Research of Viral Agent in Free-living Pigeon Feces (*Columba livia*) in the City of São Paulo, SP, Brazil, for Transmission Electron Microscopy

Investigación de Agentes Virales en Heces de Palomas de Vida Libre (*Columba livia*) en la Ciudad de São Paulo, SP, Brasil, Através de Microscopía Electrónica de Transmisión

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SUMMARY: The pigeon feces are vehicle of diseases both for humans and other animal species. In these birds, the most important viral diseases of the digestive tract are transmitted by the paramyxovirus, adenovirus and coronavirus. Avian paramyxoviruses have been isolated from a variety of species of free living and domestic birds worldwide, with several symptoms and clinical signs and economic losses. Paramyxoviruses belong to the *Paramyxoviridae* family and *Avulovirus* genus that includes nine serotypes (APMV 1 to 9). Avian adenoviruses belong to the *Adenoviridae* family and *Aviadenovirus* genus. In pigeons, cause classical adenovirus and necrotizing hepatitis. The respiratory and enteric tracts are common targets of coronavirus. They belong to the *Coronaviridae* family and to 3a and 3c groups. In this study, we described the presence of viral agents in free-living pigeon feces (*Columba livia*) from the city of São Paulo, Brazil. The feces were processed by negative staining technique (rapid preparation) for transmission electron microscopy. In this technique paramyxoviruses particles, pleomorphic, roughly spherical or filamentous, measuring 100 to 500 nm of diameter containing an envelope covered by spikes, with characteristic helical herring-bone-like nucleocapsid, measuring 15 to 20 nm in diameter, were visualized in 45 (79%) out of 57 feces samples. In 2 (3.5%) samples, paramyxovirus and adenovirus particles were simultaneously visualized. Adenovirus particles were isometric, spherical, characterized as "complete" or "empty", measuring between 70 and 90 nm in diameter. Paramyxovirus and coronavirus particles were detected in 3 (5.2%) samples. Coronaviruses were pleomorphic with a diameter of 75-160 nm containing a solar corona-shaped envelope, with projections of approximately 20 nm of diameter. Seven (12.3%) samples were negative for viral particles.

KEYWORDS: Paramyxovirus, Adenovirus, Coronavirus, Pigeons, Transmission electron microscopy.

INTRODUCTION

Pigeons are birds of European origin found worldwide. Due to their high population growth, they act as important hosts in the spread of diseases in humans and other animal species, and transmission occurs via feces (Haag & Gurdan, 1990; Orlandella *et al.*, 1992, Toro *et al.* 1999). In nature, they play an important role as insect control and replanting of seeds eliminated by feces to the soil (Museu de Ciências Naturais).

In these species, the most important viral diseases of the digestive tract are transmitted by the paramyxovirus or adenovirus (Marlier & Vindevogel, 2006) followed by coronavirus (Qian *et al.*, 2006).

Avian paramyxoviruses are non-segmented single stranded RNA negative polarity, enveloped with helical symmetry of the nucleocapsid. Virions are pleomorphic, usually roughly spherical, 150 nm or more in diameter, but filamentous forms are common (Alexander *et al.*, 1993).

They belong to the *Mononegavirales* order, *Paramyxoviridae* family, *Paramyxovirinae* sub-family and *Avulavirus* genus which include 1-9 serotypes being Newcastle disease the serotype 1 (ICTV, 2009).

According to Alexander *et al.* (1991), the serotype 7 is most frequently isolated among Columbiformes.

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Avian paramyxoviruses have been isolated from a variety of free living species and domestic birds worldwide causing different symptoms and clinical signs resulting in economic losses (Leeuw & Peeters, 1999).

Pigeons are particularly susceptible to avian paramyxovirus type 1 APMV-1 (Pennycott, 1994) and to other strains adapted to their organism (Ujvari *et al.*, 2006), as the cNDV classic and pigeon pAPMV 1 strains (Marlier & Vindevogel).

Newcastle disease can be classified into velogenic, mesogenic and lentogenic depending on the pathogenicity of its strain. This can be viscerotropic, pneumotropic or neurotropic (Marlier & Vindevogel).

The transmission of a velogenic strain in pigeons can occur in sick chickens during outbreaks (Vindevogel *et al.*, 1972).

Regarding the velogenic cNDV strain, the major symptoms are watery, and hemorrhagic diarrhea, conjunctivitis, rhinitis, dyspnea, congestion of the pharynx and larynx, tremors in the neck and wings, torticollis, paralysis and loss of healthy balance. The morbidity rate reaches 70% and mortality above 90% (Vindevogel *et al.*, Ritchie *et al.*, 1994).

