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Detection of avian influenza antibodies and antigens in poultry and some wild birds in Kogi state, Nigeria

NO Ameji[1]*, L Sa'idu[2] & PA Abdu[1]

1. *Department of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria*
2. *Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nigeria*

*Correspondence: Tel.: +2348035907570; E-mail: amejivet@yahoo.com

Abstract
The global spread of HPAI (H5N1) between 2005 and 2006 was blamed on movement of migratory wild birds and trade in live poultry across continents from infected regions. A survey was carried out to detect the presence of avian influenza (AI) antibodies in wild birds and AI viruses in poultry and wild birds from Kogi state, Nigeria. Haemagglutination inhibition (HI) test and enzyme link immunosorbent assay (ELISA) were used to detect AI antibodies in some species of apparently healthy wild birds during the survey. Using HI test, the wild birds were negative for AI (H5) antibodies but ELISA detected AI (NP) antibodies in Black Stork (*Ciconia nigra*) with an overall seroprevalence of 4.5% and mean titre of 24.50±2.400 EU. Cloacal swabs from the same species of wild birds that were tested for antibodies and 710 oropharyngeal swabs from poultry were tested for AI viruses using RT-PCR with primers targeting the AI matrix proteins but were negative for AI viruses. The detection of AI (NP) antibodies in wild birds but failure to detect the viruses showed that the exposure might not be recent. We recommend that poultry should be prevented from contact with wild water birds and a broad based surveillance for AI viruses in poultry and wild birds should be carried out in Kogi state, Nigeria.

Keywords: Avian influenza, Black stork, ELISA, HI, RT-PCR
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Introduction

Avian influenza viruses (AIVs) have been isolated from over 100 species of wild birds most of which are wild aquatic birds such as gulls, terns and shorebirds or waterfowls such as ducks, geese and swans (Swayne, 2008; Krauss & Webster, 2010). Most of the AIVs found in wild birds are low pathogenic avian influenza (LPAI) and do not cause diseases in the reservoir hosts (Krauss & Webster, 2010). The role of free-flying wild birds in the spread of AIVs is established in the spread of HPAI (H5N1) from Asia to Europe and Africa in 2006 (Kilpatrick *et al.,* 2006). It was also evident in the isolation of HPAI (H5N1) viruses from apparently healthy Tree Sparrows (*Passer montanus*) in China in 2003 and 2004 (Kelly *et al.,* 2008). In addition, large-scale outbreak of HPAI (H5N1) was reported in a zoological institution in Cambodia that affected a variety of species of captive wild birds including eagles and owls (Desvaux *et al.,* 2009).

The maiden outbreak of HPAI H5N1 in 2006 in Nigeria was blamed on trade in poultry and migratory wild birds as likely sources of introduction (Brown, 2006; Ducatez *et al.,* 2006). The involvement of wild birds in the epidemiology of AIVs in Nigeria was further supported by the detection of AIVs (H5N2) by means of molecular tests in apparently healthy white-faced whistling-duck (*Dendrocygna viduata*) and spur-winged goose (*Plectropterus gambensis*) in 2007 (Gaidet *et al.,* 2008; Snoeck *et al.,* 2011). Based on these reports and the resurgence of HPAI H5N1 in two northern states of Nigeria in 2008 after a long absence, it was suggested that terrestrial wild birds might also be major reservoirs of AIVs (Columba-Teru *et al.,* 2012).

Furthermore, the recurrent reports of HPAI outbreaks in poultry farms and live bird markets (LBMs) in some states of Nigeria in 2015 have widened the speculations about the possible role of wild birds in the maintenance and spread of AI infection (Vetline, 2015). Wild birds are capable of excreting abundant viruses especially AIVs in their feces before and after the onset of clinical signs or even asymptomatically (Chen *et al*., 2005). In addition, Si *et al.* (2013) reported that exchange of avian pathogens is enhanced if poultry markets or free ranging poultry farms are close to wild birds or waterfowls' habitats such as lakes, wetlands, breeding or over-wintering sites. Kogi state has two major rivers, Benue and Niger passing through it as well as wetlands in most Local Government Areas (LGAs) which serve as resting points for resident and migratory wild birds (Anon., 2009; Abdu, 2010).

