



Serological evidence of influenza A/H9 in indigenous birds and level of awareness at live bird markets, Plateau State

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Abstract

Avian influenza is a zoonotic disease that can adversely affect humans and animals. Nigeria first reported an outbreak of avian influenza which was caused by subtype H5N1 in 2006, thereafter virological and serological surveys revealed the importance of local birds in live bird markets and the community at large in the epidemiology of avian influenza in the country. In the present study, 276 serum samples were collected for serological testing over five months from apparently healthy local birds in live bird markets within two Local Government Areas of Plateau State, to determine antibody prevalence to avian influenza A virus. The detection of influenza A antibody was carried out using an Enzyme-linked immunosorbent assay and further tested by haemagglutination inhibition to determine the specific serotype of the influenza A virus. The result showed a prevalence of 30.4% (n=84) of antibody to influenza A, 26% (n=72) of serotype H9, 1.4% (n=4) of serotype H7, and none was confirmed to be H5 serotype. Comparatively, Jos-North had a lower relative risk with a prevalence of 18.9% (n=18) to the disease as compared to Jos-South with a prevalence of 36.5% (n=66). This study showed the presence of low pathogenic avian influenza A virus in live bird markets within the study area with the dominance of antibodies to H9. To our knowledge, this is the first serological indication of serotype H9 in Plateau State and Nigeria. Evidence of influenza A/H9 in an ecological niche known for the circulation of subtypes H5Nx may complicate the epidemiology and control of avian influenza in the region and Nigeria at large. The level of awareness by the live bird market operators about avian influenza (AI) was relatively low as indicated by the questionnaire survey conducted. Live bird market operators and poultry farmers need to maintain a high level of biosecurity and limit mixing local birds with commercial poultry to prevent the transmission of the virus which may have adverse effects on poultry production, national and international trade, the economy and public health.

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Introduction

Avian influenza (AI) is a highly infectious disease caused by type A influenza viruses. This virus belongs to the family *Orthomyxoviridae* which are single-stranded 8-segmented negative sensed RNA that encode at least 10 viral proteins (Machalaba *et al.*, 2015; Bevins *et al.*, 2016). The disease constitutes a major threat to the poultry industry worldwide (Meseko & Oluwayelu, 2019). The *Orthomyxoviridae* family is divided into four main genera viz; A, B, C, D and two other genera found in fish and arthropod (Payne, 2017). Avian influenza belongs to the genus of influenza A which is the most widespread among other influenza viruses with members infecting avian and mammalian species (Thomas *et al.*, 2019). Influenza A viruses are further classified by subtypes based on the glycoproteins, haemagglutinin (HA) and neuraminidase (NA) proteins. Presently, there are 18 subtypes and 10NA subtypes (Kumar *et al.*, 2018; Tindale *et al.*, 2020). Divergent avian influenza A viruses HA H17, H18, and NA N10 are recently isolated from bats (Lamb & Roberts, 2014; Oluwayelu *et al.*, 2015; Akanbi *et al.*, 2016a). Only H1, H2 and H3 are commonly found in humans but viruses with H5, H7 and H10 HA have been isolated from humans but without extensive human to human transmission of the viruses (Lamb *et al.*, 2014). The HA and NA are capable of eliciting subtype-specific immune responses which protect within subtypes and partially protect across subtypes (Tindale *et al.*, 2020).

The influenza A viruses infect humans and many different animals, the virus can also evolve into new subtypes of influenza A virus which may have the ability to infect humans and have a sustained human to human transmission, which can cause an influenza pandemic (Markets *et al.*, 2020; Steensels *et al.*, 2020). However, certain subtypes of influenza A virus are specific to certain species, except waterfowls, which are host to all known subtypes of influenza A viruses (Meseko *et al.*, 2020).

