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A population prevalence study on influenza infection in dogs in Southern Italy

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SUMMARY

Despite several data on influenza infection in dogs, the first natural outbreak of canine influenza virus, closely related to H3N8 equine subtype, dates back to 2004 in Florida. Subsequent studies highlighted the role of dogs in adaptation of H5N1 to mammals and the susceptibility of dogs to different subtypes of influenza. A prevalence study was carried out on 562 sera collected from pet and kennel dogs in the south of Italy. A c-ELISA test was employed and c-ELISA-positive, c-ELISA-doubtful and random c-ELISA-negative samples were also tested in subtype-specific HI test using H3N8 and H3N2 strains. c-ELISA detected a positivity of 3.56%. HI performed with the H3N8 revealed 2 positive samples and when performed with the H3N2, HI revealed 47 positive samples. c-ELISA proved to be a sensitive and specific technique. HI is a specific method only when the test antigen is homologous to the circulating virus and, because non-specific-hemagglutination inhibitors may be present in dog sera, false positives can result. The study emphasizes that due to their close contact with humans dogs must be a target for testing. Furthermore because it remains to be determined how long antibodies to influenza virus persist in canine sera, the observed prevalence might be underestimated.

KEY WORDS: Dog, ELISA, HI test, Influenza, Prevalence.

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INTRODUCTION

With the exception of highly pathogenic avian influenza (HPAI), influenza A virus is primarily a respiratory pathogen. HPAI viruses belong to the *Orthomyxoviridae* family and are characterized by a segmented genome, with eight separate strands of RNA, coding for eleven proteins, responsible for a great antigenic diversity. Three major types of influenza virus exist, A, B and C, but only types A and B cause widespread outbreaks. Influenza A viruses are classified in subtypes on the basis of the antigenic differences between two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA).

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To date, influenza A viruses of 18 HA and 11 NA subtypes have been described (Tong *et al.,* 2012, 2013). Only subtypes H1-H16 and N1-N9 have been isolated from aquatic birds. H17N10 and H18N11 have only been recovered from bats (Tong *et al.,* 2013). H1, H2, and H3 subtypes, and N1 and N2 subtypes have established stable lineages in the human population since 1918 (Nicholson *et al.,* 2003).

The interspecies transmission of a mammalian influenza virus to an unrelated mammal species is uncommon. Nevertheless, several data confirm both that human type A influenza viruses may be of significance as an aetiological factor for tracheobronchitis in dogs, and that infection of dogs with human type A influenza viruses may be of importance in the epidemiology of human influenza (Paniker and Nair 1972; Kang *et al.,* 2013).

Influenza A/Hong Kong/68 virus was isolated in Russia from one of six affected dogs and specific haemagglutination inhibiting (HI) antibodies were found in three cases (Pysina and Surin

1972). HI antibodies to A/Hong Kong/68 influenza virus were detected in 3.7% and 5.9% of dogs free from respiratory disease in Germany and in Holland respectively, and antibodies to influenza virus were found in percentages between 47% and 88% of animals from five kennels experiencing acute kennel cough (Bibrack 1975). Serum samples collected from dogs in Ohio in 1978 and tested for antibody to influenza viruses revealed that 26.3% of 80 clinically ill animals and 19.5% of 41 normal animals had low levels of HI antibodies to influenza A/Texas/77 (Houser and Heuschele 1980).

Despite all these data, there was no evidence of sustained circulation in the canine host and there were no reports of natural influenza outbreaks in dogs until the discovery of H3N8 *canine influenza virus* (CIV), closely related to H3N8 equine influenza virus, in Florida in 2004 (Crawford *et al.,* 2005). A retrospective study carried out in United Kingdom demonstrated that a severe respiratory outbreak among English foxhounds in 2002, independent of that in Florida, was caused by an equine influenza A virus H3N8 (Daly *et al.*, 2008).

