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ORIGINAL ARTICLE

Epidemiology and Clinicopathological Manifestation of Resurgent Highly Pathogenic Avian Influenza (H5N1) Virus in Nigeria, 2015

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SUMMARY

In January, 2015 the National Veterinary Research Institute, Vom, Nigeria received some chicken carcasses from the Kano state Ministry of Agriculture. The carcasses were from a backyard-commercial poultry farm and a live bird market (LBM) in Kauna and Sabon gari, Kano state, northwestern Nigeria respectively. The farm kept different types of chickens of various ages and stages and was experiencing high mortality of 350 birds daily with eventual 100% mortality observed in the older birds (54weeks). In a concurrent incidence, reports were received of unusual high mortality of birds brought from the northern part of the country at two LBMs in Onipanu and Mushin, Lagos state, southwestern Nigeria. A total of 8 chicken carcasses from the Kano suspicion were subjected to postmortem examination and testing. One broiler-chicken (4weeks old), 4 layer-chicken (22 weeks old) and 3 indigenous bred-chickens (from LBM) showed severe pathological lesions consistent with highly pathogenic avian influenza (HPAI). Moribund birds from the Lagos suspicion had cyanotic comb and wattles, torticollis and paralysis of the limbs. Parenchymatous organs, nasal and trachea swabs were collected from the dead and moribund birds respectively. The specimens were analyzed by RT-PCR and virus isolation in embryonating chicken eggs. All samples were found to be positive for HPAI (H5N1) subtype. This marks the re-introduction of HPAI (H5N1) subtype into Nigeria for a second time in the space of 9-years. So far, over 542 cases (January to December, 2015) have been confirmed positive for HPAI (H5N1) in 20 states of the country. Possible circumstances surrounding the resurgent and spread are discussed herein.

Key words: Epidemiology, Resurgent HPAI H5N1, Pathology, Nigeria.

INTRODUCTION

Exactly 9-years ago, highly pathogenic avian influenza (HPAI) H5N1 strain was detected in a commercial poultry farm in Kaduna state, north central Nigeria for the first time on the African continent (Joannis *et al.,* 2006; De Benedictis *et al.,* 2007). Subsequently, the virus spread to approximately 70% (25 out of 36) of the states of the country including the Federal Capital Territory (Abuja) within a short period of time with several waves of the outbreaks observed across the country during the 2006-2008 episode (Joannis *et al.,* 2008; Fusaro *et al.,* 2009; Métras *et al.,* 2013). The disease also disseminated to eleven other African countries and has since become endemic in Egypt affecting both humans and animals (ELbayoumi *et al.,* 2013; Métras *et al.,* 2013). As a follow up to reported outbreaks in both commercial and scavenging rural poultry, the Avian Influenza Control Project in the Federal Livestock Department under the country Ministry of Agriculture in conjunction with other stakeholders conducted targeted, active surveillance in high risk areas especially live bird markets (LBMs). During the course of the surveillance activities in July, 2008 a new sub-clade of HPAI (H5N1 clade 2.2.1.1) hitherto unreported in Africa was recovered from Gombe a northeastern state in Nigeria (Fusaro *et al.*, 2009). After two years of intense surveillance, testing and culling of infected farms with adequate compensation, and strict biosecurity measures, the disease was eventually stamped-out of Nigeria in 2008. Following the introduction and subsequent control of HPAI in Nigeria (Akanbi and Taiwo 2014a; 2014b) there has not been any official report of HPAI (H5N1) in Nigeria since July, 2008.