The primary mode of transmission in human cases of avian influenza virus infection is from bird to human through direct or close contact with infected birds or contaminated surfaces (Meseko & Oluwayelu, 2019). Even though some studies have shown the prevalence of various strains and subtypes of Avian influenza antibodies in the serum of apparently healthy birds in live bird markets in some parts of the country (Meseko *et al.*, 2018b), there is a dearth of this information in many other parts of the country, including Plateau State.

This research aims to detect antibodies to the avian influenza A virus in local birds, at LBMs in Jos-north

and south, Plateau State, Nigeria using enzyme-linked immunosorbent assay, and haemagglutination inhibition test. Knowledge of the seroprevalence of avian influenza in Jos, Plateau State will support public health workers with relevant data that will help in the planning of Avian Influenza control programmes at the human-animal interface in these areas.

Materials and Methods

Study area

Jos city with coordinates 9.8965°N, 8.8583°E is in Plateau State, which is in the middle belt of Nigeria, Plateau is situated in the tropical zone of Nigeria, at a higher altitude resulting in a near temperate climate with an average temperature of between 13 and 22°C. Harmattan winds bring about the coldest weather between December and February. The warmest temperatures usually occur in the dry season in March and April. The mean annual rainfall varies between 131.75 cm (52 in) in the southern part to 146 cm (57 in) on the Plateau. Although the state is best known for its mining production, agriculture is the major occupation of the people. It is the administrative capital and largest city in Plateau State and has a major concentration of poultry farms in Nigeria (Akkermans *et al.*, 2015; Chinyere *et al.*, 2020). Poultry farmers on the Plateau, practice either the deep litter system or the battery cage system of management, while a mixed management system is practised at live bird markets. This study was conducted in Jos-North and Jos-South Local Government Area of Plateau State, Nigeria. In Jos North, two major live bird markets (LBMs) are known, which are; Chobe LBM with coordinates 9°55'59"N and 8°52'55"E and Kasuwan Kaji LBM with coordinates 9°56'12"N and 8°53'15"E. Jos-South houses two major LBM which includes Kwararafa LBM in Bukuru with coordinates 9°47'20" N and 8°51'52" E and LBM at "British America" close to the Jos abattoir with coordinates 9°53'39"N and 8°53'18"E.

Study design

A cross-sectional study was carried out to detect the presence of avian influenza antibodies in mixed species of indigenous birds within the study area. A pretested questionnaire was also used to obtain information about avian influenza from live bird markets operators in the study area.

Determination of sample size

The sample size for the study was determined using the formula by Cohen (1988):

$$N = Z^2Pq/L^2$$

Where:

N = Number of samples to be taken from a population

Z = Z-score (for a two-tailed test at 95% confidence interval) = 1.96

P = Prevalence of AI antibodies = 18.7% (Ameji *et al.*, 2017)

$$q = 1 - P = 1 - 0.187 = 0.813$$

L = a measure of the confidence interval = allowable error (5%) = 0.05.

Substituting the values into Cohen's formula gives.

$$N = (1.96^2 \times 0.187 \times 0.813) / 0.05^2 = 233.6$$

N=234.

The sample size was increased to 276 to reduce sampling error.

Questionnaire

Validity and reliability of the questionnaire: The questionnaires were validated by experts in National Veterinary Research Institute and evaluated before testing for validity. The reliability and validity index expected was calculated using Karl Pearson's coefficient of correlation; the same one that was used to test the overall data validity.

Cronbach's alpha (coefficient alpha) method of estimating reliability was used to estimate the reliability of the test. It was found to be 0.96. To make sure the questionnaire was free from vague and unclear items, the draft questionnaire was examined by advisors and by experts on the area for comment. The questionnaire was for the marketers. It was carefully constructed as it was the heart of the study. The researchers developed them from scratch with the help of professionals as supervisors in the final stage of their development. To ensure that the questionnaires captured data accurately, the wordings were carefully constructed to avoid ambiguity.

