

Evidence of transmission and risk factors for influenza A virus in household dogs and their owners

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Background The possible transmission of influenza A virus between dogs and humans is important, as in Mexico City there are approximately 1.2 million dogs. We present the first evidence of influenza A virus infection in household dogs in Mexico.

Objectives The objective of this study was to identify the presence of antibodies against influenza A virus in dogs and their owners, as well as the presence of RNA of influenza A virus in nasal exudates of dogs and, thereby, assess the possible transmission of the virus between humans and dogs.

Methods Serum samples from household dogs and their owners were analyzed to detect the presence of antibodies against three subtypes of human influenza virus (H1N1pdm09, H1N1, and H3N2), as well as subtype H3N8 of equine influenza. We analyzed dog nasal exudates to detect influenza viral RNA. The relationship between the seropositivity of dogs and various factors (age, sex,

constantly at home, and seropositivity of owners) was statistically analyzed.

Results Seroprevalence for human influenza in dogs was 0.9% (1 of 113), and it was 4% (5 of 113) for equine influenza. In humans, seroprevalence was 22% for subtype H1N1pdm09, 20% for subtype H1N1, and 11% for subtype H3N2. No significant association ($P > 0.05$) was found between seropositivity and any of the assessed factors. Furthermore, no viral RNA was detected in the nasal exudate samples.

Conclusions Results revealed seroprevalence of the influenza virus in household dogs in Mexico City. It can be assumed that dogs are currently becoming infected with different subtypes of influenza viruses.

Keywords Canine influenza, cross-species transmission, human influenza, risk factors.

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Introduction

The constant evolution of the influenza virus favors genetic variability and transmission to other species. Interspecies transmission of influenza viruses is a difficult process because there are barriers that hinder infection; however, diverse factors such as population growth and consumption habits have reduced these barriers by increasing contact among species.¹ Antigenic drift and antigenic shift generate structural changes in the virus and allow evolution and variability,² thereby encouraging infection and adaptation in different animal species.³ The clinical disease associated with the influenza virus in dogs has only been recently observed. In 2004, an equine-origin subtype H3N8 influenza A virus was isolated from dogs with respiratory disease,⁴ and, in 2008, an avian-origin subtype (H3N2) was also isolated.⁵ Subsequently, the circulation of these subtypes has been documented in the canine population in diverse countries.^{6–9}

The influenza virus in these animals can lead to health problems related to respiratory syndromes and complications due to bacteria, and although horizontal transmission among dogs has been demonstrated, the rate of infection does not appear to be altered by age, breed, or sex.^{10,11} In their respiratory tract, dogs possess specific receptors for infection with human and avian influenza viruses,¹² thereby suggesting that dogs could play an important role in interspecies transmission of different subtypes of influenza A virus. Although no transmission of the virus from dogs to humans has been reported, dogs could represent a new bridging species for the infection.¹³

There is serological evidence of the presence of subtypes H1N1 and H3N2 of human influenza virus in dogs; a virus similar to subtype H1N1pdm09 of humans has also been isolated from dogs.^{14,15} In 2012, a new subtype H3N1 was identified as a result of reassortment in the canine genes of human H1N1pdm09 and canine H3N2 influenza viruses.¹⁶

The possible transmission of influenza A virus between dogs and humans is important, as in Mexico City there are approximately 1.2 million dogs, of which 1 million live in close contact with humans and other animals.¹⁷ The objective of this study was to identify the presence of antibodies against influenza A virus in dogs and their owners, as well as the presence of RNA of influenza A virus in nasal exudates of dogs and, thereby, assess the possible transmission of the virus between humans and dogs.

Material and methods

Study population

The experimental protocol and sampling methods were approved by the Committee of Science and Bioethics in Research of the Instituto Nacional de Enfermedades Respiratorias (INER B35-12). We utilized a prospective convenience sampling method. The study was limited to dog owners and none had horses. Between July and November 2012, 113 blood samples were collected from household dogs in Mexico City, as well as a blood sample from each owner. We used the Cannon and Roe¹⁸ formula to calculate the sample size and taking as reference the seroprevalence reported in previous studies for canine influenza in other countries^{10,19} and human influenza virus in dogs.¹⁵ Age, breed, and sex of the dog were not considered as exclusion or inclusion criteria. Seventeen nasal exudate samples were also collected from dogs with suggestive signs of respiratory disease. A sterile cotton swab was used to collect the sample from each animal, and the swabs were immediately placed in 1 ml of transport medium at 4°C after sampling.

Viral subtypes

Three human influenza strains were used: A/Mexico/LaGloria-3/2009 (H1N1)pdm09 (GenBank accession no. CY077595), A/Mexico/INER1/2000 (H1N1) (GenBank accession no. JN086908), and A/Hong Kong/8/68 (H3N2) (ATCC VR1679 strain) as well as two equine influenza strains: A/equine/2/Miami/63 (H3N8) (ATCC VR317 strain) and A/equine/Kentucky/97 (H3N8) (GenBank accession no. AF197249).

