Brazilian Journal of Microbiology (2005) 36:88-93
ISSN 1517-8382

INVESTIGATION OF INFLUENZA IN MIGRATING BIRDS, THE PRIMORDIAL RESERVOIR AND TRANSMITTERS OF INFLUENZA IN BRAZIL

Adélia Hiroko Nagamori Kawamoto[1]*; Dalva Assunção Portari Mancini[1]; Luiz Eloy Pereira[2]; Aurora Marques Cianciarullo[3]; Aurea Silveira Cruz[2]; Andrea Luppi Fernandes Dias[1]; Rita Maria Zucatelli Mendonça[1]; José Ricardo Pinto[1]; Edison Luiz Durigon[4]

[1]Laboratório de Virologia, Instituto Butantan, São Paulo, SP, Brasil; [2]Seção de Vírus Transmitidos por Antrópodos, Instituto Adolfo Lutz, São Paulo, SP, Brasil; [3]Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil; [4]Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil

Submitted: February 02, 2004; Returned to authors for corrections: February 28, 2005; Approved: March 28, 2005

ABSTRACT

Birds are the most important reservoirs of the influenza virus. Its maintenance in its natural hosts, including man, allows the influenza virus to reassorts its strains. The recent report of an avian influenza A (H5N1) virus in humans, was in a child with fatal respiratory illness in China, 1997. The current study was conducted to elucidate the transportation of the influenza by birds that migrate, annually, through the both Northern and Southern hemispheres, with special attention paid to the *Vireo olivaceus* [Juruviara(BR) or Red-eyed vireo(USA)] species, which travels from the USA to Brazil, and vice versa, and the *Elaenia mesoleuca* [Tuque(BR) or (USA)] species that flies over the entire Southern Hemisphere. There are two species of birds, which breed and migrate in São Paulo State, Brazil, and which were demonstrated to carry Influenza virus, were selected. The viral particles isolated were observed by electron microscopy. The influenza virus was detected by the House Duplex/PCR and Gloria molecular biology tests. The results demonstrated that the *Elaenia mesoleuca and Vireo olivaceus* bird species are carrying the Influenza virus whilst crossing both the Northern and Southern hemispheres. To understand the role that these migrating birds may play in epidemic influenza, in Brazil, characterization of avian influenza subtypes will be done.

Key words: migrating birds, Brazilian avian influenza virus

INTRODUCTION

Fifteen subtypes of hemagglutinin (HA) and nine subtypes of neuramidase (NA) have been described for the influenza virus type A, until now. Since 1933, serarchaeology studies and virus isolation have demonstrated that only the H1, H2 and H3 virus subtypes are maintained in humans out all subtypes have been isolated from avian species (20). Sequence analysis of the influenza viruses has shown that the common ancestor for these three types of the influenza originated from the avian virus (16). The accumulated evidence indicates that the avian reservoir of all the influenza subtypes recognized have been recorded in birds in most of the possible virus combinations. In addition, the fact that South American migrating birds have been infected by the Influenza virus during the summer in North America supports this theory (21).

Accordingly, it has been assumed that the same avian influenza viruses may play a role in the evolution of new pandemic strains among humans. Emerging viral strains may cross the species barrier, where they can cause disease and may, on occasion be fatal in humans (19).

The emergence of new strains of the influenza virus has been determined by interspecies transmission, principally from migrating birds (6).

Hatta and Kawaoka (7) reported, after the fatal case with A(H5N1), in China, 1997 (19), that avian influenza viruses

*Corresponding Author. Mailing address: Av. Vital Brazil, 1500, Butantã. 05503-900, São Paulo, SP, Brasil. Tel.: (+5511) 3726-7222 – Extn. 2152. E-mail: labvirol@butantan.gov.br; dapmancini@butantan.gov.br

reappeared in poultry markets of Hong Kong, in 2002, without human infection, but that the acquisition of properties of human viruses by the avian viruses, currently circulating in Southeast China, might result in a pandemic.

Webster (22), suggested that international based surveillance provides clues as to how to reduce interspecies transmission of influenza, due to the separation of aquatic birds from other "land based" birds. Animal husbandry practices may also influence in this situation.

Perkins and Swayn (12), investigated the ability of the zoonotic influenza A/Chicken/Hong Kong/220/97(H5N1) to increase the pathogenicity to other animals. This viral strain is carried by different species of birds.

