Serological Analysis and Detection of the Influenza Virus in Anseriformes from São Paulocity, Brazil

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Abstract

Migratory waterfowl, particularly birds of the Anseriformes order, are considered to be the main reservoir of the influenza virus. All 16 haemagglutinin and 9 neuraminidase were isolated from these birds where the influenza virus is shed in faeces and oropharyngeal secretions. From these aerosols, the virus can be transmitted to other hosts, including man, especially in environments that favour the interaction of species. In São Paulo, these environments are represented by the park, which in addition to providing leisure environments for people, are home to a huge diversity of animal species, thus leading to important epidemiological and public health issues. To identify the presence of the influenza virus circulating in domestic Anseriformes present in the São Paulo park, we evaluated 30 oropharyngeal (oro) and 35 cloacae (cl) samples from 65 duck birds (Cairina spp). The swabs were characterized by the RT-PCR technique using the Promega GoTaq kit, with a pair of primers for the matrix protein of the influenza virus M1 and M2, and then routing for an ultra-structural analysis. Forty six collected sera sample were analysed using the haemagglutination inhibition (HI) technique for subtypes H1N1, H3N2, H7N7, and H3N8 for the influenza A virus. The serology showed that over 90% of the animals exhibited antibodies against the serotypes tested. On agarose gel, electrophoresis evident bands were observed in 24 specimens (10 oro and 14 cl), with a length of approximately 300bp. Through ultra-structural analysis of the oropharyngeal and cloacae samples were detected spherical and oval particles, with or without spikes that surrounded the particles, measuring around 80nm (nanometer), which are compatible with the influenza virus. Our results suggest the presence of the influenza virus in water fowls residing in São Paulo City Park. The number of animals with a potential virus circulation is much higher, when considering that viral clearance is variable throughout the day. Because the birds in São Paulo Park are in constant contact with free-living wild animals and humans, it is important to implement monitoring system to prevent the occurrence of an epidemic.

ABBREVIATIONS

HI: Haemagglutination Inhibition; oro: Oropharyngeal; cl: Cloacae; RT/PCR: Reverse Transcriptase / Polymerase Chain Reaction; AIV: Avian Influenza virus; PBS: Phosphate Buffer Solution; DEPEC: Diethyl Pyrocarbonate; DNTB: Dinitrothiocyanobenzeno; DNTP: Desoxirribonucleotides Phosphate; TEB: Trisphosphate / Tetraacetic Acid Buffer.

INTRODUCTION

Avian influenza is an infectious disease caused by the influenza virus of the family Orthomyxoviridae. It is a cosmopolitan virus isolated in migratory waterfowls, especially the Anatidae family from the order of Anseriformes [1]. It is a systemic disease which can be highly lethal [2] and is caused by an influenza Type A virus, whose strains are classified as high or...
low pathogenicity, in accordance with the capacity to cause mild or severe illness in animals. The transmission between different species of birds occurs by direct or indirect contact of poultry with migratory wild fowl, which are the natural reservoirs of the virus, and have been the main cause of an epidemic [3]. The genetic recombination between viral strains in mixed infections is provided by a segmented RNA genome of the influenza virus, and this favours the emergence of antigenic variants that cross the interspecies barrier, and can cause serious epidemics in populations of immunologically unprotected animals [4].

The Avian Influenza Virus (AIV) has been isolated from water (which can be preserved during the freezing winter, awaiting the return of the birds) and faeces at the margin of the lakes that are home to communities of migratory wild ducks in Canada. Although AIV can infect a huge diversity of species and different classes of birds and mammals, Thus, a current hypothesis considered aquatic birds, especially those of the order Anseriformes, including duck, goose, and swan, as being major reservoirs of influenza in nature[5,6].

The sequence of events, and the temporal relationship between epidemics in birds and humans, strongly suggests a direct transmission between species, without the involvement of an intermediate host. The simultaneous infection of humans, or pigs, with human and avian influenza viruses, can theoretically generate new viruses with a pandemic potential, as a result of the genetic recombination between these subtypes. Such hybrid viruses may be capable of expressing surface antigens of avian viruses to which the human population has no immunity [7]. Through waterfowl, viruses end up affecting poultry, particularly those of economic importance, such as chickens, ducks, turkeys, geese, and others [1].

