Identification and characterization of influenza virus isolated from Brazilian snakes

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It has been reported that the reptilians can harbor pathogenic microorganisms asymptomatically and serve as potential reservoir of infection to humans, domestic animals and other reptilians. In this present study the aims was to search for an influenza virus in unknown hosts, among the heterothermic animals. Using samples collected from diseased snakes belonging to both genuses: Bothrops and Crotalus, kept in captivity at the Butantan Institute, Brazil, methodology was used for isolation, identification and characterization of the influenza virus. Several repetitions of the snakes' pulmonary washings was done with MDCK cells, observing the cytopathic effects (CPE) daily.The fluids harvested were evaluated by a Hemagglutination test (HA) and Serological tests such as the Influenza Rapid Test (Gloria) and Hemagglutination Inhibition (HI) using antibody anti-influenza A and B types patterns. The characterization of influenza isolates it was performed by the RT/PCR techniques, with primer PcDNA(flu) that amplifly a 189 bp fragment of NS1 gene. Electron Microscopy (EM) was employed to observe the viral particle ultrastructure. Results obtained through those methods showed that virus replicated in MDCK, showing CPE, with mean titer of 60HAU was comparable with the influenza virus. This virus identification was verified by the antibody to influenza viruses A and B patterns which recognized the antigen of the isolates, by both the Gloria and HI tests . The PCR product characterized the isolates as an the influenza virus that was compatible with the influenza virus used as control. Positive samples observed in EM revealed particles measuring 40 to 120 nm, with an envelope surrounded by spikes. These particles were similar to the human influenza virus particles.Based on these data and also considering the fact that these snakes had respiratory symptoms, it is possible to conclude that these animals could be a susceptible host and a harbour to the influenza virus. The present unpublished work was performed to collaborate with the global search for the influenza host circulation.

Keywords: Snakes; Influenza virus; Isolation; Identification.

Introduction

Respiratory diseases in snakes are complex, because if they are not immunocompetent to the infectious agents that are exposed, certainly these animals will have to confront a battle. The major cause of the snakes morbility is related to respiratory disease provoked by viruses. The viruses that are most cited belong to the *Paramyxoviridae*. Within this virus family, the *Paramyxovirus* as Parainfluenza – III, Senday virus, as well the Mumps and Measles virus are the most frequent isolates from the different species of snakes. It has been also reported that the reptilians can harbor pathogenic microorganism asymptomatically and serve as potential reservoirs of infection to humans, domestic animals and other reptilians.[1,2]

By the phylogenetic analyses, the ophidian *Paramyxovirus* identified by its nucleotide sequences, could be a factor for proposing a new genus for reptilian *Paramyxovirus* within the *Paramyxoviridae*.

This is because of the divergence observed in these virus antigens, and particularly the low homoly of the available sequences. [3,4,5]
In our previous work with heterothermic animals, such as snakes (genus *Bothrops* and genus *Crotalus*) and toads and frogs (genuses *Bufo* and *Rana*) all animals investigated were shown to have receptors in theirs red cells and antibodies specific to the influenza virus.[6]

The importance of investigating the unknown hosts of the influenza virus is the consideration of its recombinant feature that occurs, during the process of viral transmission, between both animals and humans. This present work aimed the isolation, identification and characterization of the influenza virus in two differents genuses of snakes, the *Crotalus durissus terrificus* and *Bothrops neuwiedi.*

Material and methods

Viral strains Pattern
Influenza virus- supplied by Bethesda (USA)Type A: A/Chile/1/83 (H1N1) (ANT#12) and A/Philippine/2/82 (H3N2) (ANT#11X79).
Type B: B/USSR/100/83 (ANT#13).

Anti-sera Pattern
Anti-sera to Influenza supplied by Bethesda (USA): Anti-A Ref: S-22 L-2 A/Chile/1/83 (H1N1) and Ref: S1670 A/Philippine/2/82 (H3N2); Anti-B Ref: S-26 B/URSS/100/83.

Animals [6]
Tree adult snakes: *Bothrops neuwiedi* (a and b) and *Crotalus durissus terrificus*(c) belong to Herpetology Department at Butantan Institute, São Paulo, Brazil, were used. The respiratory disease symptoms were present in both genuses.

Samples of sera [6]
The samples were harvested by the tail vein of the snakes using disposable needles: 25X7mm. After blood coagulation and retraction, the serum was removed and kept at –70°C until the time came to perform the serologic test.

Virus isolation [7]
The snakes' washing pulmonary samples were inoculated in monolayers of MDCK, NCI-H292 and VERO cells. These systems were incubated at 33-35°C during 3 to 7 days. Daily CPE (Cytopathic Effect) was observed. MDCK cells were choised considering theirs better virus sensitivity. After this, the fluids were harvested for HA test. The positives samples were submitted to the serologic tests as HI and Influenza A/B Rapid Test (Gloria) using influenza antisera patterns. These tests were performed as follow described.