HPAI is known to be a fatal disease of gallinaceous birds, causing up to 100% mortality (Klopfleisch *et al.,* 2006; Alexander 2008). The source of the 2006-2008 outbreaks caused by multiple strains of HPAI (H5N1) were attributed to commercial poultry trade (Fasina *et al.,* 2009) and wild birds (Ducatez *et al.,* 2006; Fusaro *et al.,* 2010). Altogether, around 939,620 commercial type chickens on 127 farms (Akanbi and Taiwo, 2014a) and 14,512 local chickens on 80 backyard farms were lost to HPAI H5N1 between 2006 and 2008 (Akanbi and Taiwo, 2014b). This significantly affected the economy not only because a sum of N631 million (US$5.43 million) was paid as compensations to farmers (Durosinlorun *et al.,* 2010), but also because the poultry industry contributes huge resources to the national economy and this emphasizes the importance of commercial poultry (Fasina *et al.,* 2007). In addition, a case of human infection was documented (Nasidi *et al.,* 2007) but no antibodies were detected in laboratory testing of poultry workers (Ortiz *et al.,* 2007) during the 2006-2008 HPAI (H5N1) outbreaks. Given that Nigeria lies within the migratory routes of wild birds from Asia and Europe coupled with the poorly regulated poultry trade within the sub region, the country remains at risk of re-introduction of HPAI (H5N1). This paper is therefore aimed at reporting the epidemiology, clinic-pathologic characteristics and examines the circumstances surrounding the re-introduction of the resurgent HPAI (H5N1) into Nigeria.

MATERIALS AND METHODS

History and Spatial Distribution

On the 24th December, 2014, at a LBM in Sabon gari, Kano state, large number of deaths in chickens, geese and turkeys were recorded. This incidence was reported to the authorities of the Ministry of Agriculture and whole carcass samples of the birds were collected and sent to National Veterinary Research Institute, Vom, (NVRI) Plateau state. The following signs were observed

before death; respiratory distress, watery faeces, cyanotic comb and haemorrhagic shanks. Daily mortality was said to exceed 300 birds, and majority of the birds were sourced from Kanya market, Jigawa state, Nigeria and from Adare town in Maradi governorate in Niger republic (Figure I).

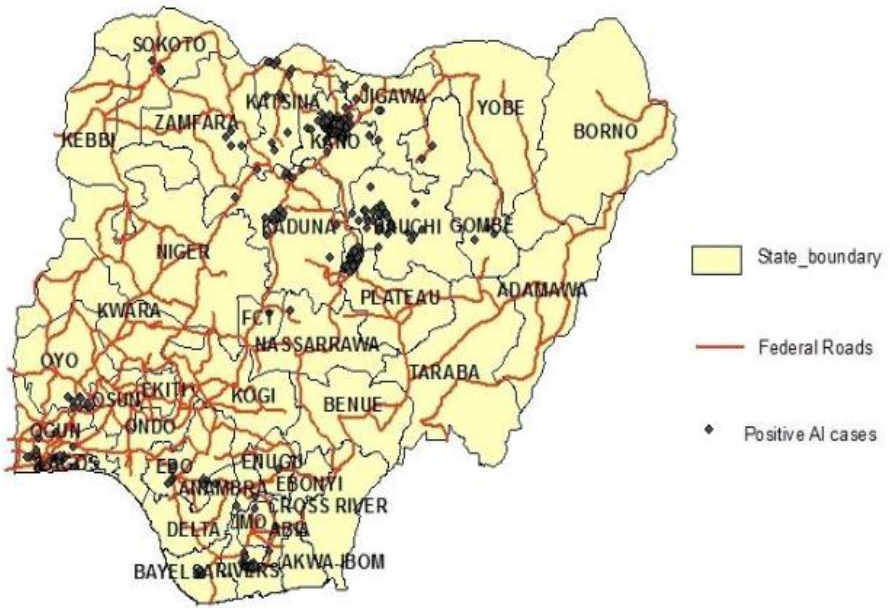

Figure I: Spatial distribution of HPAI H5N1 outbreaks in poultry, Nigeria, January-December, 2015

In a similar scenario, a poultry farm in Kauna (12.037N, 8.487E), Kano state, northwest Nigeria with a poultry population (4 week-old broiler) of about 3000 observed a sudden, high mortality of more than 200 birds on the 1st of January, 2015 and 350 birds on the 2nd, and more than 300 birds on the 3rd in its laying flock of 22 week old birds. In addition, 100% (n=500) mortality was observed in a 54 week-old laying flock kept on the same facility.