Infected bird’s can spread the virus in their faeces and respiratory secretions for 10 to 14 days after infection. The transmission of AIV from an infected bird to another bird, likely occurs mainly from respiratory secretions and digestive, directly or indirectly, the latter especially for personnel choosing clothes, shoes, and boots, etc. Free-living birds may transmit an infection directly and in advertently share the authoring environment with migratory water fowl, which are the natural reservoirs of AIV, and this favours the emergence of antigenic variants that cross the interspecies barrier, and can cause serious epidemics in populations of immunologically unprotected animals [4].

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Every year, with the approach of autumn, millions of birds leave their breeding in the cold (wintering grounds), in search of places with warmer temperatures and with a greater amount of food available, and then return to their home areas during spring and summer, thus completing their biological cycle. Brazil is a country that is on route of many species of migratory birds, both the northern visitors (birds like Nearctic), which have their breeding sites in the northern hemisphere, and the southern (Neo tropical birds), which breed in areas of the Southern Hemisphere [10].

The city parks are green areas in the city of São Paulo that serve as transit areas, or rest homes, for wildlife [11]. The birds represent a large portion of the animals that inhabit the city parks. Almost half of the people resident or visitor cohabits, with a population of 11 million people all of the year round and, in search of conditions to complete their life [12]. Among the species of birds, ornamental waterfowl constitute a considerable proportion of the animals that inhabit the park, maintaining a close contact with the population that frequents these sites, together with other species of animals that inhabit and pass through the same place, which can characterize a risk potential of zoon sis, among them, by the influenza virus.

Faced with a high serology for the influenza viruses presented by the main species present in ornamental waterfowl [13], this work contributes to the identification of the transmission cycle of the disease, analysing whether or not the virus is released by these animal species.

It was our objective to analyse the potential release of the influenza virus in Anatidae (ducks) in the parks of São Paulo city, Brazil, through the faeces and respiratory secretions, and to know that most viral subtypes, circulating between these birds, could consequently result in infections between man and animal.

**MATERIALS AND METHOD**

**Animals**

Samples were obtained from oropharyngeal and cloacae swabs of 65 domestic ducks (Cairina spp) belonging to the collection of São Paulo city park, Brazil, through the Technical Division of Veterinary Medicine. Each swab was placed in a well labelled (animal identity, sample collection method, type of sample) Eppendorf tube containing 0.2 mL of PBS (buffered saline). These samples were kept refrigerated during transportation to the Virology Laboratory, Division of Scientific Development of the Butantan Institute, Brazil, where they were kept at temperatures of 196°C (liquid nitrogen) [14]. The samples were tested by Reverse Transcriptase/Polymerase Chain Reaction (RT/PCR), followed by nucleotides sequencing, in order to determine the presence of influenza antigens in the saliva and animal faeces, as the virus affects both the respiratory and digestive tracts of these animals.

**Haemagglutination Inhibition (HI)**

Forty six sera samples were obtained from duck’s wing vein and were inactivated at 56°C for 30 min and treated with 20% Kaolin in a phosphate buffer solution (PBS) 0.01M pH 7.2, in order to eliminate non-specific antibodies. Duplicate dilutions were carried out in series in a V buttons microplate. The antigen of the influenza virus, containing 4haemagglutinatingunits was added; after one hour of a reaction at room temperature, 0.05% rooster erythrocytes, 0.25µL was added to them. Reading was processed after 30 min, being the reciprocal of the last dilution, which elicited the haemagglutinating inhibition which was considered as the antibody titre. Those serials that presented antibody titres equal for or superior to 4 HIU were considered as positive, for all constant reagents [14].
ULTRA-STRUCTURAL ANALYSIS

Electron Microscopy

Ten-microlitre drops, containing the non-purified oropharyngeal and cloacal samples, were collected from twenty two ducks that were adsorbed with 2%parlodion and carbon-coated nickel grids for 1min at room temperature. The excess was then removed with filter paper and negatively stained with 2% uranyl acetate. The grids were allowed to air-dry prior to examination with a Zeiss EM 109 transmission electron microscope operated at 80 kV. Micrographs were taken with various magnifications with an Olympus MegaViewG2 digital camera and with TEM CE software for the TEM Imaging Platform [15, 16].