Kogi state is yet to report any outbreak of HPAI H5N1 since 2006 but there had been reports of the detection of AI antibodies in poultry at different times in the state (AICP, 2008; Ameji *et al.,* 2011; Vetline, 2015; Ameji *et al.,* 2015). Ameji *et al*. (2011) had reported the detection of antibodies to AI by agar gel precipitin test (AGPT) in chickens from six LGAs of Kogi state. In addition, AI (H5) and AI (NP) antibodies were reported in poultry from 12 LGAs of Kogi state after seven years of the absence of HPAI H5N1 outbreaks in Nigeria (Ameji *et al*., 2015). The presence of AI antibodies in poultry in Kogi state which did not report HPAI H5N1 outbreak, indicated exposure to AIVs requiring further investigations. This study was designed to determine AI antibodies in wild birds and the prevalence of AIVs in poultry and wild birds in Kogi state, Nigeria. Available literature indicates that this is the first report of the presence of antibodies to AI in wild birds in Kogi state, Nigeria.

Materials and Methods

Study area

The study area is Kogi state which lies between Latitude 6°44' - 7°36'N and Longitude 7°49' - 8°27'E situated at a height of about 789 km above sea level and covering a land area of 29,833 Km^2. The state is bordered by Federal Capital Territory (FCT) and Niger state on the north; Benue and Nasarawa states on the east; Ondo, Ekiti and Kwara states on the west; Edo, Anambra and Enugu states on the south.

Kogi state has a total of twenty-one (21) Local Government Areas (LGAs) with a human population of 2,099,046 and major economic activities of the people being farming, fishing and trading (KGSADP, 2009). The poultry population is estimated to be 3,685,211 with 91.5% being rural or backyard poultry and the rest being commercial poultry (Adene & Oguntade, 2006).

Sampling method and sample size

Multistage, simple random and convenient sampling methods were used for survey locations, poultry and wild birds respectively from 12 LGAs out of the 21 LGAs of Kogi state, Nigeria.

Poultry were sampled from backyard farms, rural households and live bird markets (LBMs) during the survey. The sample size for the study was determined using the formula of Cohen (1988):

$N = Z^2Pq/L^2$. Sample size for AI survey in poultry from the 3 sampling units = 234 x 3 = 702

Data on population size of wild bird species and prevalence of AI in wild birds are scanty in Nigeria, a problem adjudged to be a major challenge for designing disease surveys in wild birds in most countries (Wilking *et al.,* 2009). Hence, the sample size was not predetermined but was thought reasonable to limit the sample size to available number of wild birds captured as done by other workers (Columba-Teru *et al.,* 2012; Assam, 2014).

Ethical consideration for wild bird survey: Approval for wild birds' survey was given by the ethical committee, Department of Veterinary Medicine, Ahmadu Bello University, Zaria. One hundred species of wild birds from 8 families were captured alive using mist net and locally made glue traps then, were released into the wild in the field after sampling and being marked on the feet with an indelible marker to avoid resampling. The families of wild birds that were captured and sampled include *Accipitridae, Muscicapidae, Apodidae, Hirundinidae, Psittacidae, Ciconidae, Sylvidae and Columbidae.*

Collection of blood from wild birds: Blood sample was collected from a wild bird after proper restraint with the use of a 23 or 24 gauge needle (depending on the size of bird) attached to a 2 ml syringe to draw 0.5-1 ml of blood from the jugular vein of the wild bird. The blood collected was allowed to stand for two hours for clotting to occur and the serum decanted into a bijou bottle, labeled and stored under ice then transported to the Laboratory within 48 hours and kept at -20 °C until used.

Cloacal and oropharyngeal swabs sampling: The oropharyngeal and cloacal swabs were collected after proper restraint of a bird by an assistant with

care being taken to avoid harm to the birds and placed in a cryo-vial of viral transport medium containing 10,000 units/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml gentamycin, 5000 units /ml mycostatin and 1% bovine serum albumin in phosphate buffered saline (PBS) (OIE, 2009). The swab samples were maintained at 4 °C in ice pack upon collection and stored in liquid nitrogen at -70 °C within 4-6 hours until they were processed.

Detection of avian influenza antibodies in wild birds
Sera from only 44 out of 100 species of wild birds caught were analyzed due to inadequate amount of sera obtained from some species after clotting.

