Tropical Biomedicine 36(4): 1054–1060 (2019)

Serological and molecular surveillance for influenza A virus in dogs and their human contacts in Oyo State, Nigeria

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Received 4 January 2018; received in revised form 23 January 2019; accepted 25 January 2019

Abstract. Evidence of influenza A virus (IAV) infection in dogs, a major companion animal of humans, suggests the possibility that they may constitute a new source for transmission of novel influenza viruses to humans. The potential public health risk posed by this possibility of interspecies spread of IAV between dogs and humans necessitated surveillance for the virus in dogs and their human contacts. Sera from 239 asymptomatic pet and hunting dogs in Oyo state, Nigeria were screened for anti-IAV nucleoprotein antibodies using competitive enzyme-linked immunosorbent assay (ELISA) while haemagglutination inhibiting (HI) antibodies in the positive sera were detected using influenza virus H3 and H5 subtype-specific antigens. Suspensions prepared from 239 and 39 nasal swabs from dogs and human contacts, respectively were tested for presence of the highly conserved IAV matrix gene by reverse transcriptase-polymerase chain reaction (RT-PCR). Only 4 (1.7%) of the 239 sera tested were positive by the ELISA. The HI test confirmed the presence of H3 influenza virus subtype-specific antibodies in one (25.0%) of the 4 ELISA-positive sera with a titre of 1:128 while none was positive for H5 subtype-specific antibodies. All the nasal swabs assayed by RT-PCR were negative for IAV nucleic acid. The detection of IAV antibodies in pet and hunting dogs in this study, although at a low rate, suggests that these dogs could play a crucial role in the zoonotic transmission of influenza viruses especially considering the close interaction between them and their human contacts. Continuous surveillance for IAV among dog populations in Oyo State (and Nigeria) is therefore advocated to facilitate early detection of infection or emergence of novel influenza virus strains that could be potentially harmful to humans and or animals.

INTRODUCTION

Influenza viruses are important pathogens of humans and many animal species including poultry, pigs, horses and marine mammals. They belong to the family *Orthomyxoviridae*, genus *Alphainfluenzavirus* and species influenza A virus. They are classified into subtypes based on two surface proteins, the haemagglutinin (HA) and neuraminidase (NA) (Webster *et al.*, 1992). Currently, 18 HA and 11 NA subtypes have been reported (Tong *et al.*, 2013; Daodu & Oluwayelu, 2018). The influenza virus has long been absent from the list of infectious agents considered as pathogens of dogs and cats as earlier experimental studies showed, although these animals are susceptible to influenza A virus infections, they did not develop clinical signs (Beeler, 2009; Harder & Vahlenkamp, 2010).

However, recent studies have shown that dogs can develop severe clinical disease upon infection with influenza viruses and play a role in the interspecies transmission of these viruses. For instance,

influenza viruses have been reported to be naturally transmitted to dogs from other species including equine (H3N8) (Crawford *et al.*, 2005; Kirkland *et al.*, 2010), avian (H5N1 and H3N2) (Songserm *et al.*, 2006; Li *et al.*, 2010), and human (pandemic H1N1/09 and H3N2) (Lin *et al.*, 2012; Sun *et al.*, 2014). Additionally, serological evidence of influenza virus infections in dogs has been provided (Holt *et al.*, 2010; Oluwayelu *et al.*, 2011, 2014). These observations indicate that dogs may play an intermediate host role in transmitting the virus to humans and other mammals, and have stimulated surveillance of dogs for influenza virus infections.

As companion animals, dogs have a special status in modern human life; the close interactions of dogs with humans and surrounding wildlife provide frequent opportunities for cross-species virus transmissions, so dogs carrying influenza viruses can pose a threat to human health (Su *et al.*, 2013; Daodu & Oluwayelu, 2016). Therefore, in a country like Nigeria with recent upsurge in keeping of exotic and local dogs as pets accompanied by increased contact and bonding between the dogs and their owners (Oluwayelu *et al.*, 2011; Daodu *et al.*, 2017), and the traditional close association between hunters and their dogs, there is need to investigate the possibility that dogs in Nigeria could serve as reservoirs for influenza A virus transmission. Hence, serologic and molecular surveillance for influenza A virus was conducted in dogs and their human contacts in Oyo State, Nigeria.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Ethics Review Committee of the Ministry of Health, Oyo State, Nigeria (AD 13/479/739). Informed consent was obtained from all the human participants before they were enrolled for the study. All applicable standard guidelines for the care and use of animals were duly followed.

Study area and sample population

The study was carried out in three senatorial districts of Oyo State, southwest Nigeria with an estimated population of 5.6 million people (NPC, 2010) between June and November, 2014.

A total of 239 apparently healthy dogs (118 males, 121 females) and 39 consenting human contacts (dog owners and handlers) were used for this study. They comprised pet (exotic breed, n=32; local breed, n=51) and hunting (156 local breed) dogs sampled from urban (n=97) and rural (n=142) areas of the State. Information on age, sex, type of management, dog use and locality were collected for each dog.