Hemagglutination (HA) [7]
The virus hemagglutination titer was estimated at room temperature using a microtiter system. Serial, two-fold dilutions of the virus (25 µL) in phosphate buffered saline, pH 7.2, were mixed with 25 µL of a 0.5% suspension of rooster red blood cells. Hemagglutination titers were measured after 1 h, unless otherwise stated, and are expressed as the reciprocal of the maximum virus dilution that causes complete agglutination.

Hemagglutination Inhibition (HI) [6]
Sera pattern duplicate dilutions were carried out in series, in "V" bottom microplates. Antigens pattern of the influenza virus and the isolates cultivated in MDCK cells, containing 4 hemagglutinating units, were added to the wells. After one-hour reaction at room temperature, 5% rooster erythrocytes were added to the wells. Reading was processed after 30 min, with the reciprocal of the last dilution, which elicited

hemagglutination inhibition, being considered as the antibody titer. Those sera presenting antibody titers of 20 HIU or superior were considered positives. A constant volume of 0.025 ml was used for all reagents

Influenza A/B Rapid Test (GLORIA gold-labeled-optically-read-immunoassay) [7]

Influenza virus isolates were characterized by Influenza A/B Rapid Test (Roche Laboratories), as follow: 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, solubilizing 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 such as bacteria or fungi, as it is informed in the package insert of the Influenza A/B Rapid Test Kit.

Electron Microscopy (EM) [7]

Negative Staining- This is a direct application method for electron microscopy. A digital pipette was used to place a drop (10µl) of viral suspension on the collodion-carbon-coated surface of a copper grid. Excess fluid was removed with filter paper, and a drop (10µl) of negative stain (PTA) was added. Excess fluid was removed with filter paper. Then, the grid was air dried overnight., examined on the electron microscope and the virus particles were photographed.

RNA Viral Extraction [7]

Viral RNA was extracted 125 µl from both influenza virus isolates and parainfluenza virus-Newcastle virus strain utilizing a mixture of 250 µL sample and 750 µL Trizol reagent (Invitrogen®), and kept at room temperature for 5 min after the addition of 200 µL Chloroform (Merck®). The samples were vortexed for 15 s and held at room temperature for 5 min, then centrifuged at 15,294 X g for 15 min at 4 °C. Four hundred microlitres of supernatant were removed avoiding the interphase, and 500 µL of isopropanol (Synth®) were added and mixed by vortexing for 5 s . The samples were centrifuged at 15,294 X g for 5 min at 4°C and the supernatant discarded. One millilitres of 75% ethanol (Synth®) was added and mixed gently followed by centrifugation at 15,294 X g for 10 min at 4 °C, the supernatant again being discarded.

Finally, 20 µL of DNAse and RNAse free distilled water and 1µl of Ribonuclease Inhibitor (RNAsin-Promega®) were added and incubated at 50 °C for 10 min. The material was stored at –70 °C until the reverse transcription technique was performed.

HOUSE DUPLEX Reverse Transcription/PCR [7]

Reverse Transcription/PCR was performed in two steps. For the first pre-transcription step, 4.0 µl of each viral RNA was added to 5.0 µl of primer P1F [(-)NDV] plus 5.0 µl of primer PcDNA [(-)flu)] and 1.0 µl of TNE, totalizing 15.0 µl. The reaction was inactivated at 95 °C for 3 min followed by 50 °C for 15 min for hybridization in a Thermal Cycler (Bio-Rad®). The pre-transcripts were kept on ice. In the second step at the 15.0 µl of the pre-transcripts were added 200 U of (RT)(SuperScriptTM II – Gibco BRLR) diluted in buffer enzyme (50mM Tris HCl pH 8.3, 7.5 mM KCL, 3 mM MgCl2); 10 mM DTT; 1.5 mM of each dNTP (dATP,dGTP,dGTP,dTTP,dCTP) Gibco BRL and 20 U of Ribonuclease Inhibitor (RNAase OUT-Gibco BRL R). The transcription was performed at 42 °C for 1 h in the same thermocycler followed by 5 min at 95 °C for RT inactivation. The cDNA was stored at –70 °C.