In São Paulo city, Brazil, 1971, 1976, Aranku (1,2), demonstrated the presence of avian influenza by serologic studies in wild, resident, migrating birds and domestic chickens which presented antibodies against A/duck/56. In Rio de Janeiro city, Brazil, 1980, the first isolation of influenza was reported from the stools of wild ducks (*Dendrocygna viaduta*) and in the cages of exotic birds. These agents isolated were antigenically related to both strains of A/turkey/Massachusset /65 (H6N2) and A/duck/England/63(H1N4) (13).

Recently, in 1996, an influenza surveillance study demonstrated a number of samples from wild, resident and migrating birds in Brazil to be positive for this virus. These interesting findings reveal the necessity for monitoring birds as reservoirs for influenza, with the potential of allowing the emergence of new viruses in the avian population with an epidemic potential for humans. This matter in question represents the aim of this present study.

In this present work it was allowed to verify in those birds isolates the presence of the influenza virus by either the House-Duplex RT/PCR Test or Influenza A/B Rapid Test (GLORIA).

MATERIALS AND METHODS

Bird capturing and sample collections

From thirthy seven birds captured, was select these species as follow: *Elaenia mesoleuca(2), Vireo olivaceus(2), Sporophila lineola(2), Sporophila caerulescens(2), Columbina talpacoti(3) and Paroaria dominicana(3),* for influenza investigation. These birds frequent the experimental camp stations located in Tiête Ecologic Park-Guarulhos, Iguape and Juquitiba, all situated in the State of São Paulo, Brazil (Fig. 1). The samples were gathered between the months of November 1997 and January 1998. *Vireo Olivaceus* was considered the most important carrier of the Influenza virus, since this species travels between both the Northern and Southern hemispheres. Whilst the *Elaenia mesoleuca* that is original from Canada, and migrated into Southern America, Brazil, Uruguay and Argentina (Fig. 2) (8).

Birds were captured with Japanese mist nets set up in different types of Brazilian habitat, according to the regulations

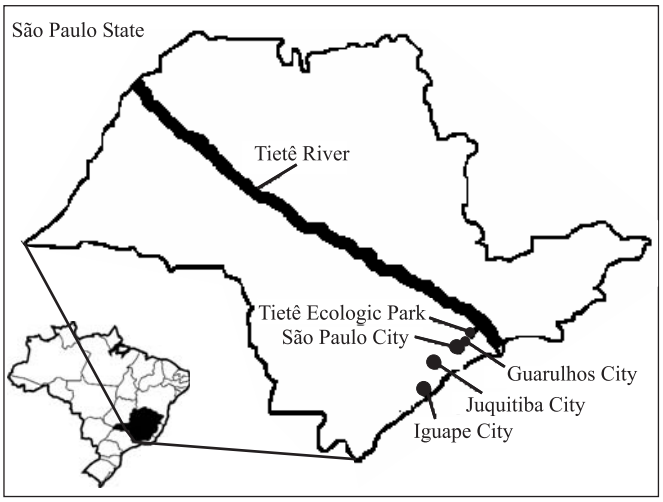

Figure 1. Birds capture collection areas.