Sampling and sample collection

Blood samples were collected from local birds after proper restraint to expose the area over the wing vein. A 21-gauge needle and 5 mL syringe were used to obtain 2 mL of blood from the wing vein. A total of 276 blood samples were collected over 5 months (March to July 2020) from apparently healthy local birds at live bird markets in the Local Government Areas in Plateau State as indicated by Figure 2. A total of 24 blood samples were collected from Kasuwan Kaji in Jos-North, 71 blood samples were collected from Chobe LBM in Jos-North, 19 blood samples were collected from "British American" LBM Jos-North and 162 blood samples were collected from Kwararafa

LBM in Jos-south. The collected blood was allowed to stand for two hours for clotting to occur and the serum was decanted into a bijou bottle, stored under ice then transported to the Regional Laboratory for Animal Influenza and Other Transboundary Diseases of the National Veterinary Research Institute (NVRI) Vom within 24 hours. These were stored at -20°C until used.

Detection of avian influenza antibodies

Detection of avian influenza antibodies by enzyme link immunosorbent assay (ELISA): Influenza A virus antibody ELISA Test Kit (IDEXX Influenza A, Hoofddorp, Netherland) was used for the detection of influenza A antibodies. This assay measures the relative level of antibodies to influenza A in animal serum. The assay is performed on 96-well plates that have been pre-coated with influenza A viral antigen. This procedure was carried out according to the OIE manual (2018).

The absorbance of the samples and controls were subsequently measured and recorded at 650nm.

Determination of specific serotype of influenza a virus from the collected samples using haemagglutination (HA) test and haemagglutination inhibition (HI) test: After ELISA was carried out, Positive samples were further analyzed by haemagglutination inhibition (HI) test for AI serotype-specific antibodies using a panel of reference antigens comprising H5, H7 and H9 viruses with four haemagglutination units of each antigen according to the OIE manual (2018).

Results

Questionnaire results

The findings from the questionnaire survey showed that 45% (45/100) of the respondents had heard about AI, 33% (33/100) comprehended what AI was; hence 39% of the respondents had heard about AI or knew what AI was, therefore 61% (61/100) did not know about the disease.

However, Table 1 showed that only 39.5% of those knowledgeable about AI could identify some clinical signs associated with suspected cases of HPAI, amongst which were haemorrhagic wattle, decreasing egg production and unusual high mortality. Furthermore, 60% (60/100) of the respondents reported seeking veterinary intervention when their flocks showed symptoms of diseases, 20% (20/100) of the respondents reported that they sell their sick birds, while 25% (25/100) reported that they slaughter for consumption and 70% (70/100) reported to attempt medicating the birds themselves before any other option.

Table 1: Questionnaire Evaluation

S/N	Questions	Yes	No
1	Have you heard about Avian Influenza (AI)?	45	55
2	Do you know what Avian Influenza (AI) is? If NO go to Q4	33	67
3	How many signs can you recognise?		
	Sneezing	30	9
	Coughing	9	30
	Ocular discharges	15	24
	Decreased egg production	6	33
	Haemorrhagic wattles	35	4
	Haemorrhagic feets	3	36
	Haemorrhagic skin of the head	2	37
	Swollen head	5	34
	Pinpoint haemorrhage on the shank	18	21
	High mortality	31	8
4	What do you do when you observe any of the above signs in Q3?		
	Self medicate	70	30
	Call/ Visit a Veterinary doctor	60	40
	Slaughter and consume	25	75
	Sell off	20	80

Table 2: Prevalence of A.I. Antibody In Local Chickens Sampled from Jos-North and Jos-South Local Government Area, Plateau State

S/N	LGA	Number tested by ELISA	Number of positive (prevalence)	Number of negative	CI
1	Jos-North	95	18 (18.9%)	77	11.9-28.6
2	Jos-South	181	66 (36.5%)	115	29.5-44.0
	Total	276	84 (30.4%)	192	25.1-36.29

Table 3: Prevalence of A.I. Antibody among local chicken samples collected from the various LBMs within Jos North and South

