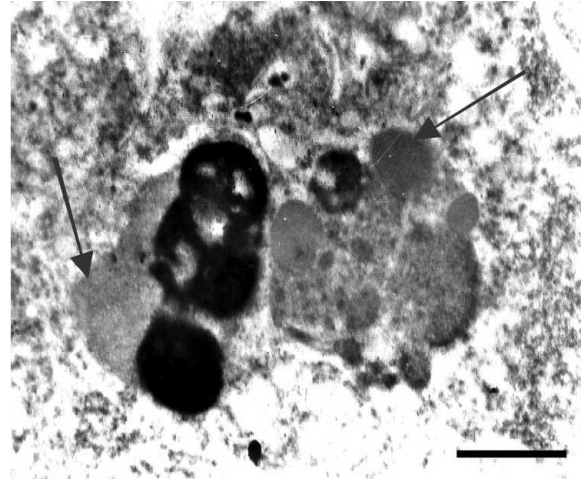


Fig. 2. Ultrathin section of the fragments of liver. Intracytoplasmic viral inclusion bodies (arrow). Bar: 800nm.

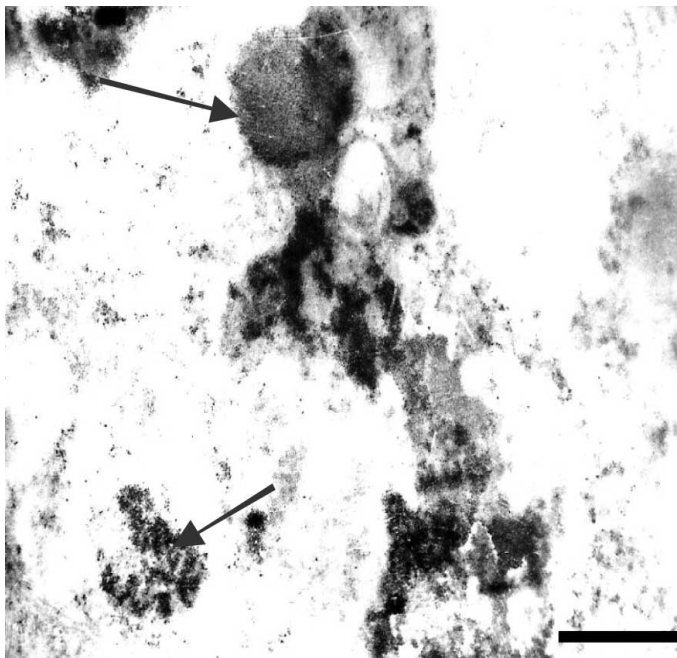


Fig. 3. Ultrathin section of the fragments of liver. Intracytoplasmic viral inclusion bodies, containing circovirus-like particles either in paracrystalline arrays or loose arrangements. Bar: 800nm.

DISCUSSION

In this study, the observation through the negative staining technique of viral particles with characteristic of circovirus, in suspensions of liver fragments of 20 (100%) *Sicalis flaveola* spp is in accordance with other reports about canaries showing spherical, non-enveloped particles with a mean diameter of 18 nm (Guercio *et al.*; Todd, 2000; Todd *et al.*, 2001; Phenix *et al.*).

Similar viral morphology was also described in other species such as geese (Soike *et al.*, 1999), pigeons (Woods *et al.*, 1993; Gough & Drury, 1996; Raidal & Riddoch, 1997), ostriches (Els & Josling) and cockatoos (Sanada *et al.*).

When using resin embedding technique, the analysis of ultrathin sections of liver fragments of 20 (100%) canaries showed the presence of viral intracytoplasmic inclusions, containing viral particles 10-14 nm of diameter with loose arrangements or paracrystalline arrays.

Other ultrastructural studies showed the presence of circovirus in the small intestine of canaries (Rampin *et al.*), bursa of Fabricius of pigeons (Woods *et al.*, 1993; Shivaprasad *et al.*, 1994, 2004), geese (Soike *et al.*, 1999) gull (Twentyman *et al.*, 1999) and in the skin of Psittacines (Latimer *et al.*, 1991; Sanada *et al.*; Kiatipattanasakul-Banlunara *et al.*).