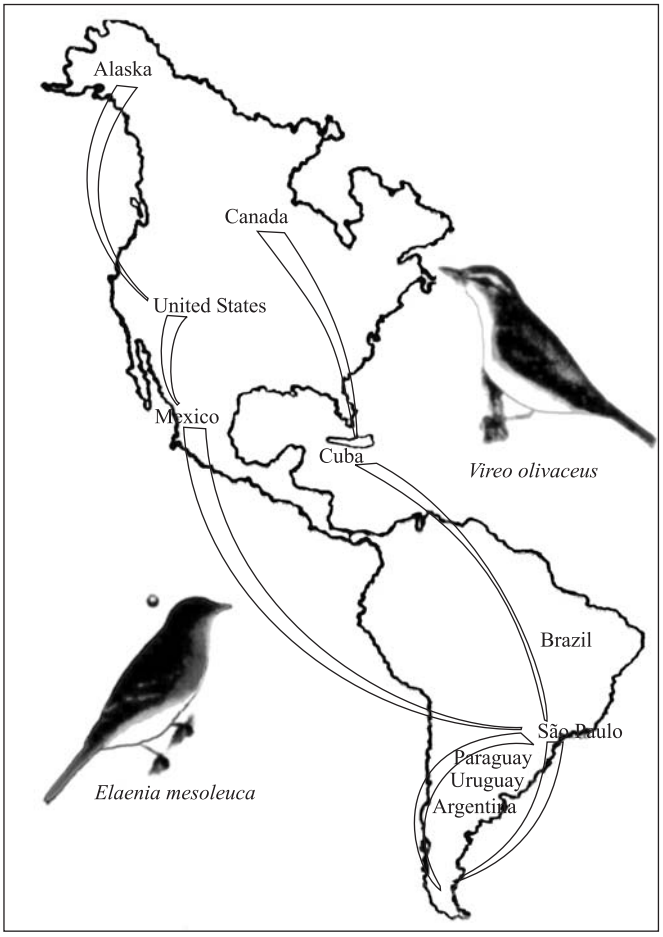

Figure 2. Geographic distribuition of the *Vireo olivaceus* and *Elaenia moseleuca* birds migration route.

of IBAMA (Brazilian Animal Protection Institute) (11). The mist net with a width of 12 meters and height of 3 meters was exposed for a period of 30 hours/net for bird capture.

A total of 69 samples were collected from 37 birds which included wild, resident and migrating species. Oral-tracheal and cloacal swabs were collected with cotton on metal sticks and placed in 199 buffer solution (pH 7.2) containing 1.0% bovine albumin, 2000 units of penicillin G, 2.5 µg of streptomycin, 6 µg of gentamicine and 25 µg of fungizone per ml and stored in a nitrogen container at -196º until laboratory manipulation.

Influenza isolation in cell culture (10)

Samples (0.2 mL) were used to inoculate two types of tissue cultures: MDCK (canine kidney) (ATCC-CCL34) and NCI H 292 ("Carcinoma Mucoepidermóides" from human lung) (ATCC-CRL 1848) in the presence of trypsin. Both cell cultures were incubated at 33ºC. The cells inoculated were observed daily. After three passages, if cytopathic effect was not detected, the hemadsorption was not seen or hemagglutinin activity was absent in both cultures, these samples were considered negative the presence of influenza virus.

Identification of isolates

Hemagglutination test(HA)(9)

Hemagglutination titers were determined at room temperature in a microtiter system. Serial two fold dilutions of virus (25 µL) in phosphate buffered Ph 7.2 were mixed with 25 µL of a suspension of 0.5% of rooster erythrocytes. Hemagglutination titers were determined after 1 h., and are expressed as the reciprocal of the maximun dilution of virus that caused complete agglutination.

Hemadsorption test (10)

The cell cultures, MDCK were tested after 2 days of the isolates inoculation. 0.1 mL of a 0.5% suspension of erythrocytes of guinea pig were deposited on the cell line infected. After 30 minutes at 4ºC, is possible to detect hemadsorption hemagglutinin.

Ultrastructural detection of virus particles - negative staining (4,5)

A digital pippete was used to place a drop (10 µL) of viral suspension on the collodion-carbon-coated surface of a copper grid. Excess fluid was removed with torn filter paper. A drop (10 µL) of negative stain (PTA) was then added. Excess fluid removed with torn filter 1 h, then placed at 4ºC overnight, and then examined in the electron microscope Zeiss EM 109, operated at 80 KV, and the virus particles were photographed.

Influenza a/b rapid test (GLORIA: gold labeled- optically- read-immunoassay)

Influenza virus isolates were characterized by Influenza A/B Rapid Test (Roche Laboratories), as follows: the test principle is based on the Roche diagnostics: GLORIA (gold-labeled-optically-read-immunoassay). In this test is detected the viral nucleoprotein and viral nucleic acid, that are released by lysing the influenza virus envelope with Lysis/Elution Solution. The test uses two pairs of monoclonal antibodies to specific influenza A and other specific influenza B. Both antibody pairs are conjugated to either biotin or digoxigenin. In the presence of the viral antigen, a sandwich complex is formed, consisting of the biotin- conjugated antibody, the nucleoprotein, and the digoxigenin- conjugated antibody. When the test strip is placed in the reaction cup, the complex migrates chromatographically, with solubilization of the colloidal gold particles incorpored in the red pad of the strip. The colloidal gold particles bind to the digoxigenin of the complex, which is then bound by the biotin to the immobilized streptavidin on the strip (positive result line). Any excess gold particles continue to migrate to the second line (control line), which then becomes visible. This indicates the correct chromatographic migration. None cross reactivity occurs with other probable respiratory viruses or other organisms as bacteria or fungi, as is informed in the package insert of the Influenza A/B Rapid Test Kit.