Avian influenza H5N1 can cross the species barrier infecting humans (Chotpitayasunondh *et al.,* 2005), domestic cats (Songserm *et al.,* 2006a) and tigers and leopards (Keawcharoen *et al.,* 2004; Thanawongnuwech *et al.,* 2005; Amonsin *et al.,* 2006). The first report of H5N1-related systemic disease in a domestic dog was described during an influenza outbreak in Thailand in October 2004 (Songserm *et al.,* 2006b). Experimental studies indicate that dogs are susceptible to H5N1 infection, though viral infectious particles could not be re-isolated from infected dogs, and transmission of virus to a contact dog did not occur (Giese *et al.,* 2008).

In 2007, a respiratory infection caused by a H3N2 avian-origin canine influenza virus was reported in a pet dog in the Republic of Korea (Song *et al.,* 2008) and at the same time an avian-origin H3N2 influenza virus was also isolated from clinically ill dogs in southern China (Li *et al.,* 2010). Evidence of avian influenza virus infection in pet dogs raises the concern that dogs may become a new source of transmission of novel influenza viruses, especially where avian influenza viruses are circulating or were detected (Song *et al.,* 2008). All these observations confirm the susceptibility of dogs to different subtypes of influenza viruses and consequently the epidemiological role of dogs should require close attention (Harder and Vahlenkamp 2010). The objective of the present study was to determine the prevalence of antibody against influenza virus in the serum samples of dogs in Southern Italy.

MATERIALS AND METHODS

Serum samples

A total of 562 serum samples from dogs older than 1 year of age were selected and employed in the present study to test antibodies to Influenza A virus. In particular, 470 serum samples were collected from pet dogs and 92 serum samples were collected from 3 different animal shelters located in areas geographically distant from each other in the province of Bari, Italy. The serum samples from pet dogs were obtained from the Clinic Laboratory of the Veterinary University of Bari, Italy (376 sera) and from a Clinical Analysis Laboratory in the province of Bari, Italy (94 sera). The selected sera from pet dogs included 94 dogs from 1997, 94 dogs from 2008, 78 dogs from 2009, 158 dogs from 2010 and 46 dogs from 2011 (470 samples). The 92 sera from stray dogs were collected from May through to December 2008. The study was conducted in accordance with the rules laid down in Italian law 116/92 and subsequent amendments, according to the Directive 86/609/CEE in the field of animal protection. Regarding the tested animals, no information was available with respect to the influenza activity or the dogs' medical history. Serum samples was stored at -20°C prior to testing.

ELISA test

A commercially available c-ELISA kit, ID Screen® Influenza A Antibody Competition Assay (ID VET Innovative Diagnostics, 167 rue Mehdi Ben Barka - 34070 Montpellier - France) based on competition principles, was employed in the study. The diagnostic kit is designed to detect antibodies directed against the internal nucleocapsid protein of the influenza A virus. The test was performed according to the manufacturer's instructions and the optical density

(OD) was read at 405 nm. Canine sera were diluted at 1:10 ratio in the dilution buffer provided with the kit. Four samples, two positive and two negative control sera provided in the kit, were included in each test microplate. Validation of the test requires that the mean value of the OD of the positive controls, ODp, is less than 30% of the mean value of the OD of the negative controls, ODn (ODp/ODn <0.3, where ODn >0.700).

HI test

c-ELISA-positive, c-ELISA-doubtful and random c-ELISA-negative samples were also tested for antibodies to influenza virus in a subtype-specific HI test. The assay was performed using two influenza A viruses:

1) a strain isolated from the nasal swabs of a horse during an outbreak of influenza in the south of Italy in 2005 and characterized as H3N8 by PCR;
2) a strain isolated in Italy from the lung of a pig in 2009 and characterized as H3N2 by PCR. Both virus stocks used as antigens were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs.