All of these sixty five samples were analysed by molecular techniques, twenty two of them by electron microscopy, and forty-six by Haemagglutination Inhibition. It was verified that samples that were evaluated by RT/PCR, presenting positivity, eleven were chosen for sequencing. Considering the difficulty in handling these samples, some were lost and there was insufficient amount among the collect samples.

RNA Extraction

One hundred μL of each cloacal or oropharyngeal sample from the sixty five duck swab samples were added to 1000 μL of triazol, mixed thoroughly by inversion, and held for 5 minutes at room temperature. 200 μL of chloroform was added, and then mixed vigorously by vortexing or by hand, for 15 seconds, and then left at room temperature for 3 to 5 minutes. They were then centrifuged at 12000g for 15 minutes at 4°C. 400 μL of the supernatant was removed and moved to another tube, avoiding proximity to the interface. Next, 500 μL of isopropl alcohol was added and mixed by inversion and kept at room temperature for 10 minutes. Then they were centrifuged at 12000g for 10 minutes at 4°C, discarding the supernatant by inversion. Then 1.0mL of 75% ethanol was added and mixed thoroughly with a vortex, centrifuged at 7500g for 10 minutes and the supernatants were discarded. Finally, they were dried in a biologically safety cabinet, with its mouth down, for about 30 minutes, or until there were no remaining droplets of ethanol. 20 μL of treated water was added with DEPEC; incubation at 50°C for 10 minutes. Then they were centrifuged at 12000g for 15 minutes at 4°C. 400 μL of the supernatant was removed and moved to another tube, avoiding proximity to the interface. Next, 500 μL of isopropl alcohol was added and mixed by inversion and kept at room temperature for 10 minutes. Then they were centrifuged at 12000g for 10 minutes at 4°C, discarding the supernatant by inversion. Then 1.0mL of 75% ethanol was added and mixed thoroughly with a vortex, centrifuged at 7500g for 10 minutes and the supernatants were discarded. Finally, they were dried in a biologically safety cabinet, with its mouth down, for about 30 minutes, or until there were no remaining droplets of ethanol. 20 μL of treated water was added with DEPEC; incubation at 50°C for 10 minutes and stored at -80°C.

Reaction of RT-PCR (obaining the cDNA - DNA produced by RNA) The following reagents were mixed gently and allowed to be chilled on ice: 1.0 μL Primer 1; 1.0 μL Primer 2; 5.0μL of the sample to be tested; 8.0 μL of water with DEPEC heated at 70°C for 5 minutes in a thermo cycler and quickly cooled on ice; in each tube, adding a mix with the following reagents: 5 μL of 5x first strand buffer; 0.6 μL RNasin; 2 μL of DNTP; 1.0 μL of RT (reverse transcriptase);16.4 μL of distilled water;incubated at 42°C for 60 minutes in the thermo cycler and packaged in -20°C.

Electrophoresis Agarose

In a conical flask, 25mL of TEB buffer was added with0.5mL of agarose and heated in a microwave for about 30 seconds, until translucent, then left to cool; then adding the content in the frame (leftfor 30 seconds for a gel formation of agarose); 10 μL of the sample were added in Eppendorf tubes with 10 μL of Load Buffer (dye race with glycerol); A comb was used as the model for the holes; after, we removed the comb from the chassis and the side plates; TBE was added on the chassis; then filling 1.0μL of each sample in the load buffer, starting at the second hole in agarose gel; On the first hole, there was 1.0μL of the positive control (100 bp); That was connected to the electrophoresis apparatus and to the voltmeter and left for the samples to run until there was 2/3 of it (approximately 1 hour); Then the unit was turned off, the gel was removed and then left it submerged in a solution of ethidium bromide for 20 minutes, it received the DNA phosphorescent; Finally, it was kept in the gel immersion in water for 5 minutes; the gel was then placed in a trans illuminator and properly covered with PFC film; then the shooting and it was analysed for results. OBS: After this time, the block and the PVC Hlm were discarded in biological waste, remaining for 72 hours in sodium hypochlorite [14].

