

*Full Length Research Paper*

## **Seroprevalence of avian origin H3N2 canine influenza virus infection in pet dogs in Shenzhen, China**

**Fu-Rong Zhao<sup>1,2#</sup>, Shou-Jun Li<sup>1#</sup>, Dong-Hui Zhou<sup>2</sup>, Ning Chen<sup>3</sup>, Yan-Zhong Zhang<sup>4</sup>, Wen-Bao Qi<sup>1</sup>, Pei-Rong Jiao<sup>1</sup>, Ming Liao<sup>1</sup>, Guang-Zhi Tong<sup>5</sup> and Gui-Hong Zhang<sup>1\*</sup>**

<sup>1</sup>College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province 510642, People's Republic of China.

<sup>2</sup>State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, Gansu Province 730046, People's Republic of China.

<sup>3</sup>Shenzhen Institute for Drug Control, Shenzhen, Guangdong Province 518057, PR China.

<sup>4</sup>Shenzhen Rui-Peng Pet Hospital, Shenzhen, Guangdong Province 518001, PR China.

<sup>5</sup>Division of Swine Infectious Diseases, Shanghai Veterinary Research Institute, CAAS Shanghai 200241, People's Republic of China.

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**Canine influenza virus (CIV) is an emerging pathogen that causes severe and acute respiratory disease in dogs. Canine influenza is caused by two subtypes of influenza. A virus: H3N2 and H3N8. In recent years, surveys of avian origin CIV infection in dogs have been reported worldwide. However, little is known about the prevalence of CIV in pet dogs in China. In the present study, the prevalence of avian origin CIV H3N2 in pet dogs in Shenzhen, Southern China was investigated using the enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) assay. Thirty-one (6.71%) of the 462 serum samples tested were seropositive for avian origin CIV by ELISA. Use of the HI test revealed the presence of anti-H3 antibodies in 28 (6.06%) of 462 serum samples. The prevalence ranged from 4.87% (HI) or 6.19% (ELISA) to 7.41% among dogs of different ages, with high prevalence in pet dogs of 1 to 3 years old, but low prevalence in pet dogs  $\leq 1$  year. The seroprevalence in female dogs was 5.21%, and in male dogs it was 7.78% (ELISA) or 6.67% (HI). These findings demonstrated that avian origin canine influenza virus infection is prevalent in pet dogs and can spread rapidly through local dog populations, which indicates its potential for becoming established in pet dogs throughout China.**

**Key words:** Canine influenza virus, seroprevalence, pet dog, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) assay.

### **INTRODUCTION**

Canine influenza virus (CIV) is a member of the influenza virus A genus in the family Orthomyxoviridae and an emerging pathogen that causes severe and acute respiratory disease in dogs (Jirjis et al., 2010; Lee et al., 2010). CIV was first identified in racing greyhounds in

Florida in January 2004 (Payungporn et al., 2008). Canine influenza is caused by 2 subtypes of influenza A virus: H3N2 and H3N8. In 2005, the H3N8 CIV is known to be an equine-derived H3N8 influenza virus and was first identified in dogs in the United States, and in 2007, the H3N2 CIVs are of avian origin and detected in dogs in Korea and China (Crawford et al., 2005; Payungporn et al., 2008; Song et al., 2011b; Lee et al., 2010; Li et al., 2010).

Regardless of subtype, avian origin H3N8 or H3N2 CIV could infect nascent individuals and causes clinical signs. The most common sign of canine influenza is a mild

\*Corresponding author. E-mail: [guihongzh@scau.edu.cn](mailto:guihongzh@scau.edu.cn).

#These authors contributed equally to this work.

respiratory disease that resembles infectious tracheobronchitis. The experimental reproduction of the disease caused by H3N2 CIV induced clinical signs including coughing, sneezing, nasal discharge, fever, and shedding of the virus in nasal discharge (Song et al., 2011a, 2009; Lee et al., 2010). In previous pathological findings, the infection produced a distinctively severe and persistent bronchopneumonia with neutrophil infiltration and apoptosis in the tracheal epithelium (Jung et al., 2010).

Epidemics of avian origin H3N2 CIV among dogs have been observed in Korea, specifically in contaminated kennels in veterinary clinics. Serologic and virological survey of the avian-origin H3N2 CIV in dogs in South Korea suggest that the epidemiological situation resembles that of equine origin H3N8 CIV currently circulating in the dog populations of the United States (Payungporn et al., 2008; Song et al., 2008; Lee et al., 2009). However, no such serological or etiological studies have been carried out in pet dogs in Shenzhen, Southern China.

The objectives of the present investigation were to examine avian origin H3N2 CIV in pet dogs in five pet hospitals in Shenzhen, Southern China under the present husbandry practice and animal welfare, and to evaluate the risk factors for CIV infection in different ages and genders of pet dogs.