Yet, in the infection with velogenic pAPMV-1 strain, with an incubation period 4-6 days to 3-4 weeks the respiratory symptoms are absent. The morbidity rate reaches 30 to 70% and mortality is less than 10% (Marlier & Vindevogel).

The ingestion of contaminated feed or feces by pigeons can be a source of infection for other avian species, particularly chickens (Alexander *et al.*, 1985).

In Brazil, other authors did not detect paramyxovirus in free-living pigeons (Carrasco, 2005; Sousa *et al.*, 2010).

Regarding adenovirus, these agents induce in pigeons two distinct forms of infection, type 1 or classical adenovirus and type 2 or necrotizing hepatitis (Marlier & Vindevogel).

The type 1 affects mainly young pigeons (Duchatel *et al.*, 2000), while type 2 affects birds aged 10 days to 6 years (Vereecken *et al.*, 1998). In both types, the usually observed symptoms are vomiting, watery acute diarrhea, weight loss, anorexia and depression, with polyuria only in type 2 (Duchatel *et al.*).

The rapid spread of infection to other poultry, with mortality preceded by secondary bacterial infection and morbidity rates reaching 100% is specific to the classical adenovirus (Duchatel *et al.*).

In type 2 or inclusion bodies hepatitis, the death of pigeons it was reported in 24 to 40 hours after the symptoms and mortality rate of 30 to 100% (Hess 1988 a, b; Vereecken *et al.*; Duchatel *et al.*; Wang & Chang, 2000).

Transmission occurs through fecal-oral route and by aerosol (Ritchie *et al.*).

The avian adenovirus belongs to the *Adenoviridae* family and *Aviadenovirus* genus, composed of seven species of viruses (ICTV, 2009). Adenovirus is icosahedral in shape and non-enveloped particles of 70 to 90 nm in diameter. The capsid consists of 240 nonvertex capsomers called hexons, each 80-10 nm in diameter, and 12 vertex casomers (pentons), each with a protruding fiber from 9 to 77.5 nm in length. The genome is a linear molecule of double-stranded DNA 36-38 kb long (Davison *et al.*, 2003). They replicate in the nucleus of the host cell producing basophilic intranuclear inclusions (Wu & Nemerow, 2004).

On the other hand, avian coronaviruses belong to the *Nidovirales* order, *Coronaviridae* family, *Gammacoronavirus* genus and to 3a and 3c groups (Woo *et al.*, 2009). The coronavirus has a pleomorphic to rounded morphology and is 90 to 200 nm in diameter. It is enveloped with club-shaped surface projections about 20 nm long. It contains a positive-strand RNA and replicates in the cytoplasm of the host cells (Cavanagh, 2005).

The respiratory and enteric tracts are common targets for coronaviruses (Miura & Holmes, 2009).

Infectious bronchitis is the main disease caused by coronavirus in poultry and the infection results in significant economic losses to the commercial chicken industry (Pohuang *et al.*, 2009). The virus was isolated in a flock of racing pigeons, carriers of infectious bronchitis, with clinical signs of ruffled feathers, dyspnea, mucus in the beak (Barr, 1988) and in pigeons with pancreatitis (Wu *et al.*, 2005; Qian *et al.*). The detection of these agents in apparently healthy pigeons suggested the inclusion of a new member in the group 3 of the *Gammacoronavirus* genus (Jonassen *et al.*, 2005).

The aim of this study was to investigate the

presence of viral agents of free-living pigeon feces (*Columba livia*), using the negative staining technique (rapid preparation) for transmission electron microscopy, since these birds live in urban habitats, allowing greater proximity to humans and other birds from commercial farms.

MATERIAL AND METHOD

Description of the case. During the year 1999, freshly stool samples of 57 free-living pigeons (*Columba livia*) were collected, of which 50 adults and 7 young birds, apparently healthy, in urban areas of Sao Paulo.

After collection feces were stored on ice to be processed by negative staining technique (rapid preparation) for transmission electron microscopy at the Electron Microscopy Laboratory of the Biological Institute of Sao Paulo, SP, Brazil, to detect possible viral agents.

Negative staining technique (rapid preparation). In negative staining technique, 57 feces 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, stabilized with carbon supporting film of 0.5% in collodium 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).

