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SHORT COMMUNICATION

Lack of Evidence of Avian-to-Cat Transmission of Avian H5 subtype Influenza Virus among Cats in Southern China

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A B S T R A C T

H5N1 is of great concern with regards to control of cross-species transmission of avian influenza virus (AIV). In southern China, H5N1 is one of the dominant enzootic AIV. It has been reported to be isolated from cats. Thus, the possibility of cross-species transmission of H5N1 from avian to cat was investigated via NT and HI assays using blood samples collected from different populations of cats in southern China. While no positive sera identified indicates lacking of evidence on cross-species transmission of H5N1 from avian to cat, the lower HI titer (1:20) with rate of 1.3% suggests that it is highly worth to monitor the sera antibodies to H5N1 in cats living in sites contaminated by H5N1 AIVs for control purposes. Our results provide initial and original reference information for future seroepidemiological and monitoring investigations of H5N1 in cats.

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INTRODUCTION

Avian influenza has attracted worldwide attention because H5N1 highly pathogenic avian influenza virus (HP-AIV) can cause fatal infections in humans and other mammals. Despite very successful efforts to eliminate or control the spread of H5N1 in poultry, viruses persisting in enzootic regions seed virus epizootics in new areas via poultry trade or wild birds. The HP-AIV H5N1 has been established as an enzootic virus in southern China, as well as other parts of the world (Kuiken *et al*., 2004; Songserm *et al*., 2006; Khan *et al*., 2012). Current circulating of the H5 subtype AIVs in China is of international concern since it is easy for the viruses to be transmitted across geopolitical boundaries via wild birds and legal or illegal poultry trade (Rimmelzwaan*et al.,* 2006). Moreover, the H5N1 virus can cross species barriers and adapt to new hosts. Direct contact with sick or diseased poultry has been implicated as a major risk factor for feline infection with H5N1 AIV (Kuiken *et al.*, 2004). Clade 2.3.2 viruses have now been detected in 9 countries since 2007, which is highly pathogenic in chickens (100% mortality). Clade 2.3.2, Clade 2.3.4 and clade 7 viruses are the main isolates in China , but the clade 2.3.2 viruses are the dominant prevalence strains, especially in southern China including Guangdong, Hunan, etc. (Guan *et al*., 2000; Jiang *et al.*, 2010; Li *et al*., 2010). Besides closely contact with domestic poultry, the special climatic and lifestyle environment in southern China provides more chances for cats to contact with other animals, such as wild aquatic birds, dogs, and humans. Whereby, it creates opportunities for interspecies transmission of the virus to cats. Meanwhile, cats exposed to the virus may act as repository for the influenza viruses due to frequent cohabitation and interactions with other animals and humans. Hence, cats carrying AIV can pose a threat to human health. To date, no serological and virological studies on H5N1 AIVs infections in cats have been carried out in China. Therefore, we initiated this serological study to uncover any evidence of H5N1 AIV (clade 2.3.2) transmission in cats in China.

MATERIALS AND METHODS

During February 2009 and December of 2011, a total of 1,680 blood samples were collected from 60 different pet hospitals and animal shelters in the Guangdong and Guangxi Provinces in southern China, where H5N1 AIV are currently circulating or have been frequently detected in the past. Of these blood samples, 480 were collected from pet cats in pet hospitals, 800 were collected from roaming catsin rural animal shelters and 400 were collected

Table 1: Survey structure in cats in southern China

Locations [a]	No. Survey structure description				
	No.	Median of age (range)	No. of female (%)	Species	(No.)
Guangdong[a]	968	5.9(0.7-11)	401(41.4)	Pet cats	(280)
				Roaming cats	(436)
				Wild cats	(252)
Guangxi[a]	712	4.5(0.5-13)	263(36.9)	Pet cats	(200)
				Roaming cats	(364)
				Wild cats	(148)

[a]shows survey sites in southern China where the sero-epidemiological study was conducted.

from wild cats in rural or suburban environment (Table 1). There are 664 females and 1016 males. All serum samples were treated with a receptor-destroying enzyme (RDE) and absorbed with erythrocytes in order to remove nonspecific inhibitors before detection. All samples were tested with a hemagglutination inhibition (HI) assay according to recommended procedures as reported in the previous study (Su *et al.*, 2013). Additionally, all samples were tested for H5N1 neutralizing antibodies using a traditional neutralization (NT) method (Kendal *et al.*, 1982). Briefly, positive sera were two fold diluted in serum-free DMEM starting at a dilution of 1:10. Then, 100 $TCID_{50}$ of virus were added to the serially diluted serum at a 1:1 ratio (V/V), followed by incubation at 37°C for 1 h. Finally, 0.2 ml of the virus-serum mixtures was transferred to 96-well monolayer plates of MDCK and incubated in 5% CO_2 at 37°C for 72 h. All samples were tested in triplicate. Pathogenic effects were observed every day and cell supernatants were tested by the HA assay to confirm the viral infection. The endpoint for neutralization was expressed as the highest dilution that prevented hemagglutination in all 3 replicates. Both HI titer of 1: 40 or greater and NT titer of 1:40 or greater were considered positive. A HPAI H5N1 virus, A/chicken/Guangdong/178/04(H5N1) (Genbank Accession: AY737296), was used for all AIV antibody detection by HI and NT assays. The H5N1 virus was isolated at College of Veterinary Medicine, South China Agricultural University (Wan *et al.*, 2005). It belongs to Clade 2.3.2, the most dominant lineage of H5N1 in southern China since 2009, which has been tested to be suitable for a detection usage (Jiang *et al.*, 2010). Bivariate χ^2 test of independence or Fisher's exact test was used to examine the association between the demographic characteristics and serological outcome. Covariates that had a $P<0.25$ in the bivariate analyses were entered into a multivariable logistic regression model. We performed backward elimination of the covariates, keeping covariates in the model which had a $P<0.05$. Final covariates were tested for goodness-of-fit.