Lagos outbreak

In December 2014 just before the Christmas festival, live birds were transported from the northern part of the country by road for sale in markets in Lagos metropolis. Shortly after, there were reports of unusual high mortality with moribund birds showing cyanotic comb and wattles, torticollis and paralysis of the limb. These signs were observed following the introduction of those birds to the live bird markets particularly in Onipanu and Mushin areas (03.423N, 06.612E). Nasal and trachea swabs were collected from two birds (one live, one dead) in virus transport medium and transported on cold chain to the National Veterinary Research Institute, Vom, Plateau state, Nigeria.

For the spatial analysis, direct farm visits to obtain coordinates of affected farms were undertaken, for terrains that could not be visited, coordinates were obtained by picking points in the affected local government areas. Coordinates were fed into Arc Map version 10.2.1

Post-Mortem and Histopathological Examination:

Post-mortem examination (PME) of the carcasses was performed and the findings documented. Parenchymatous organs (trachea, lung, heart, spleen, liver and intestine) from PME of all the chickens were collected for histopathology, virus detection and isolation.

Formalin-Fixed tissues were then embedded in paraffin, sectioned at 5 µm, mounted on clean glass slides, and stained with hematoxylin and eosin (H&E) stains for histopathological examination using low and high powered field of Carl Zeiss or Nikon binocular microscope.

Molecular Analysis:
Pooled tissues (trachea, lung, liver and spleen) were homogenized and centrifuged using standard procedure (OIE 2015). Nucleic acid extraction was carried out on the supernatant using Qiagen RNA kit (Hilden, Germany) according to the manufacturer's instruction. Thereafter, one-step RT-PCR assay targeting the matrix gene (M-gene) (Fouchier *et al.,* 2000) was performed using GeneAmp® Gold RNA PCR core kit (Applied Biosystems, Foster City, CA, USA) on 9700 Thermocycler system (Applied Biosystems, Foster City, CA, USA). The amplicons were electrophoresed at 120 volts for 30 minutes in 1.5% agarose, stained with ethidium bromide and thereafter visualized with a Gel Documentation system (Biorad). Samples that were positive for the M-gene were sub-typed with H5 protocol as previously described (Slomka *et al.,* 2007). The N1 typing of the index cases from Kano and Lagos were determined as previously described (Monne *et al.,* 2015). Subsequent determination of the N1 subtypes were carried out using the procedure of Huang *et al.,*(2013).

Virus Isolation
Virus isolation was carried out by inoculating 9-day old embryonating chicken eggs with 0.2 ml of the supernatant fluid of homogenized tissues. Egg embryo that died after 24 hours post inoculation were chilled and harvested. This was subsequently tested by haemagglutination and haemagglutination-inhibition for AIV performed according to OIE protocol (OIE, 2015).

RESULTS
Spatial Distribution of HPAI H5N1 Cases in Nigeria January to December, 2015
Following the detection of the virus in the samples from Kano and Lagos states, the virus rapidly spread to other states including Plateau, Bauchi, Delta, Anambra, Edo, Gombe, Imo, Jigawa, Kaduna, Katsina, Nasarawa, Ogun, Rivers, Sokoto, Zamfara, Enugu and Abia. It was observed that the virus rapidly spread most in Kano, Plateau and Bauchi States. In total, Twenty (20) States were affected by the virus (Figure I) and a total of 542 cases of HPAI H5N1were recorded on farm premises as at the end of December, 2015.

Necropsy Findings
A total of 8 carcasses were received and necropsied from the Kano suspicion. One broiler-chicken (4weeks old), four layer-chicken (22 weeks old) and three indigenous bred-chickens. The combs and wattles were swollen, hyperemic and haemorrhagic (5/8; 62.5%) (Figure IIA) while three were cyanotic (3/8; 37.5%). The broiler showed multifocal, ecchymotic haemorrhages on the pectoralis muscle, while all carcasses showed varying degrees of shank and foot hyperemia and haemorrhages (Figure IIB) and haemorrhagic ulcers (multifocal, ecchymotic) were observed in one local fowl. The upper palate and the oropharynx in one of the indigenous chickens had multifocal areas of ecchymotic haemorrhages, while all the other showed congestion of the distal 2/3rd of the trachea. The lungs were severely edematous, congested, and haemorrhagic (Figure IIC), with fibrinous deposits on the pleura while the air sacs were cloudy. The histopathology of the lung showed severe interstitial pneumonia with diffuse heterophilic and lymphocytic cellular infiltration and occasional airsac oedema and epithelia loss (Figure IID). Thymus in one of the birds was swollen and enlarged with multifocal

