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**ORIGINAL ARTICLE**

# Formalin-Fixed and Paraffin-Embedded Tissues of Chickens are Useful for Retrospective Studies on Pathology of H5N1 Highly Pathogenic Avian Influenza Virus (HPAI) Outbreaks in Nigeria

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## SUMMARY

In a retrospective study, histopathology and immunohistochemistry (IHC) were performed on formalin-fixed paraffin embedded (FFPE) archival tissues from chickens obtained during outbreaks of highly pathogenic avian influenza (HPAI) H5N1 that occurred in Nigeria in 2006 and 2007. Ten samples as representative of 10 outbreaks were selected, and following the detection of HPAI viral antigen in different chicken tissues using IHC, RNA was extracted from each sample and molecular analysis was performed using real-time reverse transcription-polymerase chain reaction (rRT-PCR) targeting matrix protein. Seven rRT-PCR positive samples were then subjected to conventional and rRT-PCR assays for the amplification of hemagglutinin (HA) gene. Four of them were further characterized by sequence analysis of a short HA2-part of the H5 gene. Along the 154 nucleotides sequenced, differences at 4 positions were detected in one sample. One of these mutations led to an amino acid exchange at position 544 (Ala>Thr) whereas the others were silent. The study suggests the potential application for retrospective IHC and PCR analysis of FFPE tissues from chickens involved in the AI outbreaks for pathologic studies and providing short fragment sequences which may help in the characterization of viral strains and tracing the outbreaks. This is important as archived poultry tissues can be re-examined for possibility of earlier introduction of the virus.

**Key words:** Avian influenza; FFPE; H5N1; Nigeria; Immunohistochemistry; real-time RT-PCR.

## INTRODUCTION

Avian influenza (AI) viruses belong to the family *orthomyxoviridae*, genus *influenza virus* A and can be categorized into subtypes based on the antigenicity of two surface glycoproteins (Swayne and Suarez, 2000) the hemagglutinin (HA) and neuraminidase (NA). Two subtypes of AI viruses, H5 and H7 have been associated with the highly

pathogenic form of AI (HPAI). Many isolates of avian influenza virus (AIV) subtype H5N1 are highly pathogenic avian influenza (Chamnanpood *et al*., 2011). HPAI is an acute, generalized, fatal disease characterized by systemic infections in gallinaceous poultry, waterfowl, and other avian and mammalian hosts including humans and can cause outbreaks of severe notifiable disease of zoonotic importance (Swayne and Suarez, 2000). HPAI is caused by AIVs that are extremely virulent, causing up to 100% mortality in domestic chickens (Muzaffar *et al*., 2010). During 2006 to 2008, Nigeria experienced outbreaks of HPAI of the H5N1 subtype in commercial, backyard and free ranged chickens, domestic ducks, turkeys, ostriches and geese (Akanbi *et al*., 2007, Joannis *et al*., 2008, Ekong *et al*., 2011, Akanbi *et al*., 2014, Ekong *et al*., 2017); and for a second time in poultry in 2015 (Monne *et al*., 2015, Akanbi *et al*., 2016, Ekong *et al*., 2017). It was hypothesized that several introductions of the virus from multiple sources were responsible for the spread of the virus in Nigeria (Ducatez *et al*., 2006, 2007).

In addition to, virus isolation, RT-PCR and rRT-PCR as conventional diagnostic methods, immunohistochemistry (IHC) (Klopfleisch *et al*., 2006; Akanbi *et al*., 2007; Teifke *et al*., 2007; Shenga *et al*., 2011) and in situ hybridization (ISH) have been shown to be useful to detect viral genome and antigens (Ducatez *et al*., 2007; Vascellari *et al*., 2007; Nakajima *et al*., 2013). DNA has been previously extracted from archival FFPE tissues (Goelz *et al*., 1985; Dubeau *et al*., 1986; Ludyga *et al*., 2012) and it is possible to analyze such fragments as representative of the gene and used for short fragment or partial genome sequencing (Taubenberger *et al*., 1997). A barrier to extract RNA from FFPE tissues is often significant RNA degradation due to formalin fixation and initial tissue handling (Bibikova *et al*., 2004). In this retrospective study, we investigated (i) the possibility for extraction of H5N1 AIV antigen and RNA from archival FFPE tissues, and (ii) using extracted RNA for short fragment or partial genome sequencing to confirm AI infection and to perform a retrospective analysis of HPAI outbreaks.