Sample collection

Each dog was appropriately restrained prior sampling. Blood was aseptically collected from the cephalic vein of dog into clean tubes and allowed to clot at room temperature for 3-4 hr. The sera were separated and stored in microcentrifuge tubes at -20°C until tested.

Nasal swabs were also collected from the dogs and human contacts by gently inserting a sterile swab into the nostrils and swabbing the wall to remove mucous material with it. The swab was immediately transferred into virus transport medium (containing antibiotics and foetal calf serum) in labelled micro-centrifuge tubes. The swabs were then transported on ice to the laboratory where they were stored at -80°C until analysed.

Serology

A competitive multi-species enzyme-linked immunosorbent assay (ELISA) kit (IDvet, France) for the detection of antibodies directed against the internal nucleocapsid of influenza A virus was used to screen the dog sera. All steps were performed according to the manufacturer's instructions and results were read using an ELISA reader (Optic Ivymen® System, Model 2100C, Biotech SL,Madrid, Spain) at a wavelength of 450 nm. Positive samples (S/N% $\leq$ 45) were screened by the haemagglutination inhibition (HI) test for antibodies against

influenza virus H3N8 (equine origin) and H5N2 (avian origin) using 4 haemagglutinating units of the respective antigen, according to standard protocol (OIE, 2014).

Molecular Assay -Reverse transcriptase-Polymerase chain reaction (RT-PCR)

Suspensions prepared from the collected dog and human nasal swabs were centrifuged (4°C) at 1000 rev/min for 10 min to obtain clear supernatants. RNA was extracted from the supernatants using the Aurum total RNA Mini kit (BIO-RAD, France) according to manufacturer's recommendation and amplified by conventional RT-PCR in an ABI thermal cycler (Applied Biosystems, USA). Amplification was done with the QIAGEN One-Step RT-PCR kit (Hilden, Germany) in a 50 µl reaction mixture containing 2 µl enzyme mix (including Omniscript reverse transcriptase and hot-start Taq polymerase), 10 µl 1X RT-PCR buffer, 3 µl of 10 µM of each primer, 2 µl dNTPs, 25 µl nuclease-free water and 5 µl of the RNA extract. Each amplification run contained negative and positive controls with the negative control being nuclease-free water while nucleic acid extracted from a known virus stock served as positive control. The primers (forward (M52R): 52- CTT CTA ACC GAG GTC GAA ACG- 32 and reverse (M253R): 52- AGG GCA TTT TGG ACA AAG/T CGT CTA - 32) were designed to amplify a 250 bp fragment in the influenza A virus matrix gene. The following thermal profile was used: reverse transcription for 30 min at 50°C, a single cycle initial PCR activation step at 95°C for 15 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. Final extension was for 10 min at 72°C.

Statistical analysis

Data obtained were analysed using GraphPad Prism 5.0 (GraphPad, USA) and p-values less than 0.05 were considered significant.

RESULTS

The ELISA detected IAV antibodies in 4 (1.7%) of the 239 dog sera tested. Based on dog use, 1.9% (3/156) and 1.2% (1/83) prevalence of IAV antibodies were obtained in hunting and pet dogs, respectively while on age basis, a slightly higher prevalence rate was observed in dogs more than a year old compared to those less than one year. This difference was however, not significant (Table 1). Also, there were no significant differences based on breed, sex, locality and dog use. However, analysis further showed that local dogs were 2.19 times more likely to be seropositive for CIV compared to exotic dogs. Also, the HI test for detecting influenza virus H3N8 subtype-specific antibodies revealed that only one (25.0%) of the 4 ELISA-positive sera was positive, with a titre of 1:128. This sample was from a > 2-year-old hunting dog. The positive control serum also had a titre of 1:128. However, none of the samples contained H5N2 subtype-specific antibodies.

All the nasal swabs assayed for influenza A virus RNA by RT-PCR were negative.

DISCUSSION

With an estimated population of over 400 million worldwide, dogs have been described as one of the most popular pets on earth (Coppinger & Coppinger, 2001). As companion animals, they have extensive contact with humans, sharing their spaces, playing with children, producing faecal waste, and occasionally biting and scratching. Dogs are also frequently in contact with wildlife through hunting or scavenging. The large population of dogs and their close interactions with humans therefore make them a potential risk factor for zoonotic virus transmission, as seen with rabies virus and rotavirus (Cook, 1989; Lackay *et al.*, 2008; Tsugawa & Hoshino,

Table 1. Prevalence of influenza A virus antibodies in dogs in Oyo State, Nigeria

Features	Number tested (Positive)	% positive	OR (95% CI)	p-value
Breed				
Exotic*	32 (1)	3.1	1	
Local	207 (3)	1.4	2.19 (0.22 – 21.77)	0.44
Sex				
Female*	121 (1)	0.8	1	
Male	118 (3)	2.5	0.32 (0.03 – 3.12)	0.37
Age (years)				
< 1*	69 (1)	1.4	1	
> 1	170 (3)	1.8	0.82 (0.08 – 8.01)	1.00
Dog use				
Pet*	83 (1)	1.2	1	
Hunting	156 (3)	1.9	0.62 (0.06 – 6.08)	1.00
Locality				
Urban*	97 (2)	2.1	1	
Rural	142 (2)	1.4	0.48 (0.05 – 4.71)	0.65
Total	239 (4)	1.7		

Key: * Reference value. OR – Odd ratio. 95% CI – 95% Confidence interval.