The allantoic fluids were harvested 48 h post-inoculation and clarified by centrifugation at 500 x g for 20 min. Virus titres of both H3N8 and H3N2 were determined by haemagglutination assay and virus stocks were stored at -70°C until used. Prior to testing, the sera and control samples were incubated with receptor-destroying enzyme (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 37°C, then inactivated at 56°C for 30 min. HI was performed in sterile 96-well U plates using 4 haemagglutinating units/25 µl of viruses and twofold serial dilution (log_2) of treated sera in phosphate buffered saline (PBS) starting from 1/5 to 1/2560. Plates were incubated at room temperature for 45 min and 0.5% suspension of chicken erythrocytes in PBS were added. The endpoint HI titre was defined as the reciprocal of the last dilution of serum that completely inhibited haemagglutination. The cut-off titre for seropositivity was 1/20.

Statistical analysis

Data were analysed using R Software, version 2.8.1. (http://www.r-project.org/). The differences between the proportions were determined using the chi-square test (with a significant cut-off value of 0.05), applying Yates' correction (for continuity) for dichotomous variables.

RESULTS

When the 562 dog sera were subjected to nucleocapsid specific c-ELISA, anti-influenza virus antibodies were detected in 20 samples (3.56%), and 4 samples (0.71%) resulted weakly positive/doubtful.

In particular, 4 samples (4.26%) and 1 sample (1.06%) out of the 94 canine sera collected in 1997 resulted positive and weakly positive/doubtful, respectively, for the nucleocapsid protein. Six sera (3.22%) out of the 186 serum samples collected during the 2008 resulted positive, while 1 sample (1.06%) from a pet dog was weakly positive/doubtful. Only 1 of the positive samples were from a stray dog (1.09%), while the remaining 5 positive samples (5.32%) were from pet dogs. Among the 78 sera from 2009, 4 (5.13%) resulted positive by c-ELISA. Among the 158 serum samples from 2010, 5 (3.16%) were positive for nucleoprotein antibodies and 2 (1.27%) were weakly positive/doubtful. Finally, only 1 serum (2.17%) out the 46 serum samples collected in 2011 resulted positive (Table 1).

The proportions of positive and negative effects on dogs in any particular year were compared using chi-square test: the differences between the proportions were not statistically significant (P=0.73). Risk factor analysis was not performed because of the low prevalence.

All the 20 c-ELISA-positive sera, the 4 c-ELISA-doubtful sera and 100 random negative c-ELISA sera were retested in the HI against H3N8 and H3N2 viruses. When HI test was performed with the H3N8 strain, only 1 c-ELISA-positive serum sample from 2008 and 1 c-ELISA-positive serum from 2010 resulted positive. The 4 c-ELISA-doubtful samples resulted negative. The HI titre of the positive samples was 160 for the 2008 serum sample, and 80 for the 2010 sera. The remaining c-ELISA-positive and c-ELISA doubtful serum samples, and the c-ELISA-negative sera revealed HI titres less than 10. When HI test was performed with the H3N2 strain, all the 20 c-ELISA-positive and the 4 c-ELISA-

TABLE 1 - *CIV seropositivity rates in pet and kennel dogs.*

		ELISA		*HI*		
				H3N8	*H3N2*	
Year	*Pet dogs*	*Positive (%)*	*Weakly positive/ doubtful (%)*	*Positive*	*Positive*	*Positive HI/ negative c-ELISA*[b]
1997	94	4 (4.26%)	1 (1.06%)	/	5	5
2008	94	5 (5.32%)	1 (1.06%)	1	6	3
2008[a]	92	1 (1.09%)	/	/	1	3
2009	78	4 (5.13%)	/	/	4	3
2010	158	5 (3.16%)	2 (1.27%)	1	7	8
2011	46	1 (2.17%)	/	/	1	1
Total	562	20 (3.56%)	4 (0.71%)	2	24	23

[a]kennel dogs; [b]100 sera tested among the 538 negative c-ELISA serum samples.

doubtful sera gave positive results. The HI titre was 320 and 640 for most of the c-ELISA-positive and doubtful samples. The c-ELISA positive-sample from 2011 and the c-ELISA-doubtful sample from 2010 revealed an HI titre of 80. Interestingly, 23 sera out of the 100 random negative c-ELISA serum samples resulted positive.