CLONING AND SEQUENCING

In a sequencing vector, using pGEM-T Easy Vector System (Promega) and E. coli SURE competent cells, the recombinant plasmids were purified from three colonies of each ligation reaction. The sequencing reactions were performed with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on RT-PCR products, amplified with specific primers from the RNA samples and were cloned ABI 3130 XL Plataform (Life Technologies) [17].

Sequence analysis

The DNA sequences obtained were aligned and edited using Bio Edit Alignment Sequence Editor Software and the consensus sequences were compared with the BLAST (Basic Local Alignment Search Tool) database. Comparisons that punctuated with higher scores were considered [18].

RESULTS AND DISCUSSION

DNA sequences from samples: 2, 5, 7, 8, 9, 10 revealed the following score of identity.

OBS: The oligonucleotides that were chosen for the PCR reaction can be generic, sufficiently to also produce fragments, for another organism group. The list presents the details with the most scores of identity found in the Blast.

SAMPLE 2 Influenza A virus (A/eq/Miami/1963(H3N8)) segment 7 matrix protein 1 (M1) and matrix protein 2 (M2) genes (98%).

Influenza A virus (A/eq/Detroit/2/1963(H3N8)) segment 7 matrix protein 1 (M1) gene (98%).

Influenza A virus (A/eq/Miami/1/1963(H3N8)) segment 7 (98%).

Influenza A virus (A/eq/Newmarket/1/77(H7N7)) matrix proteins M1 and M2 gene (98%) in.

Influenza A virus (A/eq/LaPlata/1/88(H3N8)) matrix proteins M1 and M2 (M) gene (98%)

Influenza A virus (A/eq/Sao Paulo/1/1969(H3N8))
Influenza A virus (A/equine/Sao Paulo/6/1963(H3N8)) segment 7 (98%)  
Influenza A virus (A/equine/Uruguay/1/1963(H3N8)) segment 7 (98%)  

**SAMPLE 5** Influenza A virus (A/equine/Miami/1963(H3N8)) segment 7 matrix protein 1 (M1) and matrix protein 2 (M2) genes (98%)  
Influenza A virus (A/equine/Detroit/2/1963(H3N8)) segment 7 matrix protein 2 (M2) gene and matrix protein 1 (M1) gene (98%)  
Influenza A virus (A/equine/Miami/1963(H3N8)) segment 7 (98%)  
Influenza A virus (A/equine/Sao Paulo/1/1969(H3N8)) segment 7 (98%)  
Influenza A virus (A/equine/Sao Paulo/6/1963(H3N8)) segment 7 (98%)  
Influenza A virus (A/equine/Uruguay/1/1963(H3N8)) segment 7 (98%)  

**SAMPLE 7** Micrococcus sp. ART55/1 (93%)  
*Clostridium saccharolyticum* strain WM1 23S ribosomal RNA (92%)  

**SAMPLE 8** Influenza A virus (A/Taiwan/969/1981(H1N1)) segment 7 matrix protein 2 (M2) gene and matrix protein 1 (M1) gene (99%)  
Influenza A virus (A/Taiwan/927/1981(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes (99%)  
Influenza A virus (A/Taiwan/927/1981(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes (99%)  
Influenza A virus (A/Taiwan/927/1981(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes (99%)  
Influenza A virus (A/Taiwan/1132/1991(H1N1)) segment 7 matrix protein 2 (M2) gene, and matrix protein 1 (M1) gene (99%)  
Influenza A virus (A/Taiwan/1037/1984(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes (99%)  

**SAMPLE 10** *Anas platyrhynchos* cytochrome b5-like (LOC101791665), mRNA (91%)  
*Gallus gallus* cytochrome b5 type A (microsomal) (CYB5A), mRNA (9)  

