

# EVIDENCE OF SWINE RESPIRATORY INFECTION BY INFLUENZA VIRUSES IN BRAZIL

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**Key words:** Influenza in swines, swine respiratory infections, influenza in Brazil

## **ABSTRACT**

The global surveillance of influenza, maintained by a network of laboratories sponsored by the World Health Organization, considers pigs as the host responsible for the interspecies transmission of influenza virus, particularly with regard to the transmission of avian strains to humans. This capacity was observed in 1918 when the most tragic influenza pandemic occurred as the consequence of the transmission of the avian influenza strain A(H<sub>1</sub>N<sub>1</sub>) to humans, through pigs. This study was carried out with the aim of investigating the potential level of the influenza reservoir in Brazilian pigs. Either oro-nasal exudates or serum samples were collected from 34 pigs from different regions of São Paulo State, BR. These samples were evaluated by two methods: Virus isolation (passage in cell cultures) and the Hemagglutination Inhibition (HI) serological test. Results from the 34 oro-nasal samples obtained by the virus isolation method showed that all these pig exudates demonstrated the presence of influenza virus. The serological survey showed that these 34 pig sera samples presented inhibitor antibodies to different influenza virus strains with the distribution of the human influenza A subtypes (H<sub>1</sub>N<sub>1</sub> and H<sub>3</sub>N<sub>2</sub>) and type B of 85.29% and 91.17%, respectively. These sera also presented higher percentages (97.05%) of antibodies to specific strains isolated from some of the positive pigs. These data reveal that numerous different influenza virus strains, detected in the Brazilian pig populations, were probably introduced by avians or even through humans, during swine husbandry practices.

## **INTRODUCTION**

Pigs have been considered the leading contender as intermediaries, as these animals may serve as hosts for productive infections of both avian and human influenza virus infections. In addition, the evidence strongly suggests that pigs have been involved in the interspecies transmission of influenza viruses (Alexander & Brown 2000). Phylogenetic data indicate that Korean influenza A (H<sub>1</sub>N<sub>2</sub>), isolated from pigs, had an avian origin (Jung & Chae 2004).

A study concerning the evolution of swine A(H<sub>3</sub>N<sub>2</sub>) influenza viruses in USA investigated isolates of the virus and showed that the internal gene complex was associated with three recent phylogenetically distinct human-like hemagglutinin (HA) molecules

(Webby et al. 2000). In Europe and Asia, the human H<sub>3</sub> and avian H<sub>1</sub> influenza viruses have been frequently isolated from pigs. Due to swine husbandry practices, the maintenance of this virus has been possible (Brown 2000, Olsen et al. 2000).

All types of hemagglutinin (HA) of human, pig, horse and aquatic bird influenza viruses, recognize sialyl lacto-series type I and II sugar chains (Sialic acid – SA) alpha 2-3(6)Gal beta 1-3(4)Glc Nac beta 1) in glycoproteins and glycolipids in target cells such as common receptor molecules. Avian and equine influenza viruses preferentially bind the terminal sialic acid alpha 2-3 Gal (SA2- 3 Gal) linkage, while human influenza viruses preferentially bind the SA2- 6 Gal linkage. This SA distribution in animal species influences the influenza virus host range. The swine trachea has receptors for both avian influenza viruses (SA2- 3 Gal specific) and for the human influenza viruses (SA2- 6 Gal specific); possibly allowing pigs to adapt the avian influenza viruses to humans and facilitate its tropism mechanisms (Suzuki 2000).

Considered as the “mixing vessel” of influenza viruses, pigs have been responsible for the majority of influenza pandemics in human populations. Since as long ago as 1918, up to recently, pigs represent a problem in the control of the influenza virus all over the world (Ito et al. 1998, Meulaman 1999, Zhou et al. 1996). A serum survey of pigs from Germany showed that antibodies against the Influenza virus could be detected in almost all the 128 pigs investigated, whether these animals displayed clinical symptoms or not (Runge et al. 1996).

In Spain, studies developed with swine influenza virus (SIV) revealed that the H<sub>1</sub>N<sub>2</sub> strain is still endemic in densely-populated pig areas in this country (Maldonado et al. 2005). Serological studies of influenza viruses in pigs in Great Britain, carried out from 1991-1992 (Brown et al. 1995) observed a prevalence of 39% of human H<sub>3</sub>N<sub>2</sub> influenza virus and 26% of classical swine H<sub>1</sub>N<sub>1</sub>. In tests with human H<sub>1</sub>N<sub>1</sub> strains, excluding isolates from pigs, the highest seroprevalence was detected in the prevailing strains from the human populations. In addition, both influenzas type B and C were detected in the HI test. These results suggest that pigs can be infected by a number of influenza viruses, some of which may have significance in the epidemiology of human influenza (Alexander & Brown 2000, Brown 2000).

