

EVIDENCE OF HUMAN-EQUINE INTERSPECIES INFLUENZA TRANSMISSION

Dalva A. P. Mancini¹, Elenice M. S. Cunha², Maria do Carmo C. S. H. Lara², Rita M. Z. Mendonça¹, Andrea L. F. Dias¹, Alessandra F. de Castro², José R. Pinto¹, Ronaldo Z. Mendonça³.

¹Laboratório de Virologia, Instituto Butantan.

²Laboratório de Raiva e Encefalites Virais, Instituto Biológico.

³Laboratório de Imunologia Viral, Instituto Butantan.

Corresponding author:

Dalva Assunção Portari Mancini, Laboratório de Virologia, Divisão de Desenvolvimento Científico, Instituto Butantan, Avenida Vital Brasil 1500, 05503-900 São Paulo, SP, Brazil.

Tel.: +55 11 3726-7222, Ramal: 2152

E-mail: dapmancini@butantan.gov.br

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ABSTRACT

High levels of antibodies against the human influenza A(H₁N₁) and A(H₃N₂) strains in horse sera have been reported. The aim of the present study was to survey the human influenza virus strains present in horses using two methodologies, viral isolation and serology. Sera and swabs (oro-nasal) were collected from horses belonging to stud and racing stables farms situated in São Paulo and Rio de Janeiro States, Brazil. The HI test demonstrated that 46 horse serum samples presented antibodies to A/Equ1/SP/56(H₇N₇), A/Equ2/SP/1/85(H₃N₈) and A/SP/1/91(H₁N₁), A/SP/2/95(H₃N₂) and type B/SP/1/91, B/SP/6/2000 antigens of influenza virus. Passages of 23 oro-nasal samples collected from race-horses and breeding horses were performed on MDCK cells. After three and five passages of the samples, it was observed that 9 race-horses and 10 breeding horses showed CPE typical of influenza on MDCK cells. These virus isolates had a mean HA titer of 4.3 HAU/25µl and 16.0 HAU/25µl, respectively and four horse samples were characterized by the Rapid Influenza A-B Test (Gloria-Roche). The HI test demonstrated that 78.26%, 76.08%, 80.43% and 93.47% of the samples reacted to A/Equ1(H₇N₇), A/Equ2(H₃N₈), A(H₁N₁) and to A(H₃N₂) respectively, and 93.47% to type B/90 and 75% to type B/2000. These results demonstrated that horses are reservoirs of the circulating influenza viruses of both the equine-specific and the non-specific strains.

INTRODUCTION

For the prediction and control of future influenza pandemics, the program of global surveillance of animal influenza has been initiated (Kida 2000). Avian influenza is cited as the emergent zoonotic virus, among other diseases such as monkey pox, Ebola and bovine spongiform encephalopathy (Blancon & Meslin 2000, Meslin et al. 2000). Interspecies transmission, via another mammalian host and reassortment of avian and human influenza viruses, is a potential mechanism for the introduction of genes of avian influenza viruses into reservoirs (Class 2000).

Current knowledge allows us to suggest that pandemic influenza is zoonotic, caused by the transfer of influenza A viruses or viral gene segments from animal reservoirs. These transmission events took place in Hong-Kong, China, in the late 1990s, making Asia the epicenter for influenza and stressing the importance of surveillance of pigs and live-bird markets in this area (Shortridge et al. 2000, Webby & Webster 2001).

Transmission of equine influenza to dogs was observed in Florida, USA in 2005 (Crawford et al. 2005).

The virus-host interaction of equine influenza viruses has been studied using cultured equine respiratory cells. The H₃N₈ subtype, replicated on these cells, provokes their death by apoptosis (Lin et al. 2001). The caspases, a group of intracellular proteases, are activated during EIV (Equine Influenza Virus) infection and contribute to EIV-mediated cell death (Lin et al. 2002).