Table 1 shows the display of the duck samples (swabs or sera) evaluated for the influenza presence by RT/PCR/sequencing, serology and ultra-structural analysis, where we observed sixty-five samples collected from theopharyngeal or cloacae from the ducks. Almost all of these sera samples tested had high positivity HI antibodies against influenza as demonstrated in Figure 1, and these results were reinforced by the twelve positive particles that were similar to the influenza virus and were
DNA sequences from samples: 2, 5, 7, 8, 9, 10 revealed the following score of identity.

**RT/PCR Transcriptase Reverse/Polymerase chain reaction.** Sequencing OR: Oropharyngeal, CL: Cloacae, IS: Insufficient Sample, LS: Lost Sample, ND: Not Done, HI: Haemagglutination Inhibition, EM: Electron Microscopy

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**Figure 1** Antibody response to influenza subtypes measured by Haemagglutination Inhibition in the duck’s sera.

- RSD=H1N1=160.5, H3N2=117.6, H7N7=105.2, H3N8=105.8

**Figure 2** Electrophoresis from RT/PCR products from the duck samples.

- n1) Z 259 or
- n2) Z 259 cl
- n3) Z 265 or
- n4) Z 267 cl
- n5) Z 275 cl
- n6) T 236 cl
- n7) T 212 cl
- n8) Z 032 or
- n9) T 235 cl
- n10) X 278 or
- n11) X 093 cl

DNA sequences from samples: 2, 5, 7, 8, 9, 10 revealed the following score of identity.

- OBS: The oligonucleotides that were chosen for the PCR reaction can be generics, sufficiently to also produce fragments for another organism group. The list presents the details with the most scores of identity found in the Blast.

It was observed that duck n. 2 (ring code Z 259) had the influenza virus in a cloacae sample, but not in the oropharyngeal sample. This suggests that the digestive tract in a duck has more receptors to the influenzavirus than the respiratory tract.

The results show that 42 animals (ducks) (93.3%, 42/45) had antibody responses by a Haemagglutination Inhibition (HI) to the sub types of influenza virus type A, whose HI titres were: RSD=260.5HIU to H1N1HIU, 117.6HIU to H3N2HIU, 105.2HIU to H7N7, and 105.8HIU to H3N8 (Fig. 1). Serologic study, verified the presence of influenza Type A, in the most of the subtype H3N8 antibodies in domestic ducks, chickens and wild...
birds [9,19]. The oropharyngeal and cloacae obtained from the different ducks, which were evaluated through RT/PCR, using the pairs of primers, for the matrix protein of the influenza virus, demonstrated that positive samples presented electrophoresis bands with a length of approximately 300bp. From eleven positive samples (cDNA), eleven were resequenced and evaluated by the method Blast, whose results confirmed that four samples were of influenza virus type A with a 98% similarity with subtypes A/H1N1, H7N7, H3N8 for the samples number 2 and 5 and a 99% similarity with subtypes A/H1N1 for the samples numbered 8 and 9. In sample number 7, any similarity with the subtypes of influenza type A were observed (Figure 2). Any high pathogenic strains of influenza virus type A, as subtypes H5 or H9, were detected. Therefore, in concordance with the observation of oligonucleotides presenting generic fragments production for any other organisms group, this appeared in sample 10, the Anas platyrhynchos cytochrome b 5-like (LOC 101791665), m RNA (91%), and Gallus gallus cytochrome b 5 (microsomal) (CYB 5A), and in sample 7, Micrococcus sp ART 55/1 and Clostridium saccharolyticum WM 1 23S ribosomal RNA with 93% and 92% of similarity, respectively.