Haemagglutination inhibition test: The inactivated AI (H5) antigen (Batch 20060212) used was produced from China and the antiserum (H5N2 Batch 1/10) used was produced by OIE/FAO reference Laboratory for AI and ND, delle Venezie, Italy. A 1% suspension of chicken red blood cells (RBC) was prepared and used as indicator in the haemagglutination (HA) and haemagglutination inhibition (HI) tests. Haemagglutination (HA) test was carried out according to the OIE (2009) protocol to determine the antigen titre, diluted to 4HAU that was used for the HI tests. The alpha HI test was also carried out as described by OIE (2009) and the results were expressed in $\log_2$.

Enzyme linked immunosorbent assay: The AI ELISA antibody test kit used was manufactured by Affinitech Ltd, Bentonville, AR 72712, USA. The reagents supplied included antigen coated microtitre plates, sample diluent, wash solution, conjugate (IgG alkaline phosphate), substrate (p-Nitrophenyl phosphate), stop solution (3.0 M NaOH), positive and negative controls which were prepared and used according to manufacturer's instructions. The ELISA plates were read using dual wavelength microtitre plate ELISA reader with 405 nm primary filter and 630 nm reference filter blanked on air. Serum with 15 ELISA Units (EU) or more was taken as positive.

Detection of avian influenza viruses in poultry and wild birds
A total of 810 swabs consisting of 710 oropharyngeal swabs from apparently healthy poultry and 100 cloacal swabs from the same wild birds that were bled for serology were collected and analyzed for AIVs.

Preparation of samples: The swab tubes were decontaminated by spraying with virkon^R (Du Pont, USA) and dried with tissue paper. The samples were then pooled based on survey locations, species and sampling units into sterile tubes. Ten swabs were pooled into one in sterile tubes and labeled before viral RNA extraction (Killian, 2008; Wakawa *et al.,* 2012).

Extraction of nucleic acid: The ribonucleic acid (RNA) was extracted from each of the pooled test samples using QIAamp Viral RNA mini kit (QIAGEN GmbH, Germany) by spin protocol according to the manufacturer's instructions. The QIAamp Mini spin column was discarded and the eluted RNA was stored at -20°C until used.

Preparation of polymerase chain reaction master mix: The master mix for the 25 µl final volume for PCR was prepared from stock of reagents of nuclease free H_2O, 10x PCR Buffer, 50 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate (dNTP), 20 mM dithiothreitol (DTT), 10 µM each of oligonucleotide (M52C and M253R – forward and reverse primers), 20 U of reverse transcriptase, 5 U of ribonuclease inhibitor, 5 U of Ampli – Taq DNA polymerase (all reagents by Applied Biosystems, USA) and 5 µl of RNA extract. The AIVs forward and reverse primers targeting the M-gene of 250 bp size with the following sequences: M52C = 5'-CTT CTA ACC GAG GTC GAA AGG-3' and M253R = 5'-AGG GCA TTT TGG ACA AAG/T CGT CTA - 3' (Fouchier *et al.,* 2000) were used.

Amplification of the reverse transcription-polymerase chain reaction: The conventional one step RT – PCR was used to amplify the samples in final reaction mixture of 25µl. Thermocycling was done in GeneAmp^R PCR System 9700 (Applied Biosystems, USA) with the following cycling conditions: activation at 40°C for 20 min and initial denaturation at 95°C for 5 min (stages I & II once), denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1 min (stage III 40 times), final elongation at 72°C for 10 min and hold at 4°C until used (stage IV once).
Agarose gel electrophoresis of the amplicons: A 1.5% agarose gel was prepared. The amplicons were mixed with a loading dye (blue/green, Promega^R) on a parafilm paper in the ratio of 2:8 and loaded in the wells of the gel with one Kb plus DNA ladder (Fermentas^R) used as band marker, each lane of the

Table 1: Avian influenza antibodies in wild birds using enzyme link immunosorbent assay in the surveyed Local Government Areas of Kogi state, Nigeria

Local Government Area	No. tested	No. positive (%)	Mean antibody titre ± SEM EU
Adavi	5	0 (0.0)	-
Ajaokuta	3	0 (0.0)	-
Ankpa	10	0 (0.0)	-
Dekina	5	0 (0.0)	-
Lokoja	7	2 (28.6)	24.50 ± 2.400
Mopamuro	2	0 (0.0)	-
Ofu	2	0 (0.0)	-
Okene	5	0 (0.0)	-
Olamaboro	5	0 (0.0)	-
Total	44	2 (4.5)	24.50 ± 2.400