In the analyzed literature there are no ultrastructural citations on the presence of this virus in the liver of canaries.

The circovirus predilection for certain organs in avian species is not well established (Todd *et al.*, 2001). Histopathology tests had shown intracytoplasmic and intranuclear inclusion bodies in skin lesions and digestive tract (Latimer *et al.*; Todd, 2000).

Some authors suggest that the immunosuppressive action of the virus is commonly associated with pathological changes in lymphoid organs, mainly

bursa of Fabricius and spleen being responsible for potentially fatal disease (Todd, 2000; Phenix *et al.*).

In this study, 101 (31.66%) out of 317 Saffron finch found dead in the Wild Animal Recovery Center of the Tietê Ecological Park or showing apathy, ruffled feathers and sudden death without any other apparent symptoms or classic sign reported in the literature, such as enteritis, sinusitis, rhinitis, tracheitis, bronchopneumonia, myocarditis, nephritis, splenitis, dyspnea, anorexia, lethargy, depression, dullness, feather disorder (Phenix *et al.*; Rampin *et al.*)

There are no records of occurrence of circovirus in commercial breeds of chickens or turkeys, but these viruses

have already been found in ducks, geese and canaries from commercial breeds. Moreover, little is known about the clinical pathologic and economic impact of the disease (Hatterman *et al.*, 2003; Soike *et al.*, 1999, 2004).

The transmission electron microscopy, by means of the negative staining and resin embedding techniques, is an important instrument to detect circovirus particles (Woods & Latimer). The definite diagnosis of this infection requires observation of characteristic particles (Latimer *et al.*; Ritchie & Latimer, 1995).

This is the first report on the presence of circovirus in Saffron finch (*Sicalis flaveola* spp.).

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RESUMEN: Circovirus son agentes virales que causan enfermedad en las aves. Los principales síntomas clínicos de la enfermedad son la inmunosupresión y desórdenes en las plumas de aves jóvenes. En los recién nacidos la enfermedad se conoce como "punto negro" y se caracteriza por distensión abdominal, congestión hepatobiliar y falla en el desarrollo. También se han observado en las aves adultas infectadas, otros síntomas y signos clínicos, tales como la enteritis, sinusitis, rinitis, traqueitis, bronconeumonía, miocarditis, nefritis, esplenitis, disnea, anorexia, depresión llevando también a alta mortalidad. En abril de 2008, durante una comercialización ilegal, fueron confiscados 317 canarios venezolanos, siendo llevados al Centro de Rehabilitación de Animales Silvestres del Parque Ecológico de Tietê. Posteriormente, 101 (31,66%) aves murieron y entre éstas fueron seleccionadas 20 y enviadas al Laboratorio de Microscopía Electrónica, Instituto Biológico de São Paulo, para identificar el posible agente etiológico. Después de la necropsia, fragmentos de pulmón, hígado e intestino delgado se procesaron mediante las técnicas de tinción negativa y de inclusión en resina. A través de microscopía electrónica de transmisión, la técnica de tinción negativa permitió visualizar en las 20 muestras (100%) de suspensión del hígado, un gran número de partículas con morfología similar al circovirus, esférico, sin envoltura, isométrico, que se caracteriza como "completa" y "vacío", que miden entre 17 y 20 nm de diámetro. Mediante la técnica de cuerpos de inclusión en resina se observó en el 100% de las muestras de suspensión de hígado, cuerpos de inclusión virales intracitoplasmáticos, redondeadas u ovals, con partículas de virus dispuestos en matrices paracristalinas o sueltas en los cortes ultrafinos. Este es el primer reporte de la presencia de circovirus en canarios Venezolanos (*Sicalis flaveola* spp.).

PALABRAS CLAVE: Circovirus de Aves; Canarios; *Sicalis flaveola* spp.

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