RESULTS

Negative staining technique (rapid preparation). Under the transmission electron microscopy, paramyxovirus particles, pleomorphic, roughly spherical or filamentous, measuring 100 to 500 nm of diameter containing an envelope covered by spikes, with herring-bone like nucleocapsid, measuring 15 to 20 nm in diameter were visualized in the 45 (79%) out of 57 feces samples (Fig. 1).

The 45 stool samples were pasty with green or brown color.

In 2 (3.5%) stool samples, paramyxovirus and adenovirus particles were simultaneously observed. The adenovirus particles were seen to be isometric, spherical, non-enveloped, containing capsid of icosahedral symmetry, particles characterized as "complete" and "empty", measuring between 70 and 90 nm in diameter (Figs. 2, 3).

Fig. 1. Negatively stained paramyxovirus particles, pleomorphic, roughly spherical, containing an envelope covered by spikes (arrow). Bar: 80nm.

Fig. 2. Negatively stained adenovirus particles, showing a hexagonal shape (arrow) with distinct closely packed capsomers (arrow head). Bar: 95 nm.

In these two samples, watery feces, yellowish and with mucus was observed.

In 3 (5.2%) samples, the presence of paramyxovirus and coronavirus particles was observed (Fig. 5). The coronavirus was pleomorphic with a diameter of 75-160 nm containing a solar corona-shaped envelope, with projections of approximately 20 nm of diameter (Fig. 4).

These stool samples were watery and yellowish.

Furthermore, no type of particle was detected in 7 (12.3%) analyzed stool samples.

Fig. 3. Negatively stained adenovirus particles, exhibiting the hexagonal outline formed by the distinct capsomers (arrow). Bar: 60 nm.

Fig. 4. Negatively stained pleomorphic coronavirus particles, with a solar corona-shaped envelope (arrow). Bar: 120 nm.

DISCUSSION

In this study, using the negative staining technique (rapid preparation) for transmission electron microscopy, we observed typical paramyxovirus particles in 45 (79%) out of 57 stool samples from free-living pigeons (*Columba livia*).

These results are in accordance with data in the literature showing particles with similar morphology in pigeons (Gough & Alexander, 1983), owls (Catroxo *et al.*,

Fig. 5. Negatively stained showing the simultaneous presence of coronavirus (big arrow) and paramyxovirus (minor arrow) particles. Bar: 110 nm.

2010), ducks (Chang *et al.*, 2001) and Gouldian Finch (Zhang *et al.*, 2006).

The paramyxovirus of the Newcastle Disease (PMV-1) was also detected in pigeons by serological techniques in organ fragments (Shirai *et al.*, 1986; Meulemans *et al.*, 2002; Mushtaq *et al.*, 2006) and blood (Toro *et al.*; Tsai & Lee, 2006) as well as by molecular techniques (Collins *et al.*, 1989; Zanetti *et al.*, 2001; Aldous *et al.*, 2004).

Later, other researchers using the same techniques were not successful in obtaining the PMV-1 in free-living pigeons (Carrasco; Lillehaug *et al.*, 2005; Sousa *et al.*).

A study by Tsai & Lee in healthy birds from poultry farms, showed the presence of PMV-2 in these birds. Similarly, the birds studied by us were apparently healthy.

Additionally, experiments demonstrate that PMV-7 type, considered non-pathogenic, has been frequently isolated among Columbiforms (Gough & Alexander; Alexander *et al.*, 1991; Ritchie *et al.*). Instead, Toro *et al.* did not detect the PMV-7 in samples of free-living birds.

Free-living birds can act as carriers and reservoirs of avian pathogens (Sousa *et al.*).

In this study, concomitant presence of particles of adenovirus and paramyxovirus samples was found in 2 (3.5%) samples.

Wang & Chang observed by the negative staining technique, the presence of typical adenovirus particles in cell

culture supernatant of pigeon tissue. Also, it was demonstrated in pigeons in ultrathin sections of liver (Goryo *et al.* 1988; Sagartz & Swayne, 1991) intestine (Bergmann & Kiupel, 1982; Wada *et al.*, 1995) and in cell cultures (Takase *et al.*, 1990), intranuclear inclusions bodies containing such particles.

Additionally, analysis of liver fragments by PCR technique (Freick *et al.*, 2008) and blood by serological techniques (Tsai & Lee) enabled diagnosis of these viruses in pigeons.

In our study, we found that 2 (3.5%) stool samples positive for adenovirus and paramyxovirus were greenish, pasty and with mucus.

These characteristics of loose stools was also reported in pigeons with inclusion body hepatitis (Goryo *et al.*, 1988; Dorrestein *et al.*, 1992; Ritchie & Carter, 1995).