RNA viral extraction (3)

Viral RNA was extracted from both influenza (medium titer = 64 HAU) and Newcastle (128 HAU) virus strains by technique utilizing a mixing of 250 µL of the virus suspension and 750 µL of the Trizol reagent (Gibco, BRL) with the addition of chloroform 10% (Merck). The samples were then mixed thoroghly homogenized (sample+trizol) by vortexing for 15 sec and kept on ice for 5 min, after this they were centrifuged at 12000 g for 15 min at 4ºC. The supernatant was removed avoiding the interfase. Cold isopropanol was then added (Sigma®) v/v and mixed by vortexing for 5 sec. Samples were then incubated for 15 min on ice before centrifuging at 12000 g for 15 min at 4ºC and discarding the supernatant. To this pellet, 800 µL of cold ethanol was added (75%), Merck® and mixed by vortexing for 15 min (increasing the velocity enough to ressuspend the pellet), before centrifuging at 9000 g for 8 min at 4ºC and discarding the supernatant.

Finally, 20 µL of distilled water DNAse, RNAse free was added plus 1 µL of Ribonuclease inhibitor (RNAsin- Promega®) and triturated well with a pipette until the pellet dissolved. This material was stored at -70ºC until performance of the reverse transcription technique. The calcution RNA was based on spectrophotometric analysis at 260 nm.

House duplex reverse transcription/PCR (18)

REVERSE TRANSCRIPTION/PCR was performed in two steps. For the first pre-transcription step, 4 µL of each viral RNA was added to 5 µL of primer P1F(NDV) plus 5 µL of primer PcDNA (flu) and 1 µL of TNE, totaling 15 µL. The reaction was inactivated at 95ºC for 3 min followed by 50ºC for 15 min for hybridization in a Gene Am PCR System 2400 thermocycler (Applied Biosystems

Inc., USA). The pre-transcripts were kept on ice. In the second step at the 15 µL of the pre-transcripts were added 200U of (RT) (SuperScript™ II – Gibco BRL®) diluted in buffer enzyme (50mM Tris HCl (pH 8.3), 7.5mM KCL, 3mM MgCl2); 10mM DTT; 1.5 mM of each dNTP (dATP,dGTP,dGTP, dTTP,dCTP), dCTP) Gibco BRL and 20U of Ribonuclease Inhibitor (RNAase OUT-Gibco BRL®), totaling a volume of 40 µL. The transcription was performed at 42ºC for 1 h in the same thermocycler followed by 5 min at 95ºC for RT inactivation. The cDNA was stored at -70ºC. Rnase free water was used as control.

PCR REACTION: 10 µL cDNA were amplified in a volume of 100 µL containing 10 µL PCR buffer 10X 20mM Tris-HCl pH 8.4, 50 mM KCl, 3.0 µL 1.5 mM $MgCl_2$, 16.0 µL 1.25 mM dNTP, 49.5 µL H_2O DEPC, 2.0 µL primer P1F(-NDV), P2R(+NDV) 2.0 µl PcDNA(-; FLU), 4.0 µL primer REV(+; Flu) and 0.5 µL (2.5U) Taq polimerase. This reaction mixture was warmed at 94ºC for 5 min and 35 cycles of 1.5 min at 94ºC, 35 cycles of 2 min at 50ºC and 5 min at 72ºC. The amplified product was analyzed by 1.5% agarose gel eletrophoretic run 100V and bands stained with 0.5 µg/mL ethidium bromide, were documented by Gel Documentation System 1000 (Mode: Eagle Eye II – Stratagene – USA).

RESULTS AND DISCUSSION

Of the 37 birds studied (a total of 69 samples: 32 oral-tracheal and 37 cloacal), 10 presented positive results in one or both of types of the samples, in cell culture of either NCI-H 292 or MDCK cells, with a cytopathic effect and activing hemadsorption test. Each viral isolate cultivated in cell culture lines was tested for hemagglutination test with a suspension of 0.5% rooster erythrocytes, presenting an hemagglutinin titer of higher than 4HAU (Table 1).