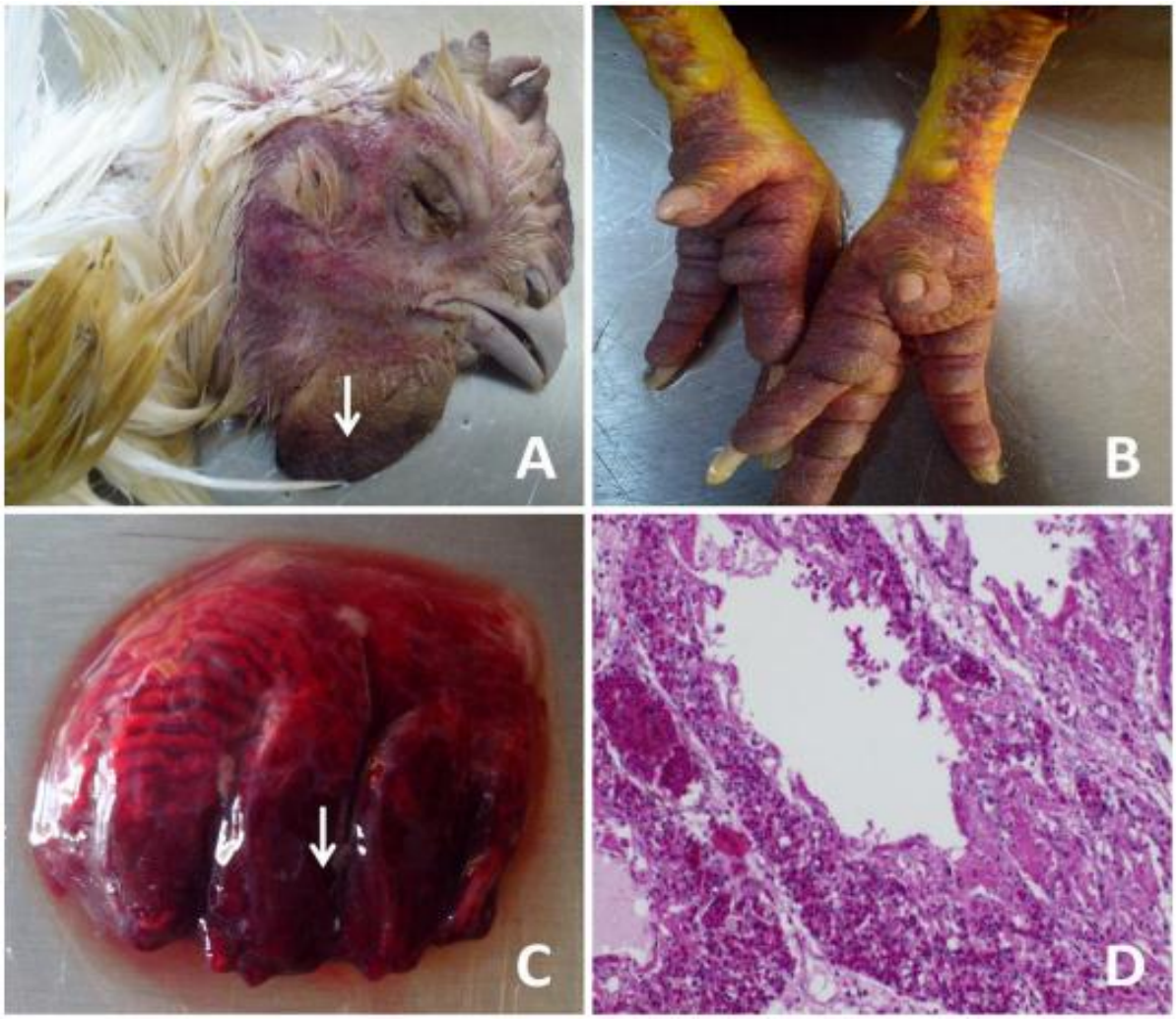

Figure II: Gross pathological lesions and histopathology (A) Swollen comb and wattle with subcutaneous hemorrhagic face (arrow), (B) shank and feet with diffuse subcutaneous hemorrhages, (C) Lung with severe congestion (arrow) and oedema, (D) Lung with parabronchiolar epithelia necrosis and desquamation, vascular congestion and expansion of the interstitium by mixed cellular infiltrates

