Research Article

Electron Microscopy, Hemagglutination Inhibition Test and Partial Sequencing Analysis of Influenza A Virus Samples Isolated from Wild Birds in São Paulo State, Brazil

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Abstract

The highly virulent avian influenza subtypes H5, H7 and H9 have caused outbreaks and epidemics in poultry and fatal infections in human beings. Wild and migratory birds may be taking part in the H17 subtypes and the N10 subtypes of the influenza virus maintenance of its transmission cycle interspecies in nature. From the bird species: Sporophila caerulescens (LE 6744) and Elaenia mesoleuca (LE 6712) – located in reserves and experimental field stations in the state of São Paulo, Brazil - were collected samples and identified the NS1 and M1 gene of influenza virus by RT-PCR and visualized the ultra - structures of viral particles measuring 30 to 120 nm by electron microscopy. Positivity to influenza type A was observed for these samples through HI test. The sequencing of NS1 and M1 regions from two influenza virus samples isolated was compared to sequences of all subtypes of influenza A available from public databases in order to verify the homology between these isolates, whose results revealed a high homology from 96% to 100% between both strains and subtypes H3N2.

ABBREVIATIONS

EM: Electron Microscopy; HI: Hemagglutination Inhibition; SPF: Specific Pathogen Free; RTPCR: Reverse Transcriptase Polymerase Chain Reaction; HA: Hemagglutinin; NA: Neuraminidase; NS1: Non Structural1; M1: Matrix1

INTRODUCTION

Influenza A virus causes respiratory illnesses in humans, producing seasonal outbreaks, epidemics and occasional pandemics, and outbreaks and epizootics in lower mammals and birds. This virus is a major public health concern because it continually circulates in mammals and birds, and its strains are subject to rapid genetic changes that can increase transmissibility and virulence in livestock, poultry and humans [1].

Migratory waterfowl as considered as ancestral reservoir for influenza A viruses, they are a primary focus of surveillance. Influenza virus type A divided into 17 subtypes of H, and 10 subtypes of N have been described to date [2]. The highly virulent avian influenza subtypes, H5N1, H7N7 and H9N2 have caused outbreaks and epidemics in poultry and fatal infections in human beings [3].

The avian influenza virus subtype H5N1 that infected individuals in China in 1997 and killed some of them did not undergo reassortment because all its genes were of avian origin [4]. The same phenomenon was also observed by [5], while conducting the study on direct transmission.

Interspecies, such as avian, swine and equines

In the city of São Paulo, Brazil, [6], showed the presence of avian influenza by serologic studies in wild, resident, migratory birds and domestic chickens, which presented antibodies against A/duck/England/56 (H11N6) from the Elaenia mesoleuca species frequently found in the state of São Paulo. In the city of Rio de Janeiro, Brazil,
it was reported, in 1980, the first isolation of Influenza from the stool of wild ducks (Dendrocygna viduata) and cages of exotic birds. These isolates presented dose antigenic affinity with both strains of A/turkey/Massachusetts/ (H6N2) and A/duck/England/63 (H11N6) [7,8]. These studies indicate the presence of Influenza A viruses and also that influenza reservoirs for all known subtypes of influenza were recorded on migratory and wild birds in most of their possible combinations. Thus, it can be assumed that the same bird flu virus might play a role in the evolution of new strains of pandemics among humans.

In previous study on influenza virus [9], observed that migratory birds carried out this influenza virus, which was detected by isolation in cell cultures, and embryo- nated eggs-SBF tested by Hemagglutination (HA) test and electron microscopy.

MATERIALS AND METHODS

Bird species: Elaenia mesoleuca and Sporophila caerulescens are frequently found in the experimental field station, located in the Tietê Ecological Park-Guarulhos, Iguape and Juquitiba, in São Paulo, Brazil.

Capture: Birds were captured in accordance with regulations of IBAMA (Brazilian Animal Protection Institute), using the Japanese technique.