The isolates that replicated in cell culture were examined by electron microscopy. Their appearance was spherical with a diameter of 35 to 90 nm. The surface of the particles was covered with short spikes, typical of the influenza viruses (Fig. 3).

The 10 hemagglutinating agents from 37 birds was tested House Duplex PCR and Gloria Tests. In both tests, the isolates from both the migrating bird species, *Elaenia mesoleuca* and *Vireo olivaceus*, tested positive for influenza virus (Fig. 4).

All isolations performed on samples from the tracheal and cloacal swabs of the birds presented positivity in 10 of 37 birds. Thus, 27% positivity is very a much higher rate than those found by Webster (20) (1.6%); Yamane (23) (4.12%); Graves (6) (2.06%) and Sinnecker (10.5%) (17). The majority of the isolates were obtained more from the cloacal (71.4%) than from oral-tracheal swabs (28.6%), also verified by these cited authors.

In the past, serological tests performed on *Elaenia mesoleuca* and on chickens revealed the presence of antibodies against influenza A/England/57 (1,2,11). In our study, we isolated Influenza A from two birds of the same species (LE6712 and LE6715) cited. These observations suggest that these birds may have been involved in the dissemination of the influenza virus during twenty eight years in the same area where they were captured in that time and recently in São Paulo State, Brazil.

The Influenza virus was isolated from two *Vireo olivaceus* migrating birds, which are native to the American continent.

Table 1. Samples collected from species of birds captured in different areas and habitats of São Paulo State, Brazil, with positive results for influenza virus(e"4hau).

Samples	Scientific name	Popular name		Collection		
		Brazilian	American	Habitat	Area	Date
LE6712(cloacal)	*Elaenia mesoleuca* M	Tuque	Olivaceus elaenia	Wood	Guarulhos	04/11/97
LE6715(cloacal)	*Elaenia mesoleuca* M	Tuque	Olivaceus elaenia	Wood	Guarulhos	04/11/97
LE 6744(oral)	*Sporophila lineola w*	Bigodinho	Lined seedeater	Coop	Iguape	12/11/97
LE 6744(cloacal)	*Sporophila lineola* W	Bigodinho	Lined seedeater	Coop	Iguape	12/11/97
LE 6745(oral)	*Sporophila caerulescens* M	Papa capins	Doble collared seedeater	Coop	Iguape	12/11/97
LE6745(cloacal)	*Sporophila caerulescens* M	Papa capins	Doble collared seedeater	Coop	Iguape	12/11/97
LE6751(cloacal	*Vireo olivaceus* M	Juruviara	Red eyed vireo	Coop	Iguape	12/11/97
LE 6781(oral)	*Columbina talpacoti* M	Rolinha caldo de feijão	Ruddy ground dove	Wood	Guarulhos	12/11/97
LE 6781(cloacal)	*Columbina talpacoti* M	Rolinha caldo de feijão	Ruddy grounbd dove	Wood	Guarulhos	12/11/97
LE6782(cloacal)	*Paroaria dominicana* R	Galo de campina	Red cowled cardinal	Wood	Guarulhos	12/11/97
LE 6783(oral)	*Paroaria dominicana* R	Galo de campina	Red cowled cardinal	Wood	Guarulhos	12/11/97
LE6783(cloacal)	*Paroaria dominicana* R	Galo de campina	Red cowled cardinal	Wood	Guarulhos	12/11/97
LE 6784(cloacal)	*Columbina talpacoti* M	Rolinha caldo de feijão	Ruddy ground dove	Wood	Guarulhos	12/11/97
LE6840(cloacal)	*Vireo olivaceus* M	Juruviara	Red eyed vireo	Wood	Juquitiba	28/01/98

R -Resident birds W - wild birds M - migrating birds.