S/N	LBMs	Number tested by ELISA	Number of positive (prevalence)	Number of negative	OP	CI
1	Kasuwan Kaji LBM	24	5(20.8%)	19	2.6125	9.2-40.3
2	Chobe LBM	71	13 (18.3%)	58	3.0673	10.5-29.6
3	British America LBM	19	0 (0.0%)	19	-	0.0-20.9
4	Kwararafa LBM	162	64 (40.7%)	96	Ref	33.2-48.8
	Total	276	84	192		

Serological test

Enzyme-linked immunosorbent assay (ELISA): From the total of 276 serum samples tested for AIV, 18.9% (18/95) samples collected from Jos-North were positive to A.I. while 36.5% (66/181) samples collected from Jos-South were positive to A.I. The prevalence of AIV antibodies in the tested local chicken sera from the different live bird markets surveyed was 20.8% (5/24), 18.3% (13/71), 0.0% (0/19) and 40.7% (66/162) for Kasuwan Kaji LBM, Chobe LBM, British America LBM and Kwararafa LBM respectively. The overall prevalence of avian influenza

A antibodies in the study areas was 30.4% (84/276). There were statistically significant differences within the LBMs (Tables 2, 3 and Figure 1).

Haemagglutination and haemagglutination inhibition test

From the Haemagglutination (HA) and haemagglutination inhibition (HI) tests carried out to determine the specific serotype of the influenza A virus from the ELISA-AIV positive samples in the study area, a total of 85.7% (72/84) of the positive samples were confirmed to be H9 serotype. A total of 4.8% (4/48) were confirmed to be H7 serotype; while

14.3% (12) of the AIV positive samples were neither positive for H9, H7 nor H5. This survey showed the possible co-existence and co-circulation of H9 and H7 serotypes of LPAI in the country because the four samples which tested positive to H7 all were positive to H9 and for the first time to our knowledge H9 serotype is detected from local birds in Plateau State.

Discussion

Avian influenza has its zoonotic potential as described by WHO (2019), but the market operators had a low level of awareness as indicated by the questionnaire survey conducted, which is believed to propagate the spread of the virus as they carry on with their daily activities, therefore awareness against avian influenza and its zoonotic potential must be created in LBMs and the community at large hence reducing the number of uninformed persons. The questionnaire also revealed that 60% of the respondents seek veterinary intervention when their birds are sick, which is the right choice

to make and this should be encouraged within the society as it aids earlier identification of causative agents and reduces drug resistance caused by indiscriminate use of drugs, as the questionnaire revealed that 70% of the respondents attempt treatments themselves before any other options. The consumption and sale of sick birds should be discouraged in the society at large as these will hasten

the spread of the AI virus, other infectious agents and may facilitate zoonosis. It was also observed in the various live bird markets that the local chickens were mixed with commercial chickens and other species of birds before or after being purchased from a local seller. This has grave implications for interspecies transmission for avian influenza.

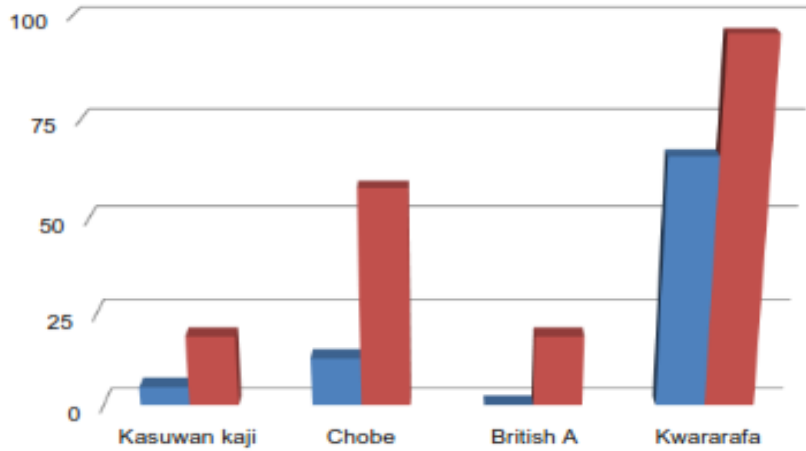


Figure 1: A graph showing the level of infection in each LBM within Jos-north and Jos-south