## MATERIALS AND METHODS

### Study area

Shenzhen is located in the very South of Guangdong province, overlooking HongKong to the South and bordering kowloon. It has an area of 1984.69 square meters, it is east to the Daya and Dapeng Bays, west to Pearl River, North to Dongguan and Huizhou and south to Hong Kong Special Administrative Regions of the People's Republic of China. It consists of 6 districts: Luohu, Futian, Nanshan, Yantian, Bao'an and Longgang which the first two are mainly urban areas. It has a mild subtropical oceanic climate with an annual average temperature of 22.3°C.

### Serum preparation

A total of 462 blood samples were collected from 5 different pet hospitals which are distributed in Futian and Luohu districts of Shenzhen city between May and July 2009. 82, 114, 127, 101 and 38 blood samples were obtained from pet dogs in Cuizhu, Futian, Honggui, Meilin and Shangbu pet hospitals, respectively. These samples were put aside for solidification followed by centrifugation at 1,000 × g for 10 min, and supernatants were transferred to new centrifuge tubes and saved at -20°C until use.

### Serological tests

#### ELISA test

The 462 serum samples described above were analyzed for CIV-specific antibodies by using a commercial ELISA Kit (Animal

Genetics Inc., South Korea) that can detect anti-nucleoprotein (NP) antibodies based on competition principles. The use of this ELISA kit for CIV detection has been previously reported (Lee et al., 2009; An et al., 2010). Briefly, ELISA plates coated with the antigen (nucleoprotein) are incubated with an equal mixture of 50 µl serum and 50 µl anti AIV antibody-HRP (Horseradish Peroxidase, 1:100 dilution in the conjugate diluent) for 30 min at 37°C. Then, the wells were washed 6 times with 350 µl of diluted washing solution. Then, 100 µl substrate was added to each well and incubated for 10 minutes at room temperature. Finally, 100 µl of stopping solution was added to each well. The absorbance of the wells was read with a bichromatic spectrophotometer at 450 nm with reference wavelength at 620 nm. Reading must be completed within 1 hour from the end of an assay. Positive and negative control sera were provided by the kit with 2 wells for each.

The mean absorbance of the negative controls was calculated, and then the PI (Percent inhibition) value of each serum was calculated, using the following formula:

$$\text{PI value} = [1 - (\text{OD sample} / \text{mean OD negative})] \times 100$$

Based on PI value, classification of each sample was as follows: PI < 50, negative (-); PI ≥ 50, positive (+).

#### Hemagglutination inhibition (HI) assay

In the present study, the A/canine/Guangdong/01/2007(H3N2) avian origin canine influenza virus strain was used in the serological tests. Genetically, this strain is highly similar to the avian influenza H3N2 virus and was isolated at the Animal Clinics of South China Agricultural University in 2007 (Li et al., 2010). The HI assay was performed according to procedures recommended by the World Organization of Animal Health (OIE). These samples were also analyzed by the HI test, which measures the ability of the sera to inhibit the hemagglutinating activity of the reference virus. The following antigens were also used for HI tested: H1N2 influenza virus (A/swine/Guang dong/06/2009/GH5) for H1, H3N2 influenza virus (A/Swine/Guangdong/01/2005) for H3, H5N1 influenza virus (A/Swan/Guangdong/197/2004) for H5, H9N2 influenza virus (A/Chicken/Guangdong/HL/2006) for H9. These viruses were isolated by the College of Veterinary Medicine, South China Agricultural University. All serum samples were treated with receptor-destroying enzyme (RDE) before testing in HI assays. Briefly, 25 µl of serial two-fold dilutions of the treated serum samples were mixed with 4 HA units of virus in V-shaped microtiter plates and incubated at room temperature for 30 min. Then, 25 µl of 0.5% (v/v) chicken red blood cells (RBCs) was added to each well and incubated at room temperature for 40 min. The HI titer was expressed as the reciprocal of highest serum dilution that completely inhibited hemagglutination of 4 HA units of the virus.

## RESULTS

In this investigation, a total of 462 pet dogs (219 females and 243 males) from Shenzhen, Southern China were examined by ELISA and HI for avian origin CIV antibodies. All serum was subjected to NP-specific ELISAs, anti-influenza virus antibodies were detected in 31 samples (6.71%). Use of the HI test revealed the presence of anti-H3 antibodies in 28 of the 462 samples (6.06%). Five samples were seropositive for avian origin H3N2 CIV by ELISA and HI test. Different levels of seropositivity were detected in different pet hospitals,

**Table 1.** Seroprevalence of canine influenza virus (CIV) in pet dogs in different pet hospitals in Shenzhen, southern China.

| Pet hospitals | Examined number | Seroprevalence (%) |               |
|---------------|-----------------|--------------------|---------------|
|               |                 | ELISA              | HI            |
| Cuizhu        | 82              | 17.07 (14/82)      | 14.63 (12/82) |
| Futian        | 114             | 2.63 (3/114)       | 2.63 (3/114)  |
| Honggui       | 127             | 6.30 (8/127)       | 5.51 (7/127)  |
| Meilin        | 101             | 5.94 (6/101)       | 5.94 (6/101)  |
| Shangbu       | 38              | 0 (0/38)           | 0 (0/38)      |
| Total         | 462             | 6.71 (31/462)      | 6.06 (28/462) |