To determine whether c-ELISA and/or HI could be used to detect specific antibody to influenza virus in canine serum samples, the relative sensitivities and specificities were evaluated. The 4 samples doubtful in c-ELISA were classified as ELISA-negative samples for purposes of the analysis. Considering the commercial c-ELISA test the gold standard, the sensitivity and specificity of HI performed with H3N8 resulted 10% and 100%, respectively, and the sensitivity and specificity of HI performed with H3N2 resulted 100% and 74%, respectively.

DISCUSSION

Recent data suggest that influenza virus plays an important epidemiological role in dogs with clinical respiratory disease, supporting the notion that commingling of animals in closed environments favours virus transmission (Crawford *et al.,* 2005; Barrell *et al.,* 2010). In Korea, an ELISA test demonstrated that the avian-origin canine influenza caused by H3N2 was significantly more prevalent in kennel dogs than in pet dogs (19% *vs* 0.5%) (Lee *et al.* 2009). However, it should be noted that the sampled dog included animals from a farm that reported an acute outbreak of avian-origin canine influenza virus before samples collection. In contrast, the remaining dog farms which had not suffered from the avian-origin canine influenza virus outbreaks had seropositivity rates of 0-6%.

The epidemiological data reported in the present study confirm the low percentage of positivity in Italy too, where c-ELISA detected antibodies against canine influenza A virus in 20 out of the 562 dog sera tested (3.56%), and 4 sera resulted weakly positive/doubtful (0.71%). There are two possible explanations for the low number of positive dogs observed. This is not a study focusing on animals with clinical signs of respiratory disease, but a population prevalence-study. Despite this, the low seroprevalence suggests that CIV is not a common respiratory pathogen encountered in Southern Italy, but confirms that dogs are susceptible to infection. The second hypothesis is that some of the tested dogs had been previously infected but no longer had detectable antibody titres, and considering that it remains to be determined how long antibodies to influenza virus persist in canine serum, this may result in an underestimation of influenza seroprevalence.

In some respects it was surprising that none

of the samples collected from kennel dogs resulted c-ELISA-positive. The seroprevalence detected in c-ELISA was low in the kennel dogs (1.09%) compared to that observed in the pet dogs (5.32%) both during the year 2008, and in general throughout the observation period (1.09 *vs* 4.04%). This finding emphasizes that the human-canine cohabitation represents a probable way for dogs to became infected and that dog influenza could arise from contact with infected owners. A possible explanation of this hypothesis might be the limited contact between dogs and men in kennels with respect to what occurs in the domestic environment and, in the absence of a specific respiratory flu infection, the kennel is a more protected area. During a human flu outbreak, the close contact between pet dogs and humans makes the interspecies transfer of this virus from man to dog and vice versa easier and more likely.

An important question regards the test employed to detect antibody to influenza virus in dogs. HI test performed with the H3N8 strain revealed 1 positive sample among the 20 c-ELISA-positive and the 4 c-ELISA doubtful serum samples, and confirmed all the 100 c-ELISA-negative sera. The HI-negative sera had titres less than 10, far below the cut-off titre for seropositivity. HI test performed with the H3N2 strain gave interesting results. All the c-ELISA positive and c-ELISA doubtful samples resulted positive, and 23 out of the 100 c-ELISA negative samples tested gave positive results.

c-Elisa is a valid approach to assess exposure to any influenza subtype in dogs, especially in those with unknown exposure history at the time of testing (De Benedictis *et al.,* 2010). Anyhow c-ELISA positive and weakly positive/doubtful samples should be confirmed in a highly sensitive and specific assay. HI performed with the H3N8 strain revealed a sensitivity and a specificity of 10% and 100%, respectively. The low sensitivity of this test with respect to c-ELISA assay, results in a poor ability to detect the true positive. On the contrary, HI performed with the H3N2 strain showed a high sensitivity (100%) and a good specificity (74%), and consequently it could be a valid test to detect the true positive. It was observed that sensitivity and specificity of HI assay are generally high when the test antigen is homologous to the circulating virus, and some researchers noted that the seropositivity rates of the dogs differed on whether an HI test or ELISA was performed. The relatively high number of positive samples detected in HI test performed with H3N2 strain confirms this observation, allowing us to hypothesize and partially to confirm that infected owners could be a possible source of infection for domestic pets.