## MATERIALS AND METHODS

### Cases

Ten formalin-fixed paraffin embedded tissue samples as representatives of ten outbreaks (A-J, in 2006 and 2007) of HPAI-H5N1 in commercial chickens in Nigeria were obtained from the archive of the Central Diagnostic Laboratory, National Veterinary Research Institute (NVRI), Vom-Jos Plateau State, Nigeria. Fresh samples from these cases had been previously characterized as H5N1 HPAI positive by virus isolation. Gross and histopathology studies were also conducted on all cases. Sample tissues from organs such as comb, wattle, brain, heart, trachea, lung, spleen, liver, kidney, proventriculus, intestine and pancreas were investigated by IHC, RT-PCR, real-time RT-PCR (rRt-PCR) and the PCR products were further characterized by sequence analysis.

### Histopathology and Immunohistochemistry

Paraffin- embedded sections (5µm) were dewaxed using xylene and stained with hematoxylin-eosin for routine histopathological investigation of tissue samples from all outbreaks. For immunohistochemistry, sections were mounted on charged microscope slides (Menzel, Braunschweig, Germany), dewaxed in xylene and then in graded concentrations of alcohol. Nonspecific binding and endogenous activities in the tissues were blocked by adding 9ppm of absolute methanol and 1ppm hydrogen peroxide to the tissues in slide chamber for 10 minutes at $25^0$C before rehydrating in distil water. Heat induced antigen retrieval method was done by placing the tissue sections on glass slides and placing them in a plastic slide holder containing citrate

buffer before microwaving. After cooling for 5 minutes, the citrate buffer was decanted and Tris buffer solution (TBS) was added for 3 minutes. Irrelevant antigens i.e unspecific receptors on the tissues were blocked by adding an in-house goat serum as a blocking antibody. This blocking antibody was later washed thrice by using TBS. To detect influenza virus antigen, sections were incubated with an in-house primary rabbit antibody produced against H5N1 AIV nucleoprotein, in a dilution of 1:500 in Tris-buffered saline (TBS, 0.1 M Tris-base, 0.9% NaCl, pH 7.6)(Starick *et al*., 2006; Klopfleisch *et al*., 2006). In brief, a biotinylated goat anti-rabbit IgG1 (Vector, Burlingame, CA; diluted 1:200 in TBS) was used as linker-antibody for the avidin-biotin-complex (ABC) method. For negative control, serum from the same rabbit prior to challenge with H5N1 AIV was applied on dewaxed tissue sections. This ensured that there were no other antigens reacting with the prior challenge serum and it is different from the serum (primary antibody) generated after challenge with H5N1 Avian Influenza virus. Positive samples produced a bright red signal with an IHC kit (Vectastain Elite ABC Kit, Vector®) and the substrate 3-amino-9-ethylcarbazole (DAKO AEC substrate- chromogen system; Dako, Carpinteria, CA, USA). The sections were counterstained with Mayer's hematoxylin and sealed with aqueous medium (Aquatex; Merck, Darmstadt, Germany). Positive and negative control tissues of experimentally H5N1 HPAI infected chickens were included for each immuno-histochemistry procedure. The immunohistochemical reactions were quantitatively scored as strong, moderate and mild based on the distribution and spread of the bright red signal.

**RNA Extraction from FFPE Tissues**

RNA was extracted from 4-5µm of HPAI positive FFPE chicken tissues using the RNeasy FFPE kit, following the manufacturer's instructions (QIAGEN, Hilden, Germany). Briefly, samples were placed in 2ml micro-centrifuge tubes and 1 ml xylene was added. Following centrifugation at 10,000 rpm gravity (g) at $25^0$C for 2 minutes the xylene supernatant was removed and then 1 ml of 100% ethanol was added to the pellet to remove residual xylene. Following an additional centrifugation step at 10,000 rpm at $25^0$C for 2 minutes, the supernatant was pipetted out and then residual ethanol was removed by incubation at room temperature (25ºc) for 10 minutes. The pellet was suspended in an in-house 150 µl Proteinase K digestion (PKD) buffer. The next steps complied with the usual terms of matrix binding and elution of nucleic acids with a final volume of 14 µl RNA according to the manufacturer's instructions (QIAGEN, Hilden, Germany).