ecchymotic haemorrhages while the spleen in one was enlarged up to 5 times. Congested liver and mesenteric vessels were observed in some birds with a haemorrhagic pancreas. The intestine had multifocal up to 0.8 cm in diameter ecchymotic haemorrhages. Summary of varying degrees of lesions and necropsy findings of selected cases of carcasses received for diagnosis from January to December, 2015 suspicion across the country are as shown in Table Ia and Ib. Also, the weekly distribution of cases from January to December is shown in figure III.

Molecular Detection

More than 500 cases have tested positive for Influenza A by M and H5 gene in 20 states of the Federation, with the virus widely disseminated in Kano, Bauchi and Plateau state (Figure I). In all samples, gel electrophoresis revealed bands at 240 and 300 base pair (bp) corresponding to the expected band sizes for the M and H5 genes

Table Ia: Summary of gross morphological changes in selected HPAI H5N1 positive poultry cases in Nig 2015

S/N	Poultry Type	Age (weeks)	Flock size	No. dead*	No. culled	Gross Morphological Changes
1	Broilers	3	100	90	Flock depopulated	Facial haemorrhages, myocardial petechial haemorrhages, cloudy airsacs, mesenteric vascular congestion.
2	Broilers	4	300	280	Flock depopulated	Lungs congestion and oedema, pectoralis muscle ecchymotic haemorrhages.
3	Broilers	5	800	300	Flock depopulated	Cyanosis of face, myocardial petechial haemorrhages, mesenteric vascular congestion.
4	Pullets	14	3000	†NA	Flock depopulated	Cyanosis of combs and wattles, swollen face, congested and haemorrhagic trachea, congested and oedematous lungs, cloudy airsacs, myocardial petechial haemorrhages, ecchymotic haemorrhages on proventriculus, duodenum and cecal tonsils, petechiation of pectoralis muscle, congested and haemorrhagic spleen, hyperemic shanks.
5	Pullets	15	4611	1775	Flock depopulated	Frothy lungs, congested coronary vessels, friable liver, hyperemic and haemorrhagic shanks.
6	Pullets	16	650	614	Flock depopulated	Congested and swollen liver, haemorrhagic cecal tonsils, petechial splenic haemorrhages, pasted vent, hyperemic and haemorrhagic shank and feet.
7	Cockerels	30	3364	620	Flock depopulated	Cyanotic comb, pale carcass, myocardial petechial to ecchymotic haemorrhages, chalky pancreas, congested and enlarged kidneys.
8	Layers	17	†NA	281	Flock depopulated	Emaciation, congested lungs, hyperemic shank.
9	Layers	22	600	†NA	Flock depopulated	Lungs congestion and oedema, atresia of the ovarian follicles.
10	Layers	25	†NA	11	Flock depopulated	Cyanotic comb and wattle, oedematous lungs, cloudy pericardial sacs, multifocal splenic necrosis, haemorrhagic cecal tonsils, cyanotic shanks.
11	Layers	34	650	†NA	Flock depopulated	Cyanotic, and hyperemic combs and wattles, congested and frothy lungs, congested coronary vessels, hyperemic pancreas, pale, friable and fatty liver, swollen spleens, congested and haemorrhagic ovarian follicles, pectoralis muscle pallor, pasted vents.
12	Layers	37	800	400	Flock depopulated	Pectoralis muscles pallor, enlarged and haemorrhagic thymus, increased abdominal fat, atresia of the ovarian follicles.