Differences in clinical signs and pathology associated with adenovirus infections in pigeons suggest that more than one virus with varied organ affinity may infect these birds (Ritchie *et al.*)

The negative staining technique is considered effective because it is simple and rapid to diagnose viruses in biological samples (Doane & Anderson, 1987) besides requiring a small amount of sample (Almeida, 1983), allow detection of different viral particles in the same sample (Fenner *et al.*, 1992) and excludes the possibility of false positives (Ueda *et al.*, 1998; Hazelton & Gelderblont, 2003), advantages with which we agree. However, according to Freick *et al.*, these techniques are laborious and time consuming especially in the detection of adenovirus.

Ritchie *et al.* confirm that additional studies are needed to elucidate better correlation between the adenovirus and pigeons as hosts.

The observation of typical particles of paramyxovirus and coronavirus in 3 (5.2%) out of 57 analyzed samples, corroborates with other authors who have found coronavirus in owls (Catroxo *et al.*, 2010), quail (Circella *et al.*, 2007) and green-cheeked (Gough *et al.*, 2006).

We observed that three stool samples were positive for coronavirus and paramyxovirus and were yellowish and watery.

In turkey, coronavirus was associated with pronounced enteric tropism (Nagaraja & Pomeroy, 1997) and quail to an enteric syndrome (Circella *et al.*).

Coronaviruses have also been identified in a flock of racing pigeons with infectious bronchitis which showed signs of ruffled feathers, dyspnea, mucus in the beak (Barr) and pancreatitis (Qian *et al.*).

Other experiments showed in apparently healthy rock doves, another type of coronavirus genetically distinct from those found in infectious bronchitis (Jonassen *et al.*).

Sousa *et al.* explain that the condition of carriers of infectious bronchitis in wild and exotic birds is justified by the interaction between species or its close relationship to commercial poultry farms.

Whereas free-living pigeons are potent agents of viral hosts, we believe that frequent monitoring through techniques such as electron microscopy, contributes to the protection of public health and prevention of diseases in commercial poultry farms and preservation of the species (*Columba livia*) studied by us.

The findings of the present study constitute the first report on presence of these viruses in this avian species.

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RESUMEN: Los heces de las palomas constituyen vehículos de enfermedades importantes, tanto para el Hombre como para otras especies animales. En estas aves, las enfermedades virales más importantes del tracto digestivo son transmitidas por los paramixovirus, adenovirus y coronavirus. *Paramixovirus aviario*, en todo el mundo, ha sido aislado de una variedad de especies de vida libre y de aves domésticas, que causan variados síntomas y señales clínicas con pérdidas económicas. El *Paramixovirus* pertenece a la familia *Paramyxoviridae* y al género *Avulavirus* que incluye nueve serotipos (APMV 1 a 9). Adenovirus aviario pertenece al género de la familia *Adenoviridae* y género *Aviadenovirus*. En las palomas, causan la adenovirosis clásica y la hepatitis necrotizante. El tracto respiratorio y entérico son los albos comunes de los coronavirus. Ellos pertenecen a la familia *Coronaviridae* y a los grupos 3a y 3c. En este trabajo, se describe la presencia de agentes virales en las heces de palomas de vida libre (*Columba livia*) en la ciudad de São Paulo, Brasil. Las heces se procesaron para la técnica de microscopia electrónica de transmisión, a través de la técnica de contrastación negativa (preparación rápida). A través de esta técnica fueron visualizadas las partículas de paramixovirus, pleomórficas, más o menos esféricas o filamentosas, de 100 a 500 nm de diámetro que contiene un envoltorio cubierto con espículas, con nucleocápside con características helicoidales, midiendo de 15 a 20 nm de diámetro en 45 (79%) de 57 muestras. En 2 (3,5%) muestras, fueron observadas simultáneamente partí-

culas de paramixovirus y de adenovirus. Las partículas de adenovirus eran isométricas, esféricas, caracterizadas como "completa" o "vacía", midiendo entre 70 y 90 nm de diámetro. Fueron analizadas en tres muestras (5,2%) las partículas de paramixovirus y coronavirus. Los coronavirus son pleomórficos, con un diámetro de 75 a 160 nm, que contiene una capa en forma de corona solar con proyecciones de aproximadamente 20 nm de diámetro. Siete (12,3%) muestras fueron negativas para las partículas virales.

PALABRAS CLAVE: Paramoxivirus; Adenovirus; Coronavirus; Palomas, Microscopía Electrónica de Transmisión.

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