Antibody detection

The hemagglutination inhibition (HI) test was used to detect antibodies against equine influenza in dog sera as described by Anderson et al.²⁰ with the following modifications: sera were heat-inactivated at 56°C and adsorbed with kaolin and 5% chicken erythrocytes. Double serial dilutions were made from 1:10 to 1:1240; the titer of the antibodies inhibiting hemagglutination was expressed as the maximal dilution at which the serum inhibited the hemagglutination activity of the virus. Sera were considered positive starting at the 1:40 dilution. To detect antibodies against human influenza virus in the sera of dogs and owners, the HI test was used

according to the WHO manual,²¹ with the following modifications: the antigen was adjusted to eight hemagglutinating units (HAU). Sera were heat-inactivated at 56°C and adsorbed on kaolin and 5% chicken erythrocytes. Double serial dilutions were made from 1:10 to 1:1240, and titers of sera were considered positive at ≥ 40 . The tests with the H1N1pdm09 influenza virus were performed in a level 2 biosafety laboratory of the INER.

Detection of viral RNA

Nasal exudate samples from dogs with probable respiratory infections were analyzed to identify viral RNA of human and canine influenza. RNA extraction was achieved using the TRIzol (Invitrogen, Carlsbad, CA, USA) technique according to the manufacturer's instructions. To construct the complementary DNA, the reverse-transcriptase SuperScriptII (Invitrogen) enzyme was used. Real-time RT-PCR was used with primers addressed to a fragment of the gene NP, according to Lu *et al.*²² To identify the viral RNA of human influenza A, the primers and probe were identical to those used for the real-time RT-PCR previously described for gene M of most influenza A subtypes.²³ Primers used in this research were designed to align most type A influenza viruses originating from multiple species. To confirm the amplification of a type A influenza virus different from the human subtype pdm09, the products were subjected to agarose gel electrophoresis.

Data collection and statistical analysis

A questionnaire was completed by dog owners who had voluntarily agreed to donate their pet's blood. The questionnaire included information regarding the dog's age, breed, and sex, their vaccination status, habits of the pet: whether it cohabits with other dogs in the same home and whether it remains at home all the time or is taken out (to parks or street walking). In addition, we gathered information specific to the owners that included age, gender, and vaccination status in the last 6 months against human influenza virus. The collected information was analyzed using contingency tables. An independent test was performed to determine the association between seropositivity to the equine subtypes in dogs and predisposing factors, such as age (>2 years, <2 years), sex (male, female), and whether the dog constantly remains in the home (yes, no). We also evaluated the seropositivity against human subtypes in the dogs (positive, negative) and compared this to the seropositivity of their respective owners (positive, negative), to analyze the role of cohabitation of dogs and humans as a possible factor for infection.

To assess whether seropositivity in humans against the influenza virus was due to recent vaccination or to natural infection, we analyzed the association of the presence of antibodies (positive, negative) with the vaccination status (vaccinated, not vaccinated).

Table 1. Frequency of the antibody titers in dogs against the different subtypes of influenza A virus

Viral subtype	HAU 25 μ l				
	<20	20	40	80	160
H3N8_63	113	0	0	0	0
H3N8_97	106	2	3	0	2
H1N1pdm09	112	0	1	0	0
H1N1	108	4	0	0	1
H3N2	110	2	0	0	1

Values are expressed as hemagglutination units (HAU) at 25 μ l. Sera were considered positive when titers \geq 40 were obtained.

For both cases, a chi-square test (with continuity correction for frequencies $<$ 5) was used. Statistical significance was determined as $P < 0.05$. For all analyses, the statistical SPSS 16.0 software was used (SPSS for Windows, Version 16.0; SPSS Inc., Chicago, IL, USA).

Results

Seroprevalence

Of the 113 dogs, 48 (42.5%) were males and 65 (57.5%) were females, and the average age was 5.3 years (range 3 months to 13 years). The seroprevalence was 4% (5 of 113) for subtype H3N8 A/equine/Kentucky/97 and 0% for subtype H3N8 A/equine/2/Miami/63. We detected one dog positive to subtype H1N1pdm09 (0.9%) and one positive (0.9%) to the two seasonal subtypes H3N2 and H1N1. The frequency of the antibody titers in the dogs against the different subtypes is shown in Table 1.

Of the dog owners included in the study, 45.7% (21) were men and 54.3% (25) were women, with an average age of 36.5 and 38.6 years, respectively. The detected seroprevalence in the sera of the dog owners was 22% for subtype H1N1pdm09, 20% for subtype H1N1, and 11% for subtype H3N2.

Detection of viral RNA

The real-time RT-PCR results revealed that no samples were positive for canine influenza or influenza A.

Risk factor analysis

We found no evidence associating seropositivity in dogs with any of the assessed factors, including age ($P = 0.868$), sex ($P = 0.564$), and whether they leave the home ($P = 0.063$; Table 2). In addition, no statistically significant association existed between seropositivity against any subtype of human influenza in dogs and the seropositivity against human influenza in their owners ($P = 0.373$; Table 3).