**RT-PCR and Sequencing**

Ten RNA samples obtained from the investigated outbreaks representing twenty-six animals were analyzed by modified TaqMan one-step real-time RT-PCR (rRT-PCR) assays which specifically amplified fragments of the AIV M and H5 (HA2 part) genes (Fereidouni *et al*., 2012). In addition, an rRT-PCR assay using a probe specific for the cleavage site of the HA gene of H5N1 clade 2.2 viruses was used. For the H5 rRT-PCR test targeting the HA2-part of the hemagglutinin, each sample was sequenced in duplicate using forward and reverse primers. In addition, the sequencing of the H5 gene using four RT-PCR of overlapping regions of the gene was carried out. Direct Sanger sequencing of PCR products was done as described previously (Starick *et al*., 2008). Sequence data were assembled and consensus sequences were generated with GCG Version 11.1 (Accelrys Inc.). Sequences of AIV isolates with the highest homology to the obtained sequences in this study were chosen from NCBI Blast search

**TABLE I**: Results of Histopathology, Immunohistochemistry and RT-PCR comparison of HPAI FFPE tissues from HPAI H5N1 outbreaks in 2006 and 2007 in Nigeria

| Cases | Animal | Histopathology | [a]Immunohistochemistry | [b]rRT-PCR H5 HA2-gene | M-gene |
|---|---|---|---|---|---|
| **A/07** | 1 | Edema of comb and wattles with necrosis of vascular endothelial cells | Comb & wattle+++, Lung+, Spleen+, Kidney++ | + | + |
| **B/07** | 2-3 | Multifocal neuronal necrosis in cerebrum and necrosis of Purkinje cells in cerebellum. Necrosis of endothelia cells and epithelia of air capillaries and moderate heterophilic infiltration in the interstitium. Surface epithelium of the ventriculus, vascular endothelium in the lamina propria of proventriculus and intestine were necrotic | Brain++,Lung++, Kidney+, Intestine++, Pancreas+ | + | + |
| **C/06** | 4-6 | Multifocal neuronal necrosis in cerebrum and necrosis of Purkinje cells in cerebellum. Necrosis of proximal convoluted tubular epithelium and moderate infiltrates of heterophils | | | + |
| **D/06** | 7-10 | Edema of comb and wattles with necrosis of vascular endothelial cells | Comb & wattle++ | + | + |
| **E/06** | 11-15 | Edema of comb and wattles with necrosis of vascular endothelial cells | Comb & wattle+, Heart++, Spleen++ | | + |
| **F/06** | 16-19 | Edema of comb and wattles with necrosis of vascular endothelial cells. Necrosis of endothelia cells and epithelia of air capillaries and moderate heterophilic infiltration in the interstitium. Multifocal necrosis of the acinar cells and hemorrhages of pancreas | Comb & wattle+++, Heart++,Trachea++, Lung++, Intestine++, Pancreas++ | | + |
| **G/07** | 20 | NVL | | | |
| **H/06** | 21-22 | NVL | | | |

| | | | | | |
|---|---|---|---|---|---|
| **I/06** | 23-24 | Necrosis of endothelia cells and epithelia of air capillaries and moderate heterophilic infiltration in the interstitium. Random foci of coagulation and hepatocellular necrosis with heterophilic infiltration were observed commonly in affected livers | Comb &wattle+, Heart+, Lung++, Liver++, Intestine+ | + | + |
| **J/06** | 25-26 | NVL | | | |

[a]Immunohistochemistry: Mildly Positive +; moderately Positive ++; strongly positive +++.
[b]RT-PCR: detection +

and sequences were aligned (Zang *et al*., 2000; Chenna *et al*., 2003) using Clustal W2.

## RESULTS

### Histopathology and Immunohistochemistry

Histopathological findings seen in the different outbreaks were moderate to severe congestion in various tissues and edema of comb and wattles with necrosis of vascular endothelia. This correlates with very strong immunohistochemical demonstration of influenza virus antigen in keratinocytes and vascular endothelium of comb and wattle (Fig. Ia and b). In the cerebrum, multifocal neuronal necrosis and in cerebellum, necrosis of Purkinje cells was detected. At the areas of neuronal necrosis, strong immunohistochemical reaction of AI virus antigens was also observed (Fig. II). In the lungs, hyperemia, necrosis of endothelia cells and epithelia of air capillaries were seen and moderate heterophilic infiltration into the interstitial were evident. Foci of superficial heterophilic infiltration and epithelial necrosis were seen in the proventriculus and at the esophageal-proventricular junction. The pancreas had multifocal necrosis of the acinar cells and hemorrhages, with immuno-staining in areas of pancreatic necrosis. Random foci of coagulation and hepatocellular necrosis with heterophilic infiltration were observed commonly in affected livers. Proximal convoluted tubular epithelium showed evidence of multifocal necrosis with moderate infiltration of heterophils, and strong immunohistochemical reaction of influenza virus antigen in different organs (Table I). Neurons of cerebrum and cerebellum, trachea ciliated epithelia, respiratory epithelia, cardiomyocytes, renal tubular epithelium were also strongly immunopositive. Nucleoprotein (NP) staining in the endothelium of sinusoids, splenic vessels, and myocardial vessels was evident