Influenza virus characterization

Virus isolations were done out in cell culture of MDCK (Madin Darby Canine Kidney) and 9 day-old specific-pathogen-free (SPF) embryonated chicken eggs in inoculated into the allantoid cavity. Additionally, the viruses were passed in V buttons microplate. Antigens of influenza virus and isolated samples from birds containing 4 hemagglutinin units were added to the cavities, and after one hour at room temperature, 0.05% rooster erythrocytes were added to them. Reading was processed after 30 min. We considered as hemagglutination inhibitor titer the highest dilution of serum able to completely inhibit the hemagglutination of red blood cells. The negative control sera was processed with phosphate buffered saline and suspension of erythrocytes 0.5%, and the antigen control was performed by replacing serum with buffered saline [10]. The series presenting antibody titers equal or superior to 20HI units were considered as positive, which identified the influenza virus in birds isolate. For all reagents, volumes of 0.025 μL were constant (Table 1).

Detection of Virus Particles by Electron Microscopy

Samples were adsorbed to carbon-coated grids and negatively stained with 2% uranyl acetate. 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 (Figures 2 and 3).

Molecular Techniques

RNA viral extraction, RT-PCR and PCR Product Purification were performed according to [12] (Primers used are in the Figure 1).

Nucleotide Sequencing

From the PCR purified product, we amplified the 189bp fragment of NS1 gene and the 340bp fragment of M1 gene of influenza virus, using the KIT QIA quick SPIN HANDBOOK (QIAGen). Subsequently, we amplified these fragments in an automatic sequencer of DNA ABI PRIS model 3100 (Applied Biosystems, Inc., USA) using Sequence Navigator 1.0.1 PCR product for aligning the nucleotide sequences, which were then analyzed by the National Center of Biotechnology Information (NCBI) internet site: www.ncbi.nlm.nih.gov. We compared the sequencing of NS1 and M1 regions from two influenza A virus isolated from wild and migratory birds to sequences of all subtypes of influenza A available from public databases in order to verify the homology between these isolates [13].

Table 1: Serological identification by hemagglutination inhibition test (HI) of the birds sample compared to anti sera and antigens of Influenza A/Hong Kong/8/68 (H3N2), A/equine/Miami/65 (H3N8) and A/duck/ Ukraine/63 (H3N8). The type B-40 as negative control.

<table>
<thead>
<tr>
<th>ANTIGEN (4UHA) SERUM (DIL1:20)</th>
<th>A/Hong Kong/8/68 (H3N2) A/equine/Miami/65 (H3N8) A/duck/Ukraine/63 (H3N8) B/Lee/40</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE6712 (Elaenia mesoleuca)</td>
<td>320</td>
</tr>
<tr>
<td>LE6715 (Elaenia mesoleuca)</td>
<td>320</td>
</tr>
<tr>
<td>LE6744 (Sporophila caerulescens)</td>
<td>1024</td>
</tr>
<tr>
<td>A/Hong Kong/8/68(H3N2)</td>
<td>1024</td>
</tr>
<tr>
<td>A/equine/Miami/65(H3N8)</td>
<td>160</td>
</tr>
<tr>
<td>A/duck/Ukraine/63(H3N8)</td>
<td>80</td>
</tr>
<tr>
<td>B/Lee/40</td>
<td>&lt;20</td>
</tr>
</tbody>
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the initial 10% of the run discarded as burn-in [15] (Figure 4).

The phylogenetic analysis of the influenza virus isolated from Brazilian birds samples are represented by the numbers 6712 and 6744, whose sequences of the NS1 and M1 protein compared with sequences available in the GenBank, were used to construct a phylogenetic tree (Figure 4). Acces:(H1N1) NS 004297.1- M CY004295.1, (H2N2)NS M80945.1- M12699.1,(H3N2)1 NS CY114465.1-M DQ 508836.1,(H3N2)2 NS CY121028.1, (H3N2)3NS CY113481.1-MCY113478.1, (H3N2)4 NSCY003545 - CY003545, (H3N2)5 NSNCY035234.1-CY 035231.1,(H3N8)NS AY427300.1-M AY 427299.1,(H4N1) NS AY633288.1- (H4N6) MAY669484, A664484.1,(H5N1)NS AF509066.1/(H5N1)M AF509040.1, (H6N1) NS AF262212.1- MAF262213.1, (H7N1)