**Table 2.** Seroprevalence of canine influenza virus (CIV) in pet dogs of different ages and genders in Shenzhen, Southern China using ELISA and HI test.

| Variable    | Class  | Prevalence (%) |               |
|-------------|--------|----------------|---------------|
|             |        | ELISA          | HI            |
| Age (years) | ≤1     | 6.19 (14/226)  | 4.87 (11/226) |
|             | 1-3    | 7.41 (10/135)  | 7.41 (10/135) |
|             | > 3    | 6.93 (7/101)   | 6.93 (7/101)  |
| Gender      | Male   | 7.78 (21/270)  | 6.67 (18/270) |
|             | Female | 5.21 (10/192)  | 5.21 (10/192) |

namely 0, 2.63, 5.94, 6.30 and 17.07% of the samples from Shangbu, Futian, Meilin, Honggui and Cuizhu were CIV antibody-positive, respectively (Table 1). Among these positive pet dogs, seroprevalence varied in different age groups, ranging from 4.87% (HI) or 6.19% (ELISA) to 7.41% (Table 2). The investigation also showed that the seroprevalence in female animals was 5.21%, and 7.78% (ELISA) or 6.67% (HI) in male animals using ELISA or HI (Table 2). All samples were also tested with other antigens (H1, H3, H5, H9) for HI test, and they were all negative.

## DISCUSSION

Canine H3N2 influenza viruses of avian origin were recently isolated and found to induce disease in dogs in Korea (Song et al., 2008). Recently, four genetically similar canine influenza H3N2 viruses of avian origin were isolated in South China with severe respiratory disease (Li et al., 2010). The emergence of these canine influenza cases in China could result also from the ecological changes in China, especially as the changing of socio-economic circumstances, particularly in urban areas where dogs are continuing to be raised for food, in some circumstances. CIV replicates efficiently in the respiratory system of dogs and causes severe respiratory disease. Active replication of CIV in the canine respiratory system results in intense inflammatory responses central to the pathogenesis of H3N2 CIV (Lee et al., 2011). Most natural

cases of H3N2 CIV died from associated respiratory diseases and the carcasses were generally quickly discarded by veterinarians for quarantine purposes.

Here, we report for the first time the seroprevalence of avian origin CIV H3N2 infection in dogs in Shenzhen city, southern China. 31 of the 462 tested pet dogs were seropositive for CIV by Ab ELISA (6.71%), which is higher than that in Korea, New Zealand and Japan. The seroprevalence of avian H3N2 influenza in Korean pet dogs in 2007 was 0.48% (2/419) by ELISA (Lee et al., 2009). In 2010, 16 (5.59%) of the 286 serum samples collected from pet dogs were CIV seropositive by ELISA in Korea (An et al., 2010). In New Zealand, the 251 dogs serum samples tested was not positive for CIV antibodies by indirect fluorescent antibody (IFA) (Knesl et al., 2009). In Japan, 12 (2.1%) of the 582 serum samples collected from dogs were HI-positive against human H3 virus, only one serum each from dogs was NI-positive against N2 virus (Said et al., 2011). By comparison, 31 of 74 (42%) dogs were seropositive for antibodies against CIV H3N8 in a metropolitan animal shelter (Holt et al., 2010). In Colorado, CIV H3N8 seroprevalence was 2.9% (4/140) for dogs seen by the Community Practice service and 4.5% (5/110) for dogs seen by other hospital services ( $P = 0.48$ ) (Barrell et al., 2010). In Italy, CIV H1N1 seroprevalence was 0.7% (7/1061) for canine serum specimens in 2009 (Dundon et al., 2010).

In the present study, the seropositivity rates of avian origin H3N2 CIV in pet dogs differed depending on whether an ELISA or HI assay was performed. For

example, while ELISA test found that 6.30 and 17.07% of the pet dogs from Honggui and Cuizhu were exposed to avian origin CIV, the HI test detected seropositivity rates of 5.51 and 14.63%, respectively. While the HI assay is often used to detect antibodies against viral hemagglutinin (HA) in human and animal serum, it is not very reliable in detecting antibodies to avian influenza viruses in mammalian serum because nonspecific hemagglutination inhibitors in the sera can result in false positives (Lu et al., 1982). In addition, Lee et al. (2009) found that the HI assay detected anti-influenza H3N2 virus antibodies 2 days later than the NP-based ELISA test. These results suggest that the NP-based ELISA is a better method for the serological survey of CIV infections in pet dogs.

## Conclusions

In summary, the present survey revealed a relatively low seropositivity of CIVs in pet dogs in Shenzhen, Southern China, which raises the concerns regarding the rapid spread of avian origin CIV in pet dogs in animal hospitals in China. These findings suggest that commercial vaccines against canine influenza virus must be developed and used in pet dog population.

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