PCR Reaction: 10.0 µl of cDNA were amplified in a volume of 100.0 µl containing 10.0 µl PCR buffer 10 X 20 mM Tris-HCl pH 8.4, 500 mM KCl, 3.0 µl 1.5 mM MgCl2, 16.0 µl 1.25 mM dNTP, 48.5 µl H2O DEPC, 2.0 µl primer P1F [(-)NDV], 4.0µl 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 heated using a Thermal Cycler (Bio-Rad®) to 94 °C for 5 min followed by 35 cycles as follows: 1.5 min at 94 °C, 2 min at 50 °C and 5 min at 72 °C, and 72 °C for 10 min. The mixture was then held at 4 °C for an indeterminate period. The amplified PCR product was analyzed by electrophoresis on 1.5% agarose gel (Sygma®) run at 100 V. The bands were stained with 0.5 µg/mL ethidium bromide, documented by Gel Documentation System 1000 (Model: Eagle II – Stratagene – USA)
Primers used:
Influenza virus (Flu)
REV [(+) Flu] (5'-3'): CCCATTCTCATTACTGCTTC
PcDNA [(-)Flu] (5'-3'): AAGGGCTTTCACCGAAGAGG
Parainfluenza virus (NDV)
P1F [(-)NDV] (5'-3'):TTGATGGCAGGCCTCTTGC
P2R [(+)NDV] (5'-3'):GGAGGATGTTGGCAGCATT

Table 1. Isolation and identification of the influenza virus from Brazilian snakes

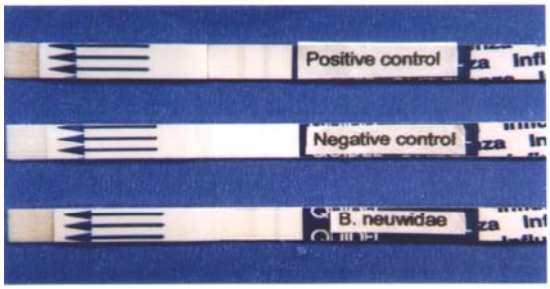
Passage Sample PW	1ª pass		2ª pass		3ª pass		4ª pass Cells		5ª pass MDCK		6ª pass		7 ª pass		8ª pass		Influenza A/B Rapid Test	EM
	CPE	HA	CPE	HA	CPE	HA	CPE	HA	CPE	HA	CPE	HA	CPE	HA	CPE	HA		
a	+	P	+	2	+	4	+	64	-	NR	ND	ND	ND	ND	ND	ND	-	+
b	+	P	+	8	+	8	+	64	+	4	+	P	+	8	+	128	+	+
c	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

P= Pure ND= Not Done PW=Pulmonary Washing EM=Electron Microscopy CPE=Cytopathic Effect HA= Hemagglutination NR= Not Reagent Snakes "a" and "b"= *Bothrops neuwiedi* Snake "c"= *Crotalus durissus terrificus*

Table 2 Recognition of the influenza types–A/B in snakes isolate using antigen/antisera patterns, by hemagglutination inhibition test.

Anti-sera to Influenza	HI TITER (HIU/µl) Virus sample isolate from Snake a	Snake b	Influenza antigens A/H_1N_1	A/H_2N_3	B
A / H1N1	320	320	1280	-	-
A / H3N2	160	640	-	2056	-
B	160	160	-	-	1280

Influenza Antigen and Anti-Sera Patterns A/H1N1 - A/Chile/1/82 A/H3N2 - A/Philippine/2/82 B-B/URSS/100/83 – Bethesda,USA

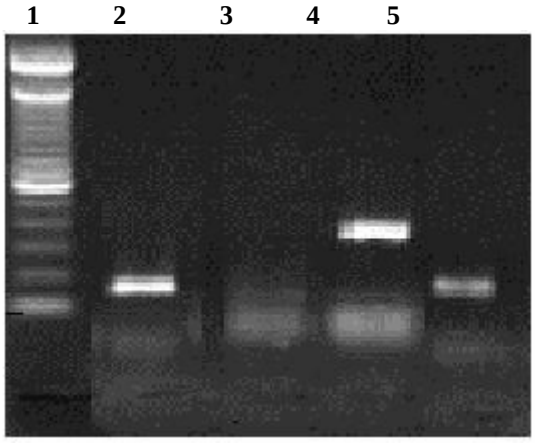


Positive reaction control [Influenza A(H3N2)]

Negative reaction (without antigen)

Positive reaction (Snake "b")