In Brazil, despite the fact that the influenza virus is a problem both for Public Health and in animal breeding, this virus has not been reported very often.

## MATERIAL AND METHODS

### Sample collection

Oro-nasal samples were collected from 34 adult pigs from different localities of São Paulo State on swabs kept in transport media until the isolation procedures. Blood samples were taken from the external jugular vein of the same pigs, using 90 x 9mm disposable needles. After blood coagulation and clot retraction, the serum was removed and kept at –70°C until the time came to perform the serologic test. No schedule of swine influenza vaccine was mentioned by the owners (Mancini et al. 2004).

### Virus isolation.

After 24h of antibiotic treatment and centrifugation at low speed (300g) the swine oro-nasal samples were inoculated on monolayers of MDCK (Madin Darby Canine Kidney) and NCI-H292 (Mucoepidermoid Carcinoma from Human Lung) cells. Both systems were incubated at 33-35°C for 3 to 7 days. Daily observation for CPE (Cytopathic Effect) was compared with a culture of influenza virus control, after which the fluids were harvested for the HA test. Samples with  $\geq 4$  UHA were considered positive to influenza virus. Then, these samples, after centrifugation at low speed, were used as antigens (Kawamoto et al. 2005).

### Virus antigen control.

Human influenza virus strains isolated by virologists from the Adolfo Lutz Institute, São Paulo, Brazil, and identified by the Centers for Disease Control & Prevention (CDC) Atlanta (GA) USA, were maintained in MDCK cell cultures through successive passages: H<sub>1</sub>N<sub>1</sub>-A/SP/1/91, H<sub>3</sub>N<sub>2</sub>-A/SP/2/95 and B -B/SP/1/91.

### Hemagglutination test (HA).

Hemagglutination titers were determined at room temperature in a microtiter system. Serial two-fold dilutions of virus (25µl) in phosphate buffered saline pH 7.2 were mixed with 25µl of a 0.5% suspension of rooster red blood cells. Hemagglutination titers (HAU/ 25µl) were determined after 1h, unless otherwise stated, and are expressed as the

reciprocal of the maximum dilution of virus that caused complete agglutination. Antigen (+): A/SP/1/95(H3N2) and red blood cell controls were performed (Mancini et al. 2004).

### **Hemagglutination inhibition(HI).**

Samples of serum were inactivated at 56°C for 30 min and treated with 20% Kaolin in phosphate buffer solution (PBS), 0.01 M, pH 7.4, in order to eliminate non-specific antibodies.

Duplicate dilutions were carried out in series, in “V” bottom microplates. Influenza virus antigen containing 4 hemagglutinating units, was added to the cavities. After one hour reaction at room temperature, 0.5% rooster erythrocytes were added to them. Reading was done after 30 min, the reciprocal of the last dilution which elicited hemagglutination inhibition being considered as the antibody titer. Those sera presenting antibody titers equal or superior to 20HIU/25µl were considered as positive. For all reagents the volume of 25µl was constant. Controls of the serum anti-influenza (+), serum not reactive to influenza (-), antigen A/SP/1/95(H3N2) and of the erythrocytes suspension were performed (Mancini et al. 2004).

## **RESULTS**

Table 1 demonstrates the results for the pig sera, as evaluated by the hemagglutination inhibition test. From 34 animal sera, antibodies to both the H<sub>1</sub>N<sub>1</sub> and H<sub>3</sub>N<sub>2</sub> strains of the human influenza type A virus, and to type B virus were detected, with mean titers of 118.82 HIU, 240 HIU, 314.54 HIU/25µl for human influenza A(H<sub>1</sub>N<sub>1</sub>), A(H<sub>3</sub>N<sub>2</sub>) and type B, respectively. The table also demonstrates that such pigs presented a mean titer of 151.17 HIU/25µl for HI antibodies to the specie specific strains of virus influenza (isolates from pigs).

**TABLE 1 – ANALYSIS OF ANTIBODIES AGAINST HUMAN AND SWINE INFLUENZA VIRUSES IN PIGS BY THE HI TEST.**

Influenza Strains	TITER – HIU/25µl							Total of positives	Mean titers	
	<20 to 40	20 to 80	>40 to 80	>80 to 160	>160 to 320	>320 to 640	>640 to 1280			N D
A (H <sub>1</sub> N <sub>1</sub> )	5	2	7	15	5	-	-	-	29	118.82
A (H <sub>3</sub> N <sub>2</sub> )	5	2	3	6	7	11	-	-	29	240.00
B	2	1	2	7	12	8	1	1	31	314.54
Swine specific isolate	1	5	10	10	8	-	-	-	33	151.17