In experimentally infected puppies with influenza H3N2 virus, H3N2 virus antibodies were detected 2 days earlier by NP-based ELISA than by HI test. Two hypotheses were proposed to explain the failure to detect HI antibodies:

1) a non homologous antigen employed in HI test;
2) the location of the antigenic determinants. If the determinants are located lower down on the stalk of the H monomer, the attachment of antibodies to such determinants *in vivo* may require processing of the H to expose these determinants.

In HI tests, these antibodies may combine with the determinants but fail to inhibit the binding of erythrocytes (Lu *et al.,* 1982). The practical consequence is that HI test with intact avian viruses fails to detect antibody and does not correlate with virus neutralization. Moreover, it is true that HI assay detects antibodies against viral HA in animal and human sera, but, because non-specific haemagglutination inhibitors in the sera may be present, it is not very reliable in detecting antibodies to influenza viruses in mammalian sera, and false positives can result (Lu *et al.,* 1982). Other researchers showed that the HI sensitivity for influenza virus can be improved by using subunit HA rather than intact virus. Rowe *et al.* (1999) employed either disrupted H5 influenza virus or purified baculovirus-expressed rH5 HA to detect antibodies by the HI assay. HI titres obtained with both methods were similar, denying that isolated HA improves the sensitivity of the HI assay.

Phylogenetic analysis of the influenza virus identified in the dogs in 2004 indicated its close relationship to H3N8 equine influenza virus and showed that it forms a monophyletic group consistent with a single interspecies virus transfer. Molecular changes observed in the H gene suggested adaptive evolution in the new host. The biologic difference between equine influ-

enza virus H3N8 and CIV H3N8 is the ability of the latter to be transmitted from dog to dog. Experimental infections have clearly showed the transmissibility of CIV among susceptible dogs (Jirjis *et al.,* 2010), although the virus is not highly contagious, probably due to the low amount of virus produced and shed by infected dogs (Deshpande *et al.,* 2009). Even if the geographic areas where the virus is currently enzootic are limited, it is reasonable to conclude that the transmission of the virus among dogs occurs readily.

Another point of reflection is the emergence of the highly pathogenic avian influenza virus H5N1 as a potential virus for a new pandemic human influenza.

Although the vehicles for the spread of the virus to distant regions are migratory wild birds, any animal with respiratory signs, including dogs due to their close contact with humans, became a target for testing. Currently CIV is not considered a zoonotic pathogen.

But all the reported data demonstrate the susceptibility in nature of dogs to human influenza A and indicate a potential role for the dog in the epidemiology of human influenza. Moreover, given the interspecies transfer of this virus from horse to dog, and considering the close contact between dogs and humans and the still unknown role of this virus in the possible future evolution of influenza viruses, genetic changes in CIVs should be carefully monitored.

The genetic interaction between human and swine influenza viruses were studied extensively. Whether this could occur in the dog is unknown, but the potential for such an accident must be accepted.

Surveillance of dogs with acute respiratory disease would seem warranted on a continuing basis to provide early detection of such an occurrence.

The seroprevalence observed in Southern Italy is low and no cases of canine influenza have been identified to date (probably because no constant monitoring is carried out!), but it should be emphasized that the present report is only a preliminary study on the epidemiology of CIV in Southern Italy. Considering the recent natural infections with influenza subtypes H3 and H5 observed in carnivores, influenza virus infection should always be considered in dogs with lower respiratory disease. These observations indicate the need:

1) for active and passive surveillance for infectious respiratory diseases of dogs;
2) to monitor dogs in animal hospitals and farms during future influenza A outbreaks;
3) to submit specimens for testing potentially infected animals, to detect the emergence of this canine pathogen of potential concern.

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