In a study regarding the influence of host species on the evolution of the nonstructural (NS) gene of influenza A virus, it has been reported that the recent equine lineages isolated after 1973, H₇N₇(Eq1) and H₃N₈(Eq2), indicate a new introduction of NS, M, and PB2 genes into horses from avian sources by genetic reassortment (Kawaoka et al. 1998, Lai et al. 2004).

Sera from horses obtained from Lagos and Ibadan, Nigeria, were examined for HI antibodies to two strains each of the H₃N₂ and H₁N₁ subtypes of influenza A virus. All the horse sera presented HI antibodies to the two strains of H₃N₂ subtype (A/Mississippi/1/85 and A/Leningrad/360/86), while 87% and 14% of the horses examined were positive for A/Taiwan/1/86 and A/Chile/1/83(H₁N₁). This study suggests that influenza A viruses circulate among horses in Nigeria and may serve as the origin of human epidemics (Olaleye et al. 1999).

Among aquatic birds the isolation of a large number of influenza A viruses, that present surface proteins antigenically similar to those of virus isolates from man, pigs and horses, provides indirect evidence for interspecies transmission (Webster et al.1980). Taking the evidence presented in the literature into consideration, together with confirmed influenza incidence in Brazil, this study was undertaken to investigate interspecies transmission and elucidate the influenza zoonosis, in an effort to improve prevention and control of the virus infection.

MATERIAL AND METHODS

Animals.

A total of 46 adult horses from both racing and breeding places were used, being 23 horse samples from Rio de Janeiro city and 23 from São Paulo city. Equine influenza vaccination was only reported for the race-horses. The race-horses presented clinical respiratory symptoms, but in breeding horses infection was subclinical.

Sample collection and virus isolation.

Samples were collected by oro-nasal swabs from the horses and were kept in transport medium 199 buffer solution (pH 7.2), with the addition of 1.0% bovine albumin, 200 units of penicillin G, 2.5 µg of streptomycin, 6 µg of gentamicine and 25 µg of fungizone per ml, at –70°C. After low speed centrifugation (300 G) these samples were inoculated on monolayers of MDCK (Madin Darby Canine Kidney) cells. This system was incubated at 33-35°C for 3 to 7 days. Daily observations for CPE (cytopathic effect) were made, and compared with cultures of the influenza (A/Eq2/SP/1/86) control in the cell culture system. After that, the fluids were harvested for HA testing. Samples presenting ≥ 4 HAU/25µl were considered positive to influenza virus. These samples, after centrifugation at low speed, were used as antigen (Kawamoto et al. 2005).

Samples of sera.

The samples were harvested from the external jugular vein of the horses, using disposable needles: 40x9mm. After blood coagulation and retraction of the clot, the serum was removed and kept at –70°C until the time came to perform the serologic test (Mancini et al. 2004).

Virus.

Laboratory adapted-influenza virus strains isolated from human and equine sources by the Adolfo Lutz Institute in São Paulo State, Brazil, were maintained in MDCK cell cultures through successive passages:

H₁N₁- A/SP/1/91

H₃N₂- A/SP/2/95

B - B/SP/1/91

B - B/SP/2/2000.

H₇N₇ - A/Eq1/SP/56

H₃N₈ - A/Eq2/SP/1/85

Hemagglutination test (HA).

Hemagglutination titers were determined at room temperature in a microtiter system. Serial two fold dilutions of virus (25µl) in phosphate buffered pH7.2 were mixed with 25µl of a 0.5% suspension from rooster red blood cells. Hemagglutination titers were determined after 1 h., unless otherwise stated, and are expressed as the reciprocal of the highest dilution of

virus that caused complete agglutination per 25 μ l (HAU/25 μ l). Titers of ≥ 4 HAU were considered as positive (Mancini et al. 2004).

Hemagglutination inhibition(HI).