ND - not done

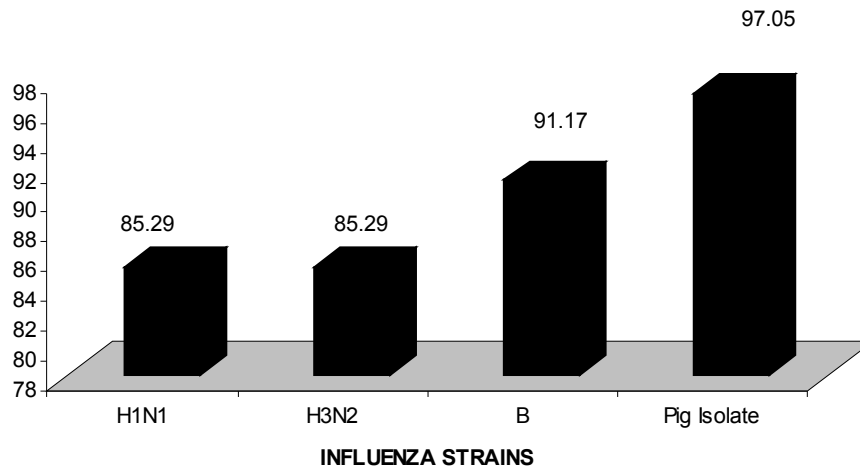
**TABLE 2 – DETECTION OF INFLUENZA VIRUS IN PIGS BY CYTOPATHIC EFFECT AND HA TEST.**

Pigs Samples	PERCENTAGE OF POSITIVE SAMPLES			
	Positive samples in NCI Cells		Positive samples in MDCK Cells	
	NCI Cells	%	MDCK Cells	%
34	18	52.94	16	47.05

Table 2 shows the data for the isolation methods, by both MDCK and NCI-H292 cells. Of the 34 pig isolate samples 18 (52.94%) and 16 (47.05%) were positive for NCI and MDCK cells, respectively.

Figure 1 demonstrates the percentage of the protection via the levels of antibodies to the influenza virus detected in pig sera, as measured by the hemagglutination inhibition test. For the H<sub>1</sub>N<sub>1</sub> and H<sub>3</sub>N<sub>2</sub> subtypes of the influenza type A, the percentage of protection was 85.29%, whilst for the type B strain a value of 91.17% was seen. With regard to the species specific strains isolated from the pigs, 97.05% presented inhibitor antibodies.

**FIGURE 1- PERCENTAGE OF PIGS WITH PROTECTOR ANTIBODY TITERS AGAINST HUMAN AND SWINE INFLUENZA VIRUSES**



## **DISCUSSION**

Data obtained in the present study demonstrate that Brazilian pigs are harbouring circulating influenza viruses in the regions of São Paulo State, Brazil. The increased positivity of the HI antibodies to the human influenza viruses observed in these pigs is similar to that reported by Runge et al. (2000) in pigs from Germany. Similarly to our study, others carried out in Europe and Asia in 2000 by Olsen et al. and Brown, respectively, also reported the isolation of H<sub>1</sub> and H<sub>3</sub> influenza viruses from pigs.

The presence of influenza type B, considered as restricted to the human population, was detected in this study and also in another study performed in pigs from Germany, by

the authors Brown, Harris and Alexander in 1995, who also reported the presence of influenza viruses type C, in the same pig herd.

The H<sub>3</sub>N<sub>2</sub> subtype was isolated from pigs in the United States by Webby et al. (2000), who showed in a phylogenetic study that this virus subtype originated from the avian and evolved to swine – H<sub>3</sub>N<sub>2</sub>, and also presents three genes from the human virus reservoir. Thus, this finding will significantly affect the efficacy of the current swine H<sub>3</sub>N<sub>2</sub> vaccines. Conversely, the new strain, produced in this manner, revealed a better protection against the specific strain as than a swine influenza virus (SIV) H<sub>1</sub>N<sub>2</sub> (Van Reeth et al. 2003).

From the data presented in this and other studies, it may be suggested that pigs are the mixing-vessel of different influenza viruses; this in turn facilitates their assortment and recombination, providing new pandemic strains of influenza virus as seen in the past. Thus, considering the information regarding the interspecies transmission of the influenza viruses, the importance of the expanding surveillance of animals known to harbor influenza strains that could spread to humans should be reinforced. Vaccines are being recommended not only for humans, but also for animals, principally due to the expected exposure of influenza viruses, on both sides, during husbandry practices.

It is important to emphasize that transmission between man and animal is the establishment of the influenza zoonotic. Currently, similar results are reported in the literature with regard to the maintenance of influenza in nature. The present study shows that pigs circulate the influenza virus in Brazil and may serve as the origin of human epidemics.

#### **ACKNOWLEDGMENTS**

The authors are grateful to Mrs Luzia da Purificação for her laboratory services and for the financial support of FAPESP-Proc. N° 00/03996-0.

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