RESULTS AND DISCUSSION

Based on the results obtained, we observed that wild and migratory birds are the most representative species for the transportation and local dissemination of the influenza virus over the Brazilian territory. These virus isolates presented from 96% to 100% homology with influenza A virus (Table1). The sequencing analysis of NS1 and M1 regions from two influenza A virus isolated

Phylogenetic Analysis from NS1 and M1 regions

Subsequent to the PCR product purification and the partial sequencing of the influenza virus from the NS1 and M1 genes obtained from LE 6712 (Elaena mesoleuca) and LE 6744 (Sporophila caerulescen) isolates, we performed multiple alignment using Clustal W Neighbor Joining (NJ) trees were constructed based on the best-fit model of molecular evolution estimated by the Model test version 3.7 with Phylogenetic Analysis Using Parsimony (PAUP) version 4b10 [14]. To determine the support for each clade, a bootstrap analysis was performed with 1000 replications.

Phylogenies of concatenated M1- NS1 sequences were constructed by the Bayesian 2001, software package for phylogenetics using a general time-reversible (GTR) model of nucleotide substitution with a proportion of invariant sites and gamma distribution of rate parameters. We ran the Differential Evolution Markov Chain algorithm search for 107 generations with

![Figure 1](image1.png)

**Figure 1** Primers used for RT-PCR and nucleotides sequencing of influenza virus.

![Figure 2](image2.png)

**Figure 2** Ultra-structural analysis of the samples LE 6712 isolates from bird replicated in embryonated egg. Particles: 30 to 120 nm (340.000X100).

![Figure 3](image3.png)

**Figure 3** Ultra-structural analysis of the LE 6744 samples isolates from bird replicated in culture cell. Particles: 30 to 120nm (340.000X100nm).

![Figure 4](image4.png)

**Figure 4** Phylogenetic Tree for concatenated M1 - NS1 sequences of influenza virus with comparison between the positive samples and the gene-bank sequencing samples using evolution TVM+G model. Obs: The samples 6712 and 6744 compared with 36 sequences of NS1 and M1 regions available in the GenBank (NCBI) was used to construct a phylogenetic tree presented homology between these isolates with H3N2 subtype - bank sequencing samples of Siena_1991, Vitoria_1990, Beijin_1989.
from these birds, in comparison with sequences of all subtypes of influenza A available from public databases, presented homology between these isolates with H3N2 subtype–Genbank samples of Siena_1991, Vitoria_1990, Beijin_1989 (figure 4). For the next steps, it will be necessary to perform the sequence analysis of others genes by PCR products in order to obtain more information on this influenza issue. By the electron microscopy were observed particles around 30 to 120 nm surrounded by spikes typical of influenza virus (Figures 2 and 3).

The molecular analysis doesn’t revealed positivity to parainfluenza virus in these evaluated birds samples.

CONCLUSION

Hence, it is possible to conclude that birds play a role as a reservoir of the influenza virus and that they have been responsible for the replacement of the avian influenza gene pool in this studied area. In the past, serological tests performed in the same species, *Elaenia mesoleuca*, revealed the presence of antibodies against influenza A [6]. These observations suggest that *Elaenia mesoleuca* may have been involved in the local dissemination of the influenza virus for more than forty years in the same area where they were captured that time and in other areas. These two species of birds are the most abundant population of in Brazil, and are also found in other neighboring countries [16].

For the next steps, performing sequence analysis of the HA, NA, PB1, PB2, PA and NP genes by PCR products is required to identify the sample subtypes.

Thus, it could assume that the same bird flu virus may play a role in providing genetic diversity to many kinds of animals such as ducks, turkey’s and chikens [17].

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