TABLE Ib: Summary of gross morphological changes in selected HPAI H5N1 positive poultry cases in Nigeria, 2015

13	Layers	41	6425	†NA	Flock depopulated	Edematous lungs, cloudy pericardial sacs and pasted vent.
14	Layers	48	3000	500	Flock depopulated	Cyanosis of combs and wattles, cloudy pericardial sac, congested and oedematous lungs, congested liver, hyperemic shank.
15	Layers	52	800	800	Flock depopulated	Comb and wattle hyperemia, hyperemic trachea and congested lungs, haemorrhagic cecal tonsils, fatty liver, swollen spleen with petechiations.
16	Layers	54	3300	1000	Flock depopulated	Lungs congestion and oedema, atresia of the ovarian follicles.
17	Layers	60	13400	†NA	Flock depopulated	Cyanosis of combs and wattles, congested trachea and lungs, cloudy airsacs, duodenal petechial haemorrhages, splenic haemorrhages, swollen thymus, hyperemia of shanks.
18	Layers	64	1500	150	Flock depopulated	Cyanosis of combs and wattles, hyperemia of shanks, congested trachea, congested and oedematous lungs, cloudy airsacs congested liver, haemorrhagic cecal tonsils.
19	Layers	‡Adult	1960	†NA	Flock depopulated	Cyanotic and swollen face, hyperemic shank and feet, congested lung, congested and friable liver, haemorrhagic cecal tonsils.
20	Layers	†NA	16000	1300	Flock depopulated	Hyperemic comb and cyanotic wattle, pectoralis muscle hyperemia, myocardial necrosis, soft, friable liver, multifocal petechial haemorrhages on spleen, hyperemic ovarian follicular, hyperemia of shank and feet.

*Number dead as at the time of reporting, †Number dead not available as at the time of submission, ‡ Age not indicated

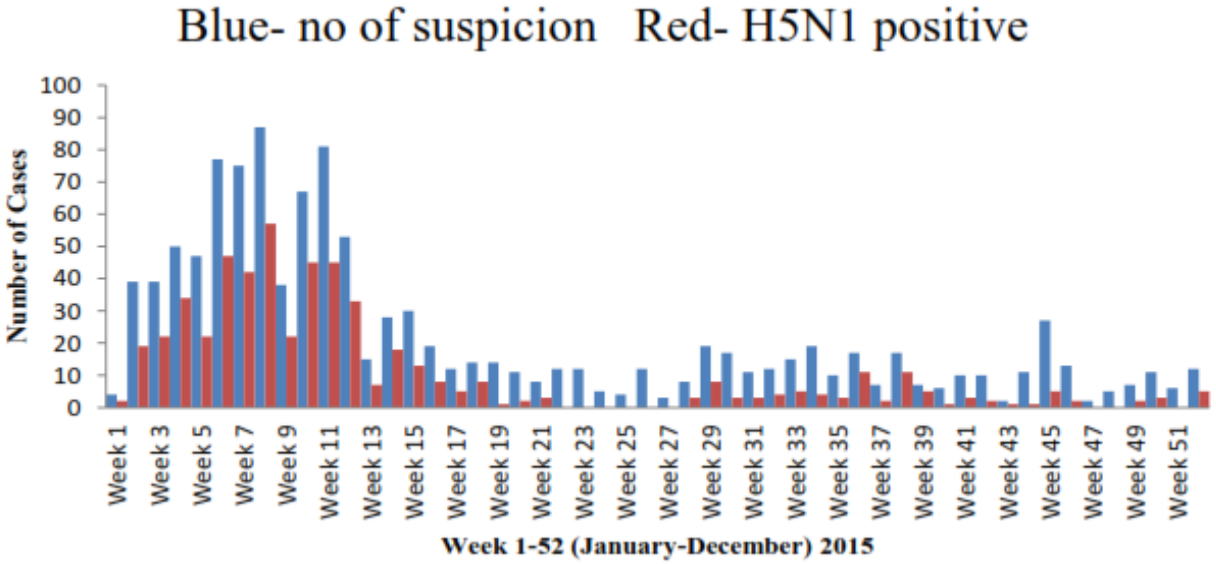

Figure III: Weekly distribution of suspected (blue bars) and confirmed (red bars) cases of HPAI (H5N1) from Nigeria, January – December, 2015. A total number of 1137 HPAI H5N1 suspicion from 20 states with 542 confirmed cases

