



Serological and molecular investigation of canine influenza virus in Plateau State, Nigeria

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Abstract

Canine influenza is a highly contagious respiratory infection of dogs caused by the Influenza A Virus (IAV), characterized by cough, sneeze, nasal secretions, and inappetence. Infections can be mild, severe or fatal. Aquatic birds constitute a natural reservoir for IAV, which is transmitted to terrestrial birds, including poultry. IAV has also emerged in other mammalian species, including humans, swine, horses, and dogs. IAV epidemics in dogs are a recent development. Commonly detected Canine Influenza Virus (CIV) strains are A/H3N2 and A/H3N8 from avian and equine influenza, respectively. Nigeria's agro-ecology witnessed widespread circulation of avian influenza since 2006, and recent outbreaks of equine influenza in 2018/2019 raise the possibility of inter-species transmission to dogs. To investigate canine Influenza in Plateau State, we collected 113 nasal swabs and 270 sera samples from dogs in clinics, live dog markets, and during dog vaccination campaigns. After extracting nucleic acid with the Qiagen kit, RT-PCR analysed swabs for the Influenza A matrix gene. Sera samples were screened by Enzyme-Linked Immunosorbent Assay before subtyping a cross-section for H3 antibody by Hemagglutination Inhibition. No matrix gene was amplified from extracted nucleic acid from the nasal swabs. Though few sera were reactive to influenza A nucleoprotein, none was positive for influenza A/H3. The H3N8 strain of equine influenza virus first caused an epidemic in dogs in 1999 in the United States. Subsequently, avian-origin H3N2 CIV emerged in dogs in China and South Korea in 2005. Past CIV epidemics arose from a single cross-species transmission of H3N8 subtype from a mammalian intermediate host and the H3N2 subtype from an avian reservoir. Even though this limited investigation did not detect CIV in Plateau State, the potential remains because of the persistent circulation of avian, swine, and equine Influenza in Nigeria, which requires more extensive virological and serological surveillance.

Keywords: Canine Influenza, Dogs, RT-PCR, Plateau State, Serology

Introduction

Influenza A viruses (IAV) belong to the family Orthomyxoviridae. They include enveloped viruses of

80-120nm in diameter with a negative-sense single-stranded RNA genome (Xie *et al.*, 2016). The genome

is made up of eight (8) segments encoding a minimum of ten (10) proteins, sometimes 14 depending on strain namely: haemagglutinin (HA), neuraminidase (NA), polymerase subunits (PA, PB1 and PB2), nucleoprotein (NP), interferon agonist (NS1), nuclear export protein (NEP), matrix protein (M1) and channel protein (M2). (Bouvier & Palese, 2008). Currently, there are 18 haemagglutinins (H1-H18) and 11 neuraminidases (N1-N11) (Henry, 2018). Viruses containing identical haemagglutinin and neuraminidase might have marked differences in their genomic segments, influencing pathogenicity and host specificity (Xie *et al.*, 2016). This variation within the genomic segment allows for both antigenic shift and antigenic drift, which may lead to the formation of a new virulent virion or a new Influenza A virus subtype. The IAVs have a diverse range of hosts, with most viruses being found as common and endemic gastrointestinal tract infections of bird populations (Yoon *et al.*, 2014). Occasionally, avian IAVs spill over to infect terrestrial bird populations or into certain mammalian populations (Webster & Govorkova, 2014). Among mammals, IAVs have caused outbreaks among humans, swine, horses, seals, mink, dogs and cats (Parrish & Voorhees, 2019). Canine influenza is a recent emerging, contagious, upper respiratory tract disease of dogs, characterized by cough, pyrexia, nasal discharge, dyspnea, and lethargy (Gibbs & Anderson, 2010). Currently, there are at least six influenza virus subtypes that have been reported to infect dogs: H3N8, H3N2, H5N1, H5N2, H3N1 and H1N1 (Crawford *et al.*, 2005). The H3N8 and H3N2 viruses can cause sustained transmission between dogs, but there is insufficient evidence that these other subtypes can be transmitted continuously in dog populations. These outbreaks in dogs suggest they could play a role in the transmission of IAV between humans and other mammalian species. Dogs are the pre-eminent companion animals to man and occupy a unique niche due to the various roles they perform which enables them to serve as an intermediate host in the transmission of IAV's between man and other mammals (Daoudou *et al.*, 2019). It is also possible that additional reassortments between canine and human viruses could occur in dogs or humans or possibly other hosts such as cats, potentially creating a new human influenza virus for which there may be little or no natural immunity. In Nigeria, there is the paucity of information on Canine Influenza, hence a need for surveillance. The recent 2018 outbreak of equine Influenza in Nigeria (Shittu *et al.*, 2020) and the knowledge that the H3N8 virus in dogs originated from horses as well as the H3N2, which is of avian origin justifies investigation into the possible exposure of dogs to AIV in the study