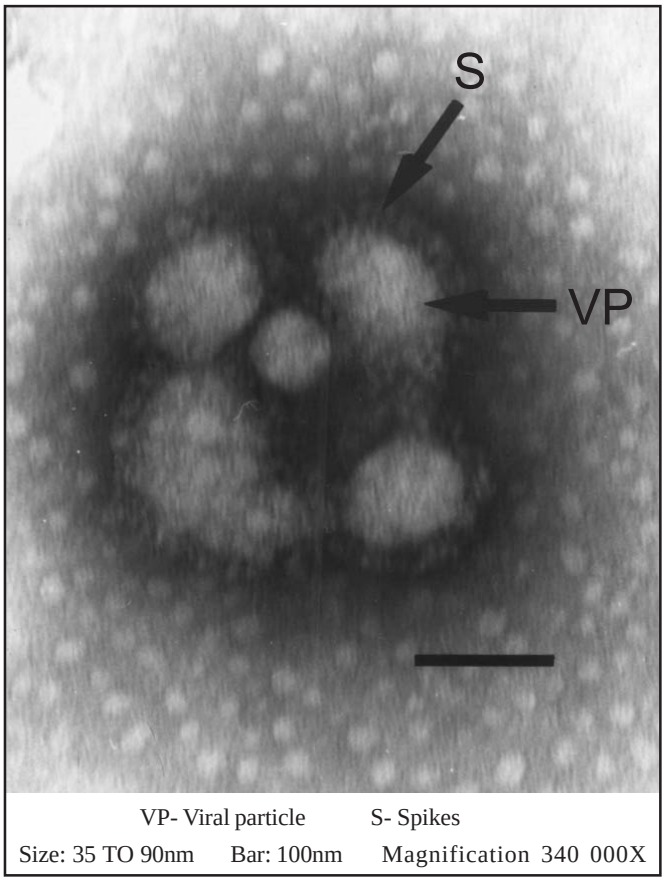

Figure 3. Micrografy of negative stain, bird sample - LE 6840.

These birds reside in Brazil from September to May, and reports of their presence in North America lead us to speculate the broad spread of the influenza virus that may occur during this journey.

Influenza can be transmitted by the faecal-oral route in untreated water, which could represent the major method of its transmission from the migrating birds to domestic fowl and pigs or even to man, as was reported by Silvanandan (15).

In addition, the presence of the domestic and lower mammalians in this study area where the avian species were infected with Influenza, may act as the reservoirs of the new pandemic strains of human influenza viruses, as suggest by Webster (21).

Whilst the number of the birds studied from each species was relatively small, the positivity demonstrated for influenza virus represented relatively large proportion of the migrating birds (4 out 10 of birds were positive for influenza). This result is in agreement with Sharp (14), who reported that these species may participate in the transport of these agents and the maintenance of its interspecies cycle in several regions and countries, at long distance.