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

Duplicate dilutions were carried out in series, in “V” bottom microplates. Antigen of the influenza virus containing 4 hemagglutinating units, was added to the cavities. After 1h reaction at room temperature, 0.5% rooster erythrocytes were added. Reading was done after 30 min, the reciprocal of the last dilution which elicited the hemagglutination inhibition, per 25 μ l being considered as the antibody titer (HIU/25 μ l). Those sera presenting antibody titers equal or superior to 20 HIU were considered as positive. For all reagents the volume of 25 μ l was constant (Mancini et al. 2004).

Influenza A/B Rapid Test (Gloria-Gold Labeled Optically-Read Immunoassay).

Influenza virus isolates were characterized by the Influenza A/B Rapid Test (Roche Laboratories), as follows. The test principle is based on the Roche diagnostics: GLORIA (Gold-labeled optically-read immunoassay). The viral nucleoprotein and viral nucleic acid that are released by lysing the influenza virus envelope with Lysis/Elution Solution, are detected. The test uses two pairs of monoclonal antibodies, one specific for influenza A and other for 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 incorporated 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. No cross reactivity occurs with other probable respiratory viruses or other organisms such as bacteria or fungi, according to the package insert of the Influenza A/B Rapid Test Kit.

RESULTS

With regard to influenza virus isolation from oro-nasal swabs collected from 24 horses from race-tracks (n=12), samples showed positivity from the 1st through the 5th passage in MDCK cells, as measured by the HA test. The same result was observed with samples collected from 12 breeding horses. The comparison of hemagglutination positivity in equine samples revealed infectivity for race-horses and breeding horses with mean titers of 4.3 and 16.0 HAU/25 μ l, respectively (Table 1).

Table 1. HA titers to influenza virus type a isolates in MDCK cells from 24 equine samples

SAMPLE	PASSAGE / TITER HAU/25 μ L	
	BREEDING HORSES 3 rd passage	RACE-HORSES 5 th passage
1	16	4
2	64	8
3	4	2
4	16	2
5	4	8
6	4	2
7	32	4
8	16	16
9	16	4
10	16	2
11	2	2
12	2	2
Mean titer HAU/25 μ l	16.0	4.3

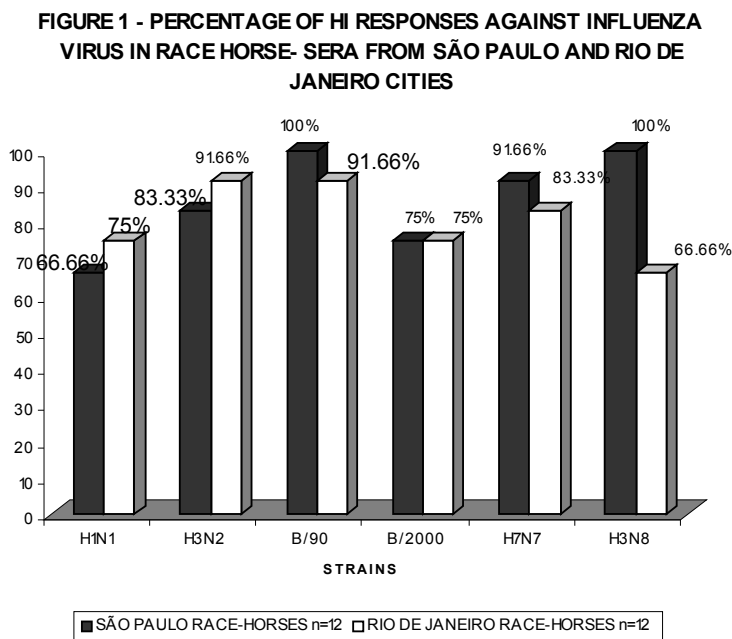
For the hemagglutination inhibition serological method, serum samples collected from 46 horses (race and breeding) were evaluated. Animals presented the following HI media titers and percentages of positivity for influenza equine-specific strains: A/Eq1(H₇N₇) 134.78 HIU (78.26%) and A/Eq2(H₃N₈) 389.13 HIU/25 μ l (76.08%). For the non-specific strains, A(H₁N₁) and A(H₃N₂), the mean titers were 105.21 HIU (80.43%) and 139.13HIU/25 μ l (93.47%), respectively, with a higher mean titer for the type B virus of 1,075.65 HIU/25 μ l (93.47%)(Table 2).