Table 2: Avian influenza antibodies using enzyme link immunosorbent assay in different wild bird species surveyed in Kogi state, Nigeria

Species	No. tested	No. positive (%)	Mean antibody titre±SEM EU
Milvus migrans	3	0 (0.0)	-
Streptopelia senegalensis	13	0 (0.0)	-
Muscicapa infuscata	2	0 (0.0)	-
Psittacula krameri	3	0 (0.0)	-
Ciconia nigra	2	2 (100.0)	24.50 ± 2.400
Hirundo spp.	15	0 (0.0)	-
Apus spp.	4	0 (0.0)	-
Hippolais polyglotta	2	0 (0.0)	-
Total	44	2 (4.5)	24.50 ± 2.400

wells had positive and negative controls. The gel was placed in the electrophoresis tank, submerged by *tris-borate ethylene diamine tetra-acetic acid* (TBE 1x) buffer after loading. The electrophoresis was run at 120 V for 35 min after which the bands were read in a Gel Doc XR^{+} with Image LabSoftware (BIO – RAD Molecular Imager^{R}, USA) viewed on a computer monitor.

Results

Avian influenza antibodies detection in wild birds

None of the sera of wild birds in any of the LGAs where wild birds were caught and sampled was positive for AI (H5) antibodies.

Two (4.5%) of the wild birds sera were positive for AI (NP) antibodies, the calculated χ^2 values were found to be significant across species and LGAs (Table 1). The seroprevalence of AI (NP) antibodies using ELISA was 28.6% for Lokoja LGA while the seroprevalence was zero for the other LGAs (Table 1). Similarly, species seroprevalence of AI (NP) using ELISA was 100% for Black stork (*Ciconia nigra*) while it was zero in the other species of wild birds (Table 2).

Avian influenza viruses in poultry and wild birds

All the samples from poultry and wild birds were negative for AI viral antigens by RT – PCR (Plate I). There was no presence of cDNA bands corresponding to a base pair of 250 bp specific for the gene generated by the pairs of primers used in the amplification of the RNA template of test samples.

Discussion

The non-detection of AI (H5) antibodies by HI test in the sera of all the wild birds surveyed indicated the absence of the AI (H5) virus infection in the wild birds investigated. It could as well be that infected birds would have died if they were infected with HPAI thereby missing the true status of infection because the survey involved only apparently healthy birds (Breed *et al.,* 2010).

However by ELISA, antibodies to AI (NP) were detected in Black Stork (*Ciconia nigra*) species of wild bird. This was an indication of the presence of

AIVs infection of other subtypes than the H5 subtype in this species. Our search indicates that, this is the first report of the exposure of free flying wild birds to AIVs with sero-conversion in Kogi state, Nigeria. Black storks and other members of the *Ciconiiformes* are medium to large wading birds found around wetlands and shallow waters in which AIVs have been isolated (FAO, 2007). The detection of AI (NP) antibodies in the entire number of Black storks surveyed, even though small, may be an indication of the susceptibility of this species to AIVs compared to other species due to their ability to live in aquatic and terrestrial habitats. This ability of dual habitats makes Black stork a potential threat as source of AIVs infection by acting as a bridge for spreading of AIVs infection from aquatic wild birds to terrestrial wild birds as well as poultry and vice versa (FAO, 2007; Stallknecht & Brown, 2008).