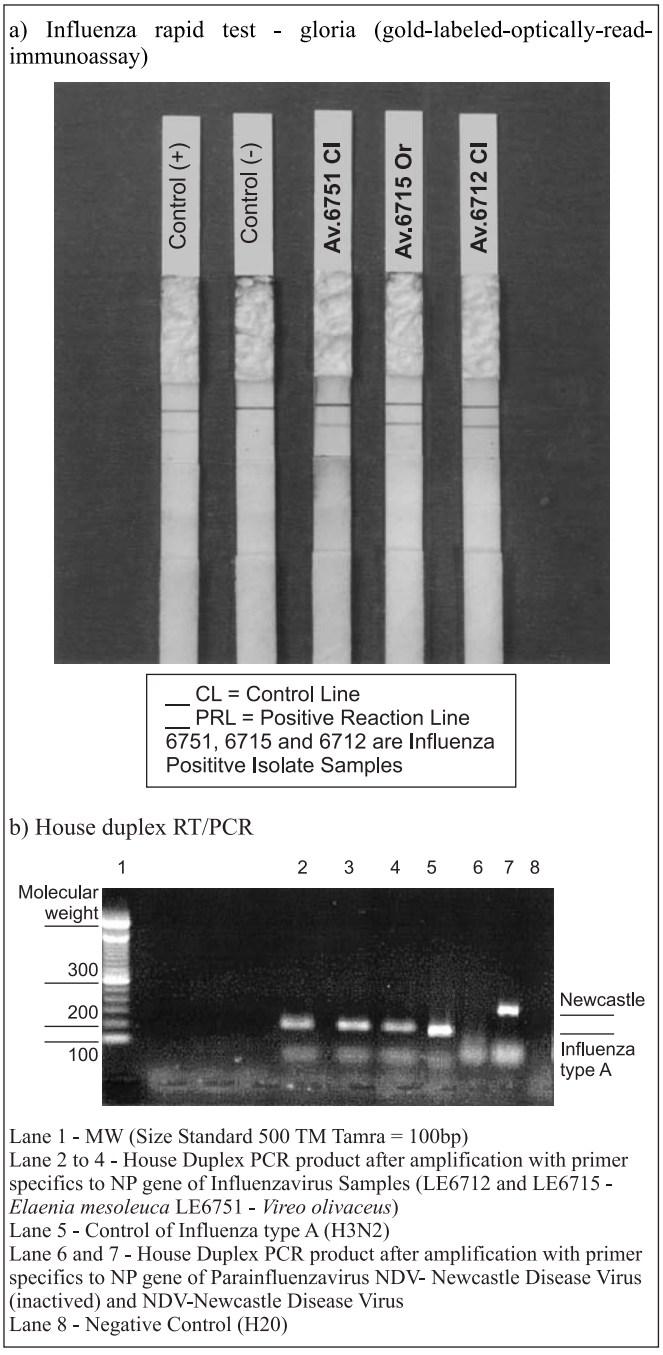

Figure 4. Influenza detection by gloria rapid test and house duplex PCR

Further study is in process in order to obtain the characterization of the subtype of these influenza isolates in these migrating birds. Molecular methods, such as nucleotide sequencing is being used to verify whether the circulating influenza is being brought to Brazil or carried out of the country.

ACKNOWLEGMENTS

The authors are grateful to Dra Akemi Suzuki and Renato Pereira de Souza for gathering of the samples. This study was supported by: Adolfo Lutz Institute, Butantan Institute and Science Biomedicine of the University of São Paulo - SP - Brazil.

RESUMO

Investigação de influenza em aves migratórias, principal reservatório e transporte de influenza no Brasil

Os mais importantes reservatórios do vírus influenza são os pássaros. A manutenção do vírus influenza em hospedeiros naturais, inclusive o homem, permite que esse vírus realize rearranjos entre as suas cepas. O recente relato de uma cepa influenza aviária A(H5N1), em humanos, se deu em uma criança com doença respiratória fatal, na China em 1977. O presente estudo foi conduzido para elucidar o transporte da influenza por pássaros que migram, anualmente, através de ambos hemisférios o do Norte e do Sul, com especial atenção voltada à espécies *Vireo olivaceo* [Juruviara(BR) e Red-eyed vireo(USA)] que viaja do USA para o Brasil, e vice-versa, e a espécie *Elaenia mesoleuca* [Tuque(BR) e (USA)] que voa por todo o Hemisfério Sul. Essas espécies de pássaros, que residem e migram em São Paulo, e que demonstram transportar o vírus influenza, foram selecionadas. As partículas virais isoladas foram observadas por microscópio eletrônico. O vírus influenza foi detectado pelos testes: House Duplex/PCR e Gloria. Os resultados revelam que os pássaros das espécies: *Elaenia mesoleuca* e *Vireo olivaceus* são transportes do vírus influenza enquanto cruzam ambos Hemisférios. Para o conhecimento da função que os pássaros migratórios podem desempenhar na epidemia de influenza, no Brasil, caracterização dos subtipos deste vírus estão sendo realizados.