Red: Negative value
Blue: Positive value

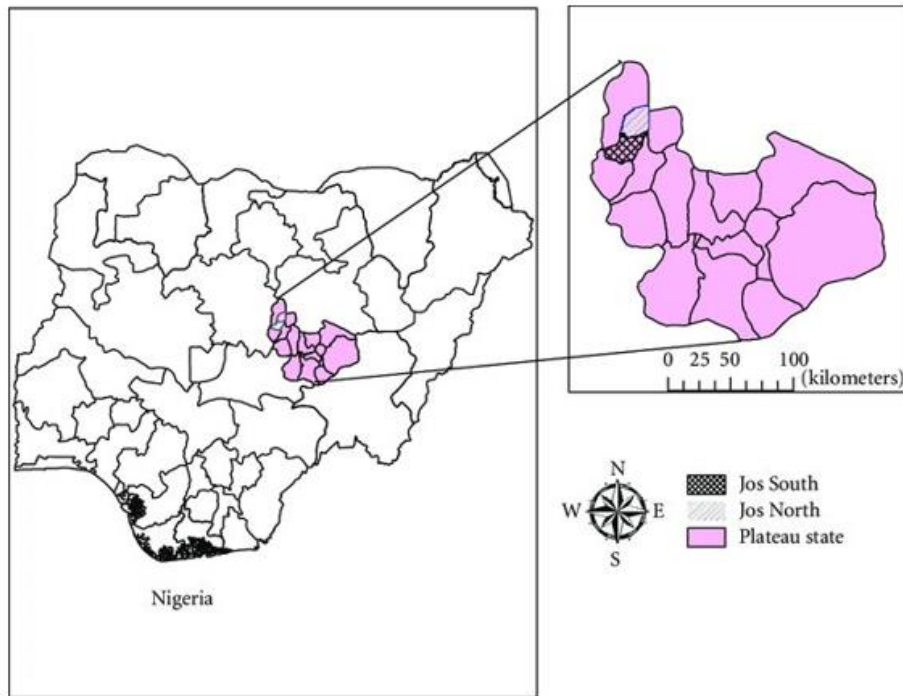


Figure 2: Map of Nigeria, showing Plateau state and Jos North and South Local Government Areas

Source: Meseko *et al.* (2012)

In this study, the detection of antibodies to low pathogenic avian influenza serotype A/H9 in local chickens indicates the presence of low pathogenic AIVs infection. Other subtypes causing highly pathogenic (HP) Avian Influenza particularly subtype H5 have often been reported in the study area and indeed Nigeria by previous researchers (Ameji *et al.*, 2020). The findings of this research indicate the first report of the detection of H9 from local chickens in Jos Plateau after a deductive literature search. This finding is of public health significance because of the threat this virus poses to the poultry industry, economy and human health particularly as they can lead to the emergence of reassortant pathogenic strain through co-circulation with other subtypes.

Due to the migratory activities of wild birds, there is a risk of dissemination of avian pathogens into the environment which may have been contracted by resident wild birds and local fowls that are extensively reared in the area. It was observed in the LBMs that both wild and domestic birds were housed together in cages, this promotes the spread of both the HPAI and LPAI virus, these findings agree with Akanbi *et al.* (2016a), it was also observed that some of these birds were housed or kept in very close proximity with other animals, such as goats, pigs, rams and cows.

Specifically, the avian influenza virus has continued to be a problem worldwide as it can spread rapidly and cause disease in domestic poultry, and some may also infect other animal hosts, including humans. Moreover, Subtain *et al.* (2011) noted that the first sign of LPAI infection in domestic poultry is often seroconversion which may be the only evidence of infection with some LPAI subtypes. The detection of antibodies to LPAIV H9 and H7 in apparently healthy local chickens in this study indicates that LPAI H9 and H7 virus subtypes presently circulate in local chickens in Jos-North and Jos-South. This study also conforms to observations by Chinyere *et al.* (2020) who earlier detected serotype H7 in Jos LBMs.