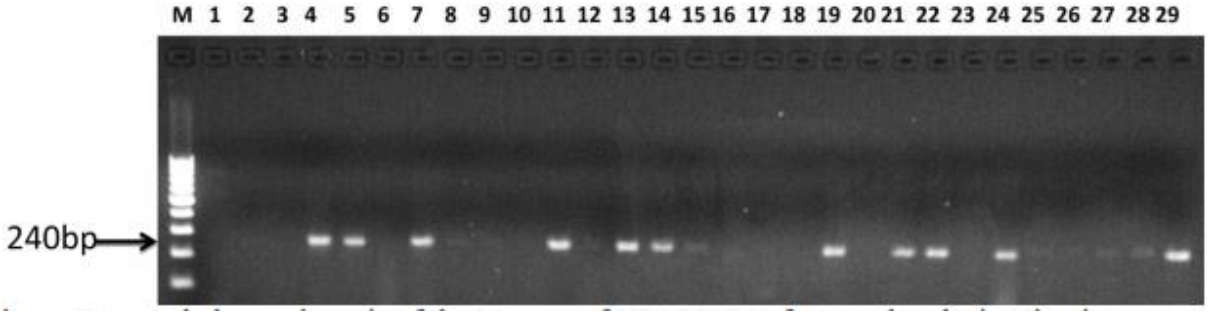

Figure IVA: Gel electrophoresis of the M-gene of HPAI H5N1 from outbreaks in Nigeria

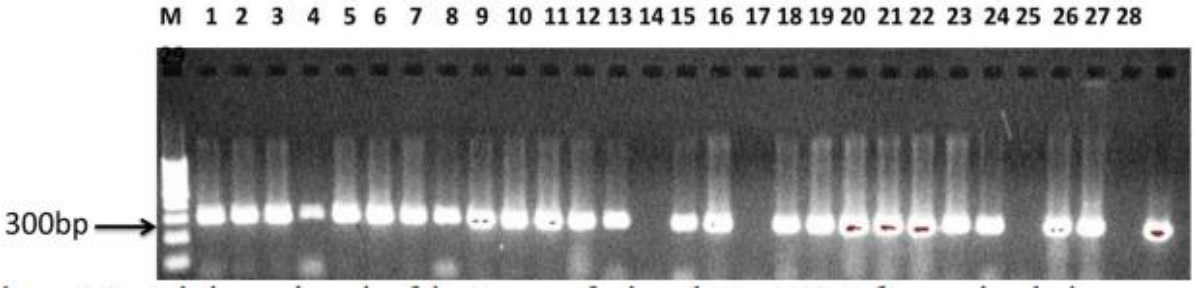

Figure IVB: Gel electrophoresis of the H-gene of selected HPAI H5N1 from outbreaks in Nigeria

respectively (Figure IVA and B). Similarly, a 137bp amplicon was observed for the N1 assay (data not shown). Some of the samples were positive for Newcastle disease virus concurrently.

Virus Isolation

Influenza virus were successfully isolated in embryonated eggs from samples that were positive by RT-PCR within 48 hours with an HA titer ranging between 7 and 10 Log_2. The isolates were also inhibited by H5 reference antiserum.

DISCUSSION

This first reported HPAI (H5N1) case in 2015 in Nigeria occurred on a farm that kept different breeds of chickens of various ages and stages of production as was the 2006 H5N1 index case. Rearing of different poultry species at different stages have been observed to influence the mortality of H5N1in the country (De Benedictis *et al.*, 2007). The high mortality and the patho-morphological changes in the chickens are consistent with HPAI which was confirmed by molecular and virological tests as well as sequence analysis. Sequence data of the virus placed it within the genetic clade 2.3.2.1c (Monne *et al.*, 2015).

Most outbreaks of H5N1 have been reported to occur during January to March (Durand *et al.*, 2015). In this outbreak, reports of high mortality were observed in December. Similarly epidemiological findings suggest that there have been unreported cases of high mortality in poultry farms and live bird markets in Kano and Lagos State during the later part of 2014. This period (November to January) in Nigeria, is usually cold, dry and windy thereby supporting virus transmission. This period is associated with increased travelling of city dwellers to their home towns/villages for Christmas and end of the year celebrations. Some of these travelers buy and take with them live poultry. This results in increased demands for live poultry during this period, which motivate live bird sellers to move from one location to another across the length and breadth of the country in search of live

poultry. Movement of apparently healthy poultry in anticipation of festivities or holidays have been identified as source of transmission (Nwanta *et al.,* 2008; Durand *et al.,* 2015).This practice easily supports the contamination of farms and introduction of infectious agent among farms. Other practices by farmers including movement and sales of infected birds and indiscriminate disposal of dead birds all contributes to the continuous contamination of poultry farms and the environment leading to lingering of outbreaks for several months.