Table 2. Association between seropositivity in dogs against equine influenza subtype A/equine/Kentucky/97 H3N8 and various factors

Variable	Category	No. of seropositive dogs	No. of seronegative dogs	P value
Age	<2 years	0	14	0.868
	>2 years	5	94	
Sex	Male	1	47	0.564
	Female	4	61	
Constantly home	Yes	2	7	0.063
	No	3	101	

Statistically significant $P < 0.05$.

Table 3. Association between seropositivity in dogs against any subtype of human influenza and seropositivity of their owners

Variable	Category	No. of seropositive dogs	No. of seronegative dogs	P value
Owner positive	Yes	1	94	0.373
	No	0	18	

Statistically significant $P < 0.05$.

Table 4. Assessment of vaccination status and seropositivity against influenza in dog owners

Variable	Category	No. of seropositive owners	No. of seronegative owners	P value
Vaccinated against H1N1	Yes	2	8	0.969
	No	7	29	
Vaccinated against H1N1pdm09	Yes	5	3	0.002*
	No	5	33	

*Statistically significant $P < 0.05$.

Assessment of the vaccination status and seropositivity in dog owners revealed that the only statistically significant factor ($P = 0.002$) associated with the presence of antibodies was vaccination in the last 6 months against subtype H1N1pdm09 (Table 4).

Discussion

Seroprevalence in dogs against subtype H3N8 A/equine/2/Miami/63 was nil. In Mexico, the presence of antibodies in

horses against different isolates of the subtype H3N8 equine influenza virus has been reported²⁴; comparatively, variation in the hemagglutinin gene of this virus has been reported in isolations performed before 1992.⁶ It can be assumed that, in Mexico, something similar occurred in the equine influenza virus and, therefore, dog sera do not present antibodies against old isolates of this subtype due to antigenic drift. Seropositivity in dogs against subtype H3N8 A/equine/Kentucky/97 was 4%. Anderson *et al.*²⁰ discussed that contemporary subtypes of equine and canine influenza viruses are phylogenetically close and therefore can be used as diagnostic antigens. The positive results in dogs suggest that interspecies transmission of the equine influenza virus has occurred. Seroprevalence of the equine influenza virus was low, but similar to that in studies with pet dogs from other countries^{10,25,26} where canine and equine influenza subtypes were used. The low seropositivity could be explained by the fact that we analyzed pet dogs, because prevalence of canine influenza in dogs held in canine control shelters has been reported to be up to 42%, and the only associated factor was the number of permanent days in the shelter.²⁷ Seroprevalence in these dogs against the three subtypes of human influenza (H1N1pdm09, H1N1, and H3N2) was 0.9%. This is similar to that reported by Dundon *et al.*,¹⁵ who found a 0.7% seroprevalence in dog sera that were collected during the circulation time of the H1N1pdm09 human influenza virus. Damiani *et al.*²⁸ also found a 0.13% seroprevalence against the H1N1pdm09 human influenza virus. Our results differ from prevalence data found in Brazil,⁹ which was 15.27%, but this study was against an isolate of an old human influenza virus, and not the 2009 variant, which could explain the difference in the proportion of positive results.

The percentage of seropositivity against the H1N1pdm09 human influenza virus detected in the owners is similar to other reports (39% and 33%) from previous studies performed in Mexico.^{29,30} Circulation of subtype H1N1 among dog owners was not associated with their vaccination status, thereby suggesting that dogs were in contact with the human influenza virus and that there is interspecies transmission.

We did not find sufficient statistical evidence to associate the seropositivity against the equine influenza virus in dogs with age, sex, or constant presence inside the home; this is similar to a previous study in which the only factor associated with seropositivity for canine influenza was their lodging in veterinary facilities in the previous 6 months.¹⁰ We did not find a statistical association between seropositivity against the human influenza virus in the dogs and their owners. Therefore, we can assume that the observed seropositivity in dog owners does not pose a risk for seropositivity against human influenza in their dogs. However, subtypes H1N1pdm09 and H1N1 of human influenza are infecting dogs and their owners. There may be factors

directly associated with the infection, but these have not yet been studied. We did not find any nasal exudates positive to RNA of human influenza A virus. In previous studies^{4,6,16,31} performed in other countries, isolates were obtained from samples of dogs with severe respiratory clinical signs. The disease is probably not frequently presented by dogs in Mexico or the number of dogs that are infected is low, and consequently, the number of dogs that develop the disease is also low. We will continue to search for a strain in Mexico City that can be analyzed and compared with other isolates.

These are the first results from household dogs in Mexico. However, it is necessary to continue to study influenza virus circulation in dogs from different places. The seropositivity results revealed that the influenza virus is currently infecting household dogs in Mexico City. We have no information regarding when this virus was first present in dogs, but it can be assumed that dogs are currently in contact and becoming infected with different subtypes of human and equine influenza viruses. We do not presently have an isolate of canine influenza in Mexico City that could generate more information related to the circulation among the canine population, but we will continue to study the implications to animal and human health.

Conflict of interests

Authors declare no conflict of interests.

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