area. (Parrish *et al.*, 2015). The study aimed to detect CIVs in dogs by quantitative real-time polymerase chain reaction (qRT-PCR) and serological methods from nasal swabs and sera, respectively.

Materials and Methods

The study was carried out in Plateau State with coordinates 10.3837°N, 8.3763°E and 8.3971°N, 9.6272°E, which has a population of about 3.5 million people who are predominantly farmers (Anon, 2019). One hundred and thirteen nasal swabs were collected in viral transport medium from dogs that showed signs of coughing and/or having nasal discharge. Two hundred and seventy sera samples from dogs at a rate of 30 samples per month for nine months. All samples were collected from January to September 2019 from veterinary clinics, live dog markets and during dog vaccination campaigns in Jos metropolis. These samples were appropriately labelled and thereafter transported to the Influenza Laboratory of the National Veterinary Research Institute, Vom, Plateau State, Nigeria, in an ice-packed box. The swab samples were stored at -80°C and serum samples were stored at -20°C until analyzed.

RNA extraction from swabs was carried out by the use of a Qiagen RNeasy Mini Kit, a high Pure RNA extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in a final volume of 50 µl and stored at -80°C.

The RNA was screened for matrix (M) gene using real-time qRT-PCR assays on a Rotor-Gene Q MDx machine, model 5-plex (QIAGEN Co, CA, Germany) in a 25 µL reaction mixture containing 5 µL of RNA, 1.5 µL of each primer, M+25 5' AGATGAGTCTTCTAACCGAGGTCG-3' and M-124 5' TGCAAAAACATCTTCAAGTCTCTG-3' (5 µM), 2.5µL of M+64 FAM probe 5' TCAGGCCCTCAAGCCGA-3' (1 µM), 12.5 2X RT-PCR master mix, 0.2 µL enzyme mix (Quantitect multiplex), and 1.8 µL of Rnase-free water with the following cycling conditions: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45 s and 60°C for 45 s (Spackman *et al.*, 2002).

The sera samples were tested using a specific commercially available indirect Influenza A Virus Antibody ELISA Test Kit (IDEXX Influenza A Test Kit) following the manufacturer's instructions. This kit is an enzyme immunoassay capable of detecting IgG antibodies to all influenza A viruses in animal serum. Each sample was diluted tenfold (1/10) with the dilution buffer and mixed thoroughly using plain microplates before being assayed. The samples were tested individually in ELISA plates that were pre-coated with influenza A antigens. The absorbance of the samples and the controls was measured and recorded at 650nm. The validity criteria of the test were determined as NCX ≥ 0.600 and PCX ≤ 0.50 for

the mean of negative control and positive control respectively. The presence or absence of antibody to Influenza A in the samples were determined by the sample to negative ratio (S/N). The cutoff for negative samples was $S/N \geq 0.50$ while the cutoff for the positive samples was $S/N < 0.60$.

Treatment of sera: Treatment of test sera to remove nonspecific agglutinins and nonspecific inhibitors was carried out according to WHO protocol (2002).