RESULTS

The serological screening of 1,680 feline serum samples revealed only one HI positive against H5N1 in roaming cats, wild cats, and pet cats, but none samples were confirmed by the NT test with the H5N1 AIV. Hence, the negative results are not likely a result of false negatives. However, the lower HI titer against H5N1 was observed, with titer of 1:20 at ratio of 1.3% (Table 2). HI titer against canine influenza virus [CIV, A/canine/ Guangdong/2/2011 (H3N2)] was also performed as a reference positive control. The neutralization (NT) assay was employed for confirmation if an HI titer of 1:20 or more was obtained. A total of 368 (368/1680) sera were found HI titer of 1: 20 or greater. Among these 368 sera, 99 samples were detected to be positive by the MN assay (NT>1:40) (Date not shown). HI titers against H9N2 AIV (A/chicken/Guangdong/V/2008) of 1:20 or more were also detected in 66 of 1680 serum samples (56 with an HI titer of 1:20 and 10 with an HI titer of 1:40). However, none of the 66 samples were positive tested by the MN assay, with NT less than 1:40. In addition, 109 of 1680 serum samples were observed with titer of 1:20 or greater against H1N1 swine influenza virus (A/swine/ Guangdong/L6/2009/H1N1). Six of 109 samples were confirmed to be positive by the NT test (NT>1:40) (Date not shown).

DISCUSSION

To date, the best way to assess the prevalence of the influenza virus is to perform the serological survey. To date, serological surveys of AIVs infection in cats are limited worldwide. In Italy, survey of feline sera collected from different cat populations during 1999 to 2005 (Paltrinieri *et al.*, 2007) and 2004 to 2008 (Piccirillo *et al.*, 2010) using a competitive enzyme-linked immunosorbent assay (ELISA) showed no evidence of AIV antibodies. In agreement, we report the lack of seroepidemiological evidence of avian H5 influenza transmission to cats in China for the first time. The results imply transmission of HPAI H5N1 from avian to cats, at least Clade 2.3.2, seems very low in southern China for now. It also suggests that the H5N1 infections in poultry may have not yet significantly affect the rural animal shelters or suburban environment. However, it should be noted that the lower HI titer against H5N1 of 20 was observed (Table 2). As H5N1 is currently the most prevalent avian influenza virus in southern China, observation of HI antibodies against H5 in different cat population is not unexpected. Since feline infection with H5N1 has been commonly reported (Kuiken *et al.*, 2004), cats in southern China may not be susceptible to 2.3.2 yet. Whether there is any infection of other clades of H5N1 to cats in southern China needs to be investigated in the future.

In recent years, numbers of wild cats and roaming cats have been increased in Guangdong and Guangxi Provinces. These cats have more possibility to contact with the AIV through air transmission or via eating the body of dead animals with AIV infectionas H5N1 continuous outbreaks of H5N1 infection among avian in Southeast Asia nations. Thus, there is a continued risk for cat infection with H5N1 virus. Moreover, companion cats are in close contact with humans. So far, although no reassortment between avian and mammalian influenza viruses has occurred in cats, but experts fear that cats might give the H5N1 AIV an opportunity to adapt to mammals. Therefore, continuous surveillance of cat infection with H5N1 AIV in southern China is essential. In future studies, our results can be used as initial and original reference information for further sero-epidemiological and monitoring investigations of H5N1 in cats in southern China.

Table 2: The hemagglutination inhibition (HI) titers against H5N1 AIV, H9N2 AIV, H1N1 SIV and H3N2 CIV among 1680 cats in southern China

Viral strains	HI titer					Sum of titer ≥ 1:20 (%)	Sum of titer ≥ 1:80 (%)	OR (95%CI)[a]	P value
	1:20	1:20	1:40	1:80	≥ 1:160	≥ 1:20	≥ 1:80		
H5N1	1658	21	1	0	0	22(1.3)	0(0)	Reference	
H3N2	1312	199	68	50	51	368(21.9)	101(6)	0.03	0.05
H1N1	1571	89	15	5	0	109(6.5)	5(0.3)	0.32	0.05
H9N2	1614	56	10	0	0	66(3.9)	0(0)	0.15	0.05

H5N1 = A/chicken/Guangdong/178/04 (H5N1); H9N2 = A/chicken/Guangdong/V/2008(H9N2); H1N1 = A/swine/Guangdong/L6/2009(H1N1); H3N2 = A/canine/Guangdong/2/2011(H3N2). [a]The statistical tests about the association between serological outcome selected HI titer≥20□OR, odds ratio; CI, confidence interval.

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