Through this finding of these cytochromes, that could be characterised on an avian species, and on the other hand, the presence of the microorganisms could signify as a co-infection between virus and infectious microorganisms. The co-infection with the influenza virus and microorganisms such as Cryptosporidium was observed and reporting that the same co-infection reduced the immunity against influenza virus in vaccinated chickens [8,20]. The influenza virus co-infecting with proteolytic bacteria, such as Streptococcus sp, Staphylococcus sp, and Pseudomonas sp, was detected in the oropharyngeal tract from individuals presenting respiratory manifestations, with grave infection complications [21]. The influenza co-infections with pathogenic microorganisms has been a kind of cause of mortality among birds, mainly chickens and turkeys [9].

Considering that ducks are cited as reservoirs and the transmitters of the influenzavirus Type A, the regular interaction with chickens in small farm, or within a live market, then there will be necessary biosecurity measures put in place in order to avoid the current circulation of this virus, aiming an economic understand animal welfare. With a base on this, it has been suggested that there be a vaccination against influenza A/H5 for domestic birds, since subtype H5 is highly pathogenic for all influenza host species, including ducks, even if they are being considered as being resistant to influenza infection by other subtypes [22].

Taking into account, the zoonotic characteristics of the influenza virus, which facilitate interspecies transmission, favouring the genetic virus recombinations among their subtypes, promoting an emerging new strain, or a re-emergent one, provoking outbreaks of influenza epidemic, in susceptible populations, without protector immunity [4].

Besides of the influenza virus identification in hosts, through serological and molecular methods, an evaluation on influenza isolates, by ultra-structural methods has been important to identify the influenza virus when comparing images of viral particles by its measurements and shapes with particle standards. Thus, through the electron microscopy examination of samples obtained from anatidae (ducks), it is observed that images of viral particles measuring 180 nm each, with shapes that were spherical surrounded with spikes on its envelope (Figure 3), and oval without visible spikes (Figure 4), respectively, from oropharingeal and cloacae isolates. These particles are compatible to the influenza virus particle, which in our previous work, using isolates from humans and birds, it was also reported [23,24].

It is important to inform that the influenza virus in circulation is not only among these homothermic animals, as this has been known for a very long time, but today, the heterothermics, like reptiles, have been cited as participating of the influenza virus reservoir, which could also be verified in our prior study of influenza virus isolation in cell cultures, certified by RT/PCR and visualized by its viral particles by the ultra-structural analysis of the reptiles [25].

**CONCLUSION**

Based on these results, and also in the literature on Anseriformes/anatides as duck (cairina sp) versus the influenza virus, is obvious to conclude that this virus is circulating among the ducks in the park in São Paulocity, Brazil, as well as in other waterfowl that are circulating the influenza virus all over the world.

It has been demonstrated that the presence of the influenza virus A subtypes such as H1N1, H3N2, H7N7, and H3N8, in over 90% of the evaluated ducks, by serology, and by the presence of the H1N1, H3N8, and H7N7 by the molecular technique of RT/PCR and nucleotide sequences, with a 98% of similarity with the influenza virus, as has been informed in several sites. The co-infection could be detected due to the generic oligonucleotide chosen for RT/PCR. Taking into account that these influenza subtypes were considered to infect mammals, such as humans, horses and dogs, it was detected in these evaluated ducks, then these hosts seem to confirm their characteristics of being a principal reservoir and the transmitter of this virus in nature. The presence of the influenza virus in ducks, visualised by ultra-
structural analysis of their isolates, once more reinforced this.

The present study has reported information on the situation of ducks in the park located in São Paulo city, Brazil, which also serves as a transit area, or as a rest home for wild life. Therefore, the influenza virus circulation is very facilitated, considering that waterfowl and other birds cohabit with humans (millions of people per/year) that use the city park for recreation. This situation could characterise the probability of potential zoonoses is, which exposes a risk in people. That is why it is of the importance to use biosecurity measures when taking care of animals and the implementation in a monitoring system, in order to prevent the direct transmission between interspecies. In conclusion of this study, it is necessary to mount a constant order to prevent the direct transmission between interspecies of animals and the implementation in a monitoring system, in order to prevent the direct transmission between interspecies.

Referring to the search of the influenza virus, which is also affecting the heterothermic animals, it should also be considered to be a more dangerous effect on virus circulation.

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REFERENCES

18. Blast Accession