Pathological lesions shown by some of the poultry are consistent with HPAI (H5N1) as reported earlier especially lesions seen on the comb and wattles (Monne *et al.,* 2015). However, other lesions such as serosal and fat haemorrhages, shank hyperemia, pancreatic necrosis and pneumonia can be seen in outbreak of velogenic Newcastle disease in unvaccinated flock as early observed in 2012 at our laboratory in Vom, Nigeria (NVRI database 2012, unpublished report).

It has also been observed that the lessons learnt and the control measures implemented for the 2006 outbreaks have been relaxed, some of which include: ban on the indiscriminate movement of poultry and poultry products in and across the country's borders and poor biosecurity measures. In addition, it has been observed that improper disposal of poultry carcasses, poor biosecurity measures including disregard for farm isolation and perimeter fencing, indiscriminate movement of poultry and poultry products, porous borders for poultry smuggling, uncontrolled and unregulated live bird market activities, indiscriminate importation of poultry and its products are some of the factors responsible for the transmission and spread of the current H5N1 outbreak. Furthermore, the circumstances surrounding resurgent of HPAI in Nigeria can be better appreciated by taking a critical look at both wild and domestic birds' ecology and pattern of distribution of poultry and poultry products in Nigeria. Though the epidemiological link between this H5N1/2015 isolate in Nigeria and previously identified strains in Asia is yet unclear, it is suggested to be probably associated with human activities, migratory bird movements, or both (Monne *et al.,* 2015).

Kano State shares border with Jigawa state, a region with known migratory bird flyway and activities (Snoeck *et al.,* 2011; Coker *et al.,* 2014) with adjoining wetlands which appears to be the major hub of current HPAI outbreaks and may be the source of virus transmission and spread to other parts of the country (Saidu *et al.,* 2008; Fusaro *et al.,* 2010) (Figure 1). The outbreaks in Lagos were mainly in live bird markets and some of the birds were brought from the northern part of Nigeria, apparently from Kano and Jigawa State adjoining the Hadejia wetlands. During the 2006-2008 outbreaks of HPAI in Nigeria, the north central and southwestern part were identified as possible sources of infection and spread to other regions (Fusaro *et al.,* 2010). Hence, interaction of wild migratory birds with backyard flocks and subsequent interstate movement of live birds are strongly suspected to be the source of the virus re-introduction into Nigeria for the second time. Effective control of HPAI in Nigeria and preventing future outbreaks and rapid spread must take into cognizance such elements as wild bird interaction with free-range domestic poultry, backyard poultry, cluster farming and live bird market chain as well as in between farm transmission. It has been predicted that minimizing contacts between commercial/free range chickens and wild birds in the north central part of Nigeria may help to avert future outbreaks (Fusaro *et al.,* 2010). Deciphering the mechanism of spread, including movement of birds and fomite is an important biosecurity step that must be strictly adhered

to in order to control transmission, devastating economic losses and the potential public health threat. The resurgence of HPAI in Nigeria by our investigation is a new introduction of a virus earlier eradicated and is a reminder that infectious pathogens especially those that are involved in transboundary transmission could occur when the conditions for introduction are favorable. Continuous surveillance and monitoring is therefore canvassed for early detection and control.

CONCLUSION

In view of all of the possible factors identified above, it is therefore concluded that sustained passive and active surveillance for HPAI H5N1 should be implemented in live bird markets, poultry farms and at border posts and other high risk areas. The established Avian Influenza desk offices during the 2006-08 outbreaks should be funded and encouraged to submit surveillance samples for laboratory diagnosis at regular intervals and especially intensify surveillance and monitoring during October - January periods of the year.

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