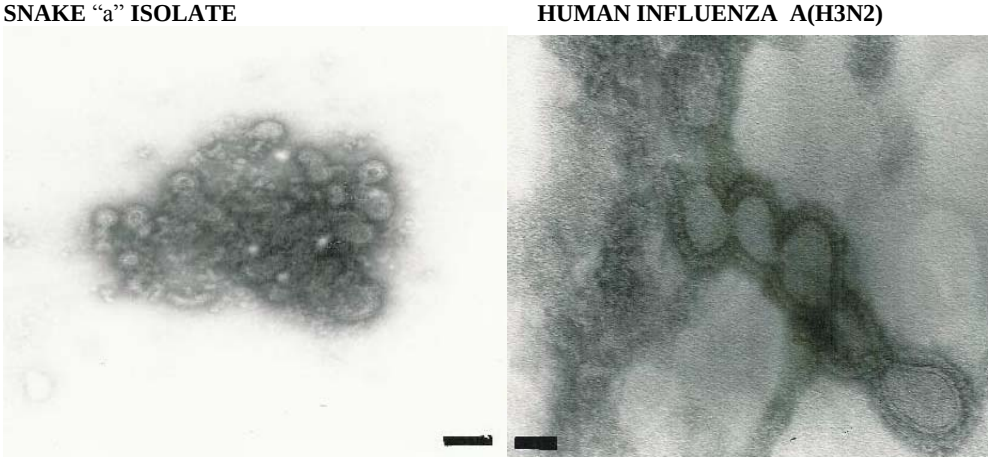
Figure 1 –Identification of the influenza virus in snakes samples by the influenza A/B rapid test (Gloria)



1-Ladder/Control 2- Influenza A(H_3N_2)
3- H_2O 4- New Castle 5- Snake"b"

Figure 2 - Characterization of the influenza vírus isolated from snake, by RT/PCR techniques.

SNAKE "a" **ISOLATE** **HUMAN INFLUENZA A(H3N2)**



Magnification : 106,500
Particles : 40 nm to 100 nm
Bar: 100 nm

Magnification: 260,400
Particles:40 to 100 nm
Bar: 100 nm

Figure 3 – Ultrastructure of particles isolated from snakes , visualized through the electron microscopy

Results and discussion

By the results obtained with both snakes' pulmonary washings (PW), it was verified that samples of both "a and b" snakes of *Bothrops* genus showing cytopathic effects (CPE) in MDCK cells, reach HA titers of 64 HAU for sample "a" and 128 HAU for sample "b". Concerning snake "c" of *Crotalus genus,* neither the CPE nor HA titer were observed with its PW sample (tab 1).

Through serologic tests as Hemagglutination Inhibition, as well as the Rapid Test (Gloria), it was observed that such fluids harvested from infected MDCK cells, showing positive HA titers, were recognized as the influenza antigen by anti-influenza (A or B types) serum patterns. The snake sample "b" was positive to the Gloria Test, revealing an influenza positive line on chromatographic strip (fig 1), and it also reacted with influenza antibodies at titers of 320 HIU, 640HIU and 160 HIU to types A (H1N1), (H3N2) and B, respectively. The snake sample "a" was not positive to the Gloria Test, but reacted with titers 320 HIU and 160 HIU, respectively, to the patterns of antibodies of both subtypes influenza A (H1N1), A (H3N2) and to type B.

Comparing results of the antigen patterns correspondent to the anti-type and subtypes of the influenza virus,it was observed that snake isolates demonstrated equivalent levels of influenza antibody recognition, by the HI test. Therefore, these snake isolates were identified as an influenza virus (tables 1 and 2).

The PCR product cDNA obtained from the snake "b" isolates by RT-PCR techniques, using primer (flu) that amplified 189 bp fragments of NS1 gene of the influenza virus, showed an electrophoretic band (180bp) correspondent to the band presented by the influenza A (H3N2) used as control. But, it was not compatible with electrophoretic band obtained by the Parainfluenza / Newcastle disease (NDV) with 200 bp (fig. 2). The Parainfluenza virus was used in the House-Duplex RT-PCR techniques, aiming to detect a possible mixed infection in these evaluated animals.

These *Bothrops* snakes isolate samples "a and b", identified and characterized as influenza virus, were examined by Electron Microscopy. The presence of particles measuring 40 to 120 nm, with spikes surrounding the envelope were visualized. The ultrastructures of these particles were similar to the human influenza virus particles (fig.3).

Conclusions

Considering these results and adding to the fact that these snakes have had respiratory symptoms, it is suggested that these animals could be a suceptible host to the influenza virus. Therefore, this finding indicates that the influenza viruses possess other hosts, beyond those natural classics reported as either aquatic or domestic avians (ducks, chickens, turkeys) as well as mammals (pigs, horses and humans).

From the literature, it is possible to conclude that reptilians, in general, can serve as potential reservoirs of infection for humans. But, nothing has been reported concerning influenza viruses harbouring these animals. Therefore, for at the first time influenza isolation is presented from heterothermic animal, such as the snakes kept in captivity at the Butantan Institute (SP, Brazil).

With this investigation, the authors of this work intend to collaborate with global influenza surveillance.

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