Forty (40) sera samples that were positive by ELISA were analyzed using a haemagglutination inhibition test according to the protocol described in OIE (2018). H3 antibody was assessed from serum samples. Briefly, 0.025mL of PBS was dispensed into all wells of a plastic 96-well microtiter plate (v-bottomed wells) except the first well in row H, and 0.025mL of serum was placed in the first well. 0.025mL of the positive control serum (with known HI titer) and negative control sera were added to two respective wells of the microtiter plates. With the aid of a multichannel micropipette, twofold dilutions of the sera were made across the plate (A1–A12). The last 0.025mL was discarded and 0.025mL of antigen containing 4HAU was added to all the wells except row H, which served as back titration.

H3N8 antigen was used. Back titration was carried out; thus, 0.025mL of antigen suspension containing 4HAU was added to each of the first two wells of row H and twofold dilution was made from H2 to the H6 and the last 0.025mL was discarded to obtain 4, 2, 1, 0.5, 0.25, and 0.125 HAU. This was mixed by tapping gently and plates were placed at room temperature for 30 minutes. 0.025mL of 1% washed chicken-RBC was added to each well. Again the mixture was placed on the bench at room temperature for 30 minutes and observed for HI reactions. This was done by tilting and observing the presence of tear-shaped streaming at the same rate as the control wells containing RBCs only.

Results and Discussion

The samples analyzed in these investigations were negative for Influenza A virus. Yet, Canine influenza was detected in previous studies. The serological test by ELISA showed some reactive sera but none was subtyped. The first detection of Canine influenza virus was reported in Florida, USA in 2004 in racing greyhounds and it later spread to nine other states in USA between 2004 -2005 (Crawford *et al.*, 2005). They also reported that the virus was first detected by serological technique in dogs which showed acute respiratory disease in shelters, and Vet clinics in both New York and Florida in 2004 and 2005.

The qRT-PCR result for all the 113 samples tested showed no amplification for matrix gene from extracted nucleic acid from the nasal swabs. The none

detection of CIV in both 113 nasal swabs and 270 sera samples from the tested dogs in Plateau State, Nigeria corroborate the report of De Benedictis *et al.* (2010) in Italy, Knesl *et al.* (2009) in New Zealand, Kruth *et al.* (2008) in Ontario, Canada and Schulz *et al.* (2014) in Germany who screened for CIV in dogs and detected zero prevalence. This is an indication that, currently, canine influenza virus (CIV) of H3N8 and H3N2 circulates only in the dog populations in the USA, China, Thailand, North America and Korea. The risk of CIV transmission from dogs to other mammals and humans appears low in Plateau State. This may imply that the CIV is yet to find a suitable host in dogs in Plateau State since all the samples tested were negative for the influenza A virus. The possibility of missing out CIV in the samples collected was low, first, because qRT-PCR was used to analyze the swab samples, which had been shown to detect viruses with titre as low as 3 EID 50 (50% egg infectious dose) in its sensitivity (Joannis *et al.*, 2008). Secondly, the sera samples were analyzed using two serological techniques (ELISA and Haemagglutination inhibition test) which are the recommended serologic test for detecting Influenza A viruses in samples (OIE, 2012). The PCR technique for viral nucleic acid detection is more sensitive (up to 100-fold) than virus isolation in eggs or mammalian cell cultures (Fouchier *et al.*, 2000). The absence of CIV in this study is an important finding for reference, knowledge and documentation for tracking the potential role of dogs in influenza A virus transmission in Plateau State.

This result also suggests that the risk of possible mixing or reassortment of the CIV in dogs with its concomitant risk to humans and other species is low, despite the unique ability of dogs to interface with humans, poultry and wildlife. It also reveals that the probability of canine influenza vaccine use in the study area is probably nil, since serologically there were no animals positive for the H3 haemagglutinin test. This result also differs from the results obtained by Oluwayelu *et al.* (2014) who obtained a 40% seropositivity to H3N8 virus in pets and hunting dogs in Ibadan and Lagos, Nigeria. It is important to note that they specifically targeted hunting dogs. Despite the result of this study, more work is required for continuous seroepidemiological surveillance of the CIV and other AIVs in Plateau state as well as nationwide.

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Nil

Conflict of Interest

The authors declare that there is no conflict of interest.

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