Palavras chave: aves migratórias, vírus da influenza em aves brasileiras

REFERENCES

1. Aranku, M.M.C; Faria, W.DE Carmo; Takeyama, D.C. Influenza Aviária em aves silvestres brasileiras I – Inquérito sorológico através de Imunodifusão. *Rev. Inst. Med. Trop. São Paulo*, 13, 292-96, 1971.
2. Aranku, M.M.C.; Pinto, A.A.; Godoy, C.V. de Franco; Hipólito, O. Influenza tipo A em galinhas: inquérito sorológico através da Inibição da Hemaglutinação e da Imunodifusão. *Rev. Inst. Med. Trop. São Paulo*, 18, 6-9, 1976
3. Claas, E.C.J.; Sprenger, M.J.W.; Kleter, G.E.M.; Van Beek, R.; Quint, W.G.V.; Masurel, N. Type-specific identification of influenza viruses A, B and C by PCR. *J. Virol. Meth.*, 39, 1-13, 1992.
4. Doane, F.W.; Anderson, N. Methods for preparing specimens for Electron Microscopy. Chapt 3. *In:* Electron Microscopy in Diagnostic Virology: A practical Guide and Atlas. Edited by Cambridge; Cambridge Univ Press., 1987, pp.21-29.
5. Fonseca, M.E.F.; Frimer, N.; Mendonça, R.E.A.; Couceiro, J.N.S.S.; Machado, R.D. A combined staining technique developed for vírus particle observation in the Electron Microscope. *Rev. Bras. Biol.*, 44, 37-40, 1984.
6. Graves, I.L. Influenza Viruses in birds of the Atlantic flyway. *Avian Dis.*, 36, 1-10, 1992.
7. Hatta, M.; Kawaoka, Y. The continued pandemic threat posed by avian influenza viruses in Hong Kong. *Trends Microbiol*., 10(7), 340-344, 2002.
8. Höfling, E.; Almeida, H.F. Aves no Campus. Ed. Universidade São Paulo, São Paulo, 3ª ed. P.80, 112, 2002.
9. Mancini, D.A.P.; Mendonça, Z.M.R.; Pinto, R.J.; Almeida, De Teixeira, S. Influenza eqüina: avaliação da resposta imunohumoral, através das reações de Inibição de Hemaglutinação e de Hemólise Radial Simples, em soro de animais vacinados com vacina comercial e experimental. *Bras. J. Vet. Res. Anim. Sci.*, 33(1), 36-40, 1996.
10. Minnich, L.L.; Ray, C.G. Early testing of cell cultures for detection of hemadsorbing viruses. *J. Clin. Microbiol.*, 25, 421-2, 1987.
11. Pereira, E.L.; Suzuki, A.; Souza, De P.R.; Souza, G.C.F.M.; Flauto, G. Sazonalidade das populações de *Vireo olivaceus* (Linnaeus, 1766) (Aves, Vireonidade) em regiões da Mata Atlântica do Estado de São Paulo. *Ararajuba*, 6, 117-22, 1998.
12. Perkins, L.E.; Swayne, D.E. Varied pathogenicity of a Hong Kong origin H5N1 avian influenza virus in four passarine species and budgerigars. *Vet. Pathol.*, 40(1), 14-24, 2003.
13. Salcedo Chaves, J.R. *Ocorrência de influenza em aves selvagens e pássaros ornamentais no Rio de Janeiro*. Rio de Janeiro, 1980. (Master Thesis, Universidade do Rio de Janeiro).
14. Sharp, G.B.; Kawaoka, Y.; Jones, D.J.; Bean, W.J.; Pryor, P.S.; Hinshaw, V.; Webster, R.G. Coinfection of wild ducks by Influenza A viruses: distribuition patterns and biological significance. *J. Virol.*, 71(8), 6128-35, 1997.
15. Silvanandan, V.; Halvorson, D.A.; Laudert, E.; Senne, D.A.; Kumar, M.C. Isolation of H13N2. Influenza A virus from turkey and surface water. *Avian Dis.*, 35, 974-7, 1991.
16. Sholtissek, C. Source for influenza pandemics. *Eur. J. Epidemiol.*, 10, 455-8, 1994.
17. Sinnecker, R.; Sinnecker, H.; Zilske, E.; Köhler, D. Surveillance of pelagic birds for Influenza A Viruses. *Acta Virol*., 27, 75-9, 1983
18. Soares, P.B.M. *Padronização da RT- PCR duplex para detecção dos vírus da influenza A e doença de Newcastle em aves migratórias*. São Paulo, 2002, 32-38p. (Master Thesis, Instituto de Ciências Biomédicas da Universidade São Paulo).
19. Subbarao, K.; Klimov, A.; Katz, J.; Regnery, H.; Lim, W.; Hall, H.; Perdue, M.; Swayne, D.; Bender, C.; Huang, J.; Hemphill, M.; Rowe, T.; Shaw, M.; Xu, X.; Fukuda, K.; Cox, N. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science*., 279, 393-6, 1998.
20. Webster, R.G.; Morita, M.; Pridge, S.; Tumova, B. Ortho-and Paramyxo Viruses from migrating feral ducks: characterization of a new group of Influenza A Viruses. *J. Gen. Virol.*, 32, 217-25, 1976.
21. Webster, R.G. Predictions for future human influenza pandemic. *J. Infect. Dis.*, 177 (suppl 1), S14-S19, 1997.
22. Webster, R.G. The importance of animal for human disease. *Vaccine*, 15 (20 Suppl.) 2, 516-20, 2002.
23. Yamane, N.; Odagire, T.; Arikawa, J.; Ishida, N. Isolation and characterization of Influenza A Viruses from wild ducks in northern Japan: appearance of HSW1 antigens in the japanese duck population. *Acta Virol.*, 23, 375-84, 1979.