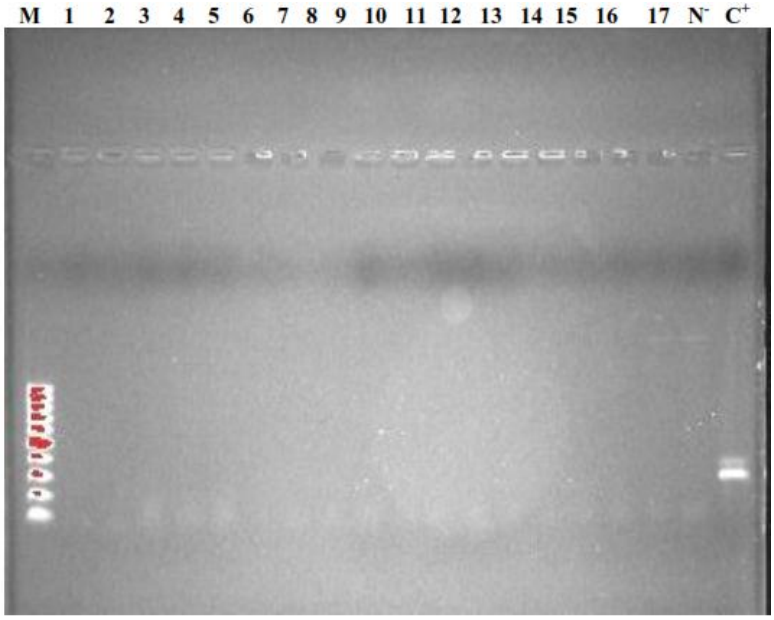

Figure I: Avian influenza virus RT-PCR results of oropharyngeal and cloacal swabs from poultry and wild birds in the surveyed areas of Kogi state (Pooled samples 1 – 17, **M**=molecular weight marker, **N**$^{-}$ = negative control, **C**$^{+}$=positive control)

Lokoja LGA was the only area where AI (NP) antibodies were detected in wild birds. Lokoja is traversed by two major rivers, Benue and Niger that converged at a point within the city to form a confluence. These two rivers run downward from some states in Nigeria where HPAI (H5N1) outbreaks were earlier reported, the water provides good habitat for the Black Storks (*Ciconia nigra*) that were positive for AI (NP) antibodies as well as other water birds. The AIVs have been known to persist in water for a long period especially under freezing and cold condition (Staliknecht *et al.*, 1990). Hence, excreted AIVs from Black Stork (*Ciconia nigra*) may persist in water and be carried away by water current over long distances to remain infective to susceptible poultry and other birds.

Conventional RT – PCR is highly sensitive and can detect viruses with titre as low as 3 EID50 (fifty percent egg infective dose) in clinical samples (Joannis *et al.,* 2008). The failure to detect any positive band of AI viral antigens by the RT – PCR from the cloacal and oropharyngeal swabs was an indication of the absence of AIVs in poultry and wild birds in the study area. The failure to detect AI viral antigens in this study was also an indication that the AI (H5) and AI (NP) antibodies detected in poultry earlier in this area were not due to recent infection (Ameji *et al*., 2015).

Sometimes the RT – PCR used might have limitations in terms of sensitivity and specificity in detecting AIVs which were 88.2% and 99.5% respectively (OIE, 2008). Starick *et al.* (2005) and OIE, (2008) suggested repeating the conventional RT – PCR several times on the samples; using rRT – PCR or nested PCR as ways to overcome the limitations of conventional RT – PCR. In the same vein, Fouchier *et al.* (2000) reported that negative RT – PCR result might not imply the absence of AIVs in the samples. However, the absence of reported cases of HPAI in the study area during the outbreak of 2006 in Nigeria coupled with the failure to detect AIVs in the samples tested by RT- PCR in this study might indicate the free status of AI (H5) viruses in poultry and wild birds in Kogi state, Nigeria.

The result of the present study is similar to the work of other workers in other parts of Nigeria that failed to detect AIVs in poultry and wild birds. Columba-Teru *et al.* (2012) surveyed wild birds in live poultry markets, live wild bird markets and around poultry farms from eight states across Nigeria where HPAI outbreaks were reported but failed to detect AIVs in 403 cloacal swabs by isolation. Wakawa *et al*. (2012) surveyed 58 affected and non-affected poultry farms in Kano state but could not detect the AI viruses by RT-PCR. Equally, Assam (2014) surveyed wild birds from live poultry markets and live wild bird markets in Kaduna state but could not detect AI viruses by RT-PCR.

We acknowledge the limitations of the effects of the small sample size of wild birds on the results of the study in reaching valid conclusions. The study highlighted the exposure of wild birds to AIVs and the presence of AI (NP) antibodies in Black stork (*Ciconia nigra*) but failed to show the circulation of AIVs in poultry and wild birds in Kogi state, Nigeria. It is therefore, recommended that poultry should be prevented from having contact with Black stork (*Ciconia nigra*). Also, more broad based surveillance targeting AIVs subtypes other than AI (H5) subtype and involving larger sample size should be carried out to determine the status of AIVs in poultry and wild birds in Kogi state, Nigeria.

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