Table 2. Species and non-species specific hemagglutination inhibiting antibodies to influenza detected in both breeding and race horses

Influenza Strains	EQUINE SERUM LEVEL – HIU								Total samples	Mean titers	% protective titer (>20)	
	<20 to 40	20 to 80	>40 to 160	>80 to 320	>160 to 640	>320 to 1280	>640 to 1280	>1280 to 5120				>5120 to 10240
*A (H ₁ N ₁)	9	9	15	6	5	2	0	0	0	46	105.21	80.43
*A (H ₃ N ₂)	3	14	7	12	6	4	0	0	0	46	139.13	93.47
**A(H ₇ N ₇)	10	13	6	9	4	3	1	0	0	46	134.78	78.26
**A(H ₃ N ₈)	11	5	7	11	4	5	1	2	0	46	389.13	76.08
*B	3	6	1	16	6	3	4	5	2	46	1,075.65	93.47

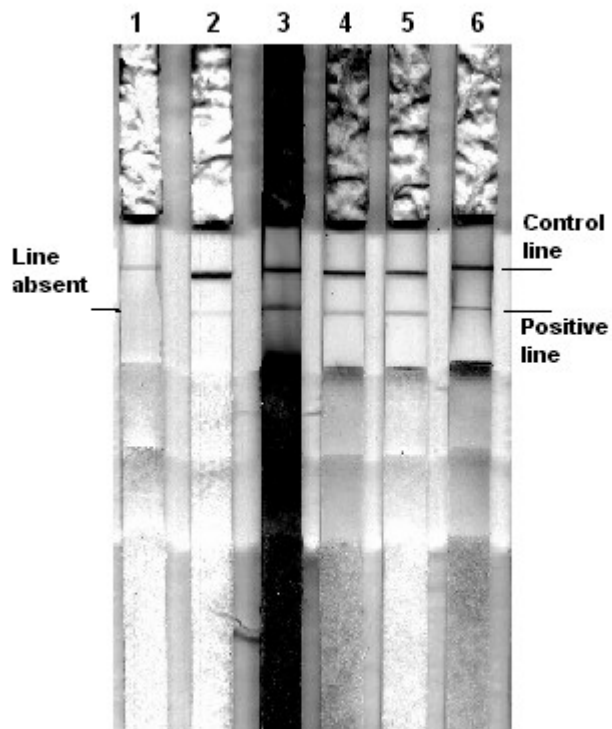
(*) Human influenza strains (**) Equine influenza strains

Analysing the 12 animals from the race-tracks of São Paulo city separately, HI responses to the influenza viruses were 75% for A(H₁N₁), 91.66% for both A(H₃N₂) and type B/90. For type B/2000 the response was 75%. The race-horses (n=12) from Rio de Janeiro city had an HI response of 66.66% to A(H₁N₁), 83.33% to A(H₃N₂) and 91.66% and 100% to A/Eq1(H₇N₇) and A/Eq2(H₃N₈), respectively. With regard to type B virus, these horses presented percentages of 100% to B/90 and 75% to type B/2000 (Fig. 1).



The Influenza A/B Rapid Test (Gloria-Gold labeled optically-read immunoassay) demonstrated that of the 9 horse isolate samples that were previously positive for the virus using the other methods cited, 4 of them had the influenza virus confirmed (Fig. 2).

**FIGURE 2 - INFLUENZA RAPID TEST
GLORIA (gold labeled optically read immunoassay)**



**1-Negative Control
2-Positive Control-Influenza
A(H1N1)
3 to 6-Equine Sample Isolates**

DISCUSSION

Based on the results obtained in this study, a high incidence of human influenza virus infection was observed among the horses evaluated from both regions of São Paulo and Rio de Janeiro States.