Since the vaccination against AI is not officially permitted in Nigeria and local chickens are not usually vaccinated, according to the report Meseko *et al.* (2020), the antibodies detected in these birds could only have resulted from seroconversion following natural infection with the viruses. Thus, the local chickens could serve as reservoirs shedding the virus into the environment, thereby playing a crucial role in the epidemiology of the disease which agrees with research carried out by Ameji *et al.* (2020). This finding is also consistent with previous reports of infection with LPAI H5N8 in Jos Plateau State, Nigeria (Ameji *et al.*, 2019) and other parts of the world such

as China, Asia and Europe. The detection of antibodies to both H9 and H7 LPAI viruses in some local chickens in this study is of public health concern because co-infection with different avian influenza viruses might provide the opportunity for re-assortment, leading to the emergence of novel reassortant subtypes with zoonotic potential. It has been reported previously that if a field virus such as AI is a low pathogenic type, there is a risk of it mutating to a highly pathogenic form after circulating in susceptible poultry (Richard *et al.*, 2017).

It is important to note that 12 of the ELISA-positive sera were negative for antibodies to H5, H7 and H9 serotypes LPAI virus, suggesting the possibility of other AIV subtypes circulating among local birds in the study area. There is therefore a need to further investigate the presence of other AIV strains present in local chickens in Jos, Nigeria using a large panel of reference AIV subtypes (H1-H18) sera as well as virus isolation and molecular identification techniques such as reverse transcriptase-polymerase chain, reaction (RT-PCR).

The detection of a higher level of seropositivity to LPAI H9 virus in this study compared to the H7 subtype is significant because, unlike aquatic birds, local chickens are not natural hosts of H9 subtype influenza virus and H9 infected poultry are asymptomatic (Meseko *et al.*, 2018b; Ameji *et al.*, 2019). This suggests a need to determine the current status of H9 in the country. Moreover, it is known that normal asymptomatic infection of avian species can silently maintain and transmit influenza virus provided there is an opportunity for genetic re-assortment with other prevalent strains (e.g H5) in the avian population and this may be promoted where different avian species are kept together as we observed in these LBMs.

Thus, mixed farming of several species of birds and other livestock practised in many areas of Nigeria reported by Oluwayelu *et al.* (2015) is a critical management risk that should be discouraged because pigs have also been implicated to be a major mixing vessel for AIV as suggested by (Meseko *et al.*, 2018b). It is important to note that also, local birds have been implicated as incubating vessels for generating influenza reassortants of human and avian origin in the field (Kapczynski, 2009). Additional studies are advocated to determine the potential role of local chickens in the zoonosis of AIV strains in Nigeria.

In this study, it was observed that the local chickens could have been exposed to the virus while scavenging for food. According to Meseko *et al.* (2018a), most evidence obtained on the prevalence

of influenza in different types of poultry and from different geographical locations supports the view that the primary introduction is from wild and feral birds. Therefore, influenza viruses are most likely to infect poultry reared in a way that allows contact with feral birds, such as on range poultry farms.

The findings of this study revealed likely circulation of LPAI H9 and H7 viruses in local birds within Jos-North and Jos-South of Plateau State, Nigeria. This study emphasizes the importance of routine surveillance for AIVs in different avian species for the prevention of AI outbreaks in the country. Live Bird Market operators need to be educated on the dangers of collecting, mixing and trading different avian species without biosecurity. The outcome of this research if taken seriously by the governing bodies will help eliminate market practices that can enhance the spread or interspecies transmission of AIVs in the country. The use of social network and market value chain analyses for future AI surveillance investigations is proposed for the development of more effective AI control strategies.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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