2008). Evidence of canine influenza infection in pet dogs, a major companion animal of humans, suggests the possibility that dogs may provide a new source for transmission of novel influenza viruses to humans (Crawford *et al.*, 2005). Consequently, close contact between dogs and humans poses a public health concern related to the possible emergence of reassortant influenza viruses in companion animals with the likelihood of zoonotic transmission to humans (Song *et al.*, 2012).

In the present study, an overall IAV antibody prevalence of 1.7% was obtained using the ELISA technique in pet and hunting dogs in Oyo state, Nigeria while a prevalence rate of 0.4% (1/239) of H3N8 subtype-specific antibodies was obtained in the sampled dogs. Although this implies a low exposure rate of dogs in the study area to influenza A virus, it however, suggests that these seropositive dogs had been naturally exposed to the virus since vaccination of dogs against influenza virus is not a current practice in Nigeria. Similar studies in Italy, the USA and Canada also reported low prevalence of IAV antibodies in dogs (Steven *et al.*, 2008; Piccirillo *et al.*, 2010; Barrell *et al.*, 2011; Anderson, 2013). Moreover, it is worth noting that none of the seropositive dogs in the present study showed any respiratory abnormality i.e. they were all asymptomatic. This implies that they could serve as potential carriers shedding the virus into the environment. In a previous study, Yamanaka *et al.* (2009) also detected IAV seropositivity in dogs showing no respiratory abnormality and suggested that the seropositivity observed might be as a result of past infection.

Although the study revealed no significant difference in IAV antibody prevalence in the tested dogs based on breed, sex, age, dog use and locality, more hunting dogs (n=3) possessed antibodies to the virus compared to pet dogs (n=1). The seropositive hunting dogs might have been infected through consumption of offal of infected animals killed during hunting (Oluwayelu *et al.*, 2011), or through mating which is largely indiscriminate and uncontrolled, thus exposing them to influenza virus in droplets and aerosols created by coughing and sneezing, and through contact with nasal discharges, either directly or on fomites (CFSPH, 2014).

Interestingly, a trace-back of the only seropositive pet dog showed that it was a male Samoyed dog, less than a year-old and kept in close confinement with two other dogs which were IAV seronegative in this study. According to the owner, the dog was obtained from a dog breeder who operates a commercial kennel that boards different exotic (imported) dogs for breeding purposes. It is possible that the pet dog contracted the virus from these exotic dogs especially considering that the two other dogs kept together with it in the same compound were seronegative. This possibility is strengthened by the observation that the greatest risk of influenza A virus infection is among dogs that reside in kennels or are exposed to transient groups of dogs, as in animal shelters or dog day care facilities (CFSPH, 2014).

The detection of influenza virus H3N8 subtype-specific antibodies in this study, coupled with previous reports (Oluwayelu *et al.*, 2011, 2014) of detection of antibodies against H3 and H5 influenza virus subtypes in dogs within the same study area, suggests that the circulating influenza A viruses in the study area include the H3 and H5 subtypes. However, this does not preclude the possibility of other IAV subtypes circulating as the dog sera in the present study were not tested against other currently identified IAV haemagglutinin subtypes (Tong *et al.*, 2012). It is likely that the ELISA-positive sera which were negative by the HI test contained antibodies to other IAV subtypes that were not screened for in this study.

The non-detection of IAV matrix gene in nasal swabs from dogs and their human contacts in this study is consistent with the findings of Yamanaka *et al.* (2009) who also could not detect IAV in nasal swabs of dogs by RT-PCR. It is possible that the dogs and their human contacts were not actively shedding the virus at the time of sample collection or that the virus exists in too low titres that were undetectable by the RT-PCR.

CONCLUSION

Although this study revealed low seroprevalence of IAV antibodies in pet and hunting dogs in Oyo State, Nigeria, it however shows that these dogs may potentially play a role in interspecies transmission and spread of influenza virus as previously reported (Song *et al.*, 2008; Zhan *et al.*, 2012). Specifically, the frequent close contact between dogs and humans raises concerns about the possibility of zoonotic transmission of the virus. We therefore advocate continuous surveillance for IAV among dog populations in Oyo State and Nigeria as whole to ensure early detection of IAV strains or emergence of novel strains that could possibly be harmful to humans and or animals.

Acknowledgement. Positive control influenza virus H3N8 antigen and antiserum were kindly provided by Professor Ed Dubovi of the College of Veterinary Medicine, Cornell University, Ithaca, New York, USA.

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