The viral isolation method was used to determine the isolates obtained from either breeding or race-horses that presented HA mean titers from the first passage onwards in cell systems. The highest infectivity rate was detected in the breeding horses, which was to be expected, since these were not vaccinated against equine influenza virus in contrast to the race-horses. Thus, these unvaccinated animals remained more susceptible to this virus, providing a good reservoir. Using the commercial test (Roche®) for influenza A/B diagnosis, some of these isolates could be confirmed as influenza positive. These MDCK cells were considered the best system for influenza virus isolation.

Webster et al. (1980) reported the isolation from aquatic birds, of a large number of influenza A strains possessing similar surface protein antigens to those of humans, pigs and horses, providing evidence for interspecies transmission. The apoptotic effect observed by Lin et al. (2001, 2002), of equine influenza strains on cell culture was caused by the activation of the caspases, a group of intracellular proteases. Such enzyme activation can increase virus pathogenicity *in vivo*. Genetic reassortment with avian sources resulted in the recently discovered equine lineages Eq1(H₇N₇) and Eq2(H₃N₈), demonstrating the introduction of NS, M and PB2 avian genes (Kawaoka et al. 1998). These interspecies transmissions reinforce the potential mechanisms for introduction of genes of the avian influenza viruses in reservoirs that can reach man (Class 2000).

The serology performed in this study, with horses from both breeding farms and race-tracks, showed that these animals presented high positivity (≥ 80 HIU) for influenza. Likewise, the incidence registered in the range of 60% to 100% among the horses, corresponding to the equine specific strains, A/Eq1(H₇N₇), and A/Eq2(H₃N₈) as well as the non-equine specific strains, A/H₁N₁, H₃N₂ and type B. The response to the type B strain in the horse sera is particularly noteworthy, since this strain is considered to be restricted to humans. These serological results from horses characterize the zoonotic viruses that may be transmitted by workers via interspecies transmission during the animals' care and feeding. In our previous study, horses that were vaccinated or not against equine influenza demonstrated similar serum responses to both groups of influenza strains (Mancini et al. 2004).

Horses with titers ≥ 80 HIU demonstrated that this was not a cross-reaction. Furthermore, although the race-horses had undergone influenza vaccination, whilst the breeding horses had not, their antibody levels and the percentages of the positivity to different influenza strains used in this experiment were very similar. With regard to the efficacy of equine influenza vaccines, subclinical infection with virus shedding has been reported to occur in vaccinated horses. Such infection may contribute to the spread of the disease (Daly et al. 2004). A high incidence of influenza virus was also verified in horses from Lagos and Ibadan, Nigeria, 1990, where 87% and 14% of these animals presented antibodies to the human influenza subtypes, H₃N₂ and H₁N₁, respectively (Olaleye et al. 1999).

Comparing the data obtained in this study to those reported in the literature, it may be concluded that the high incidence of influenza viruses among animals and humans surely represents a problem to both Public Health and Animal Protection all over the world. The occurrence of interspecies transmission is of concern since this allows the maintenance of the influenza virus in different reservoirs. These transmission events have been related to frequency in Asia, considered as the epicenter for influenza (Shortridge et al. 2000, Webby & Webster 2001). A program for global surveillance of animals, targeting the prediction and control of future influenza will be very important to Brazil, as related by Kida (2000).

Thus this study may offer a contribution to influenza global surveillance, particularly in relation to interspecies transmissions between horses and humans in Brazil. The data lead us to conclude that besides being susceptible to the virus, horses also are a reservoir of the non-equine specific influenza viruses in Rio de Janeiro and São Paulo, Brazil. Thus, interspecies transmission probably occurs between man and horse, considering the high percentage of HI antibody titers seen in the horses to human influenza strains, particularly for type B. This kind of study is necessary to provide a better understanding regarding the ecological properties of influenza, and demonstrate ways to impede the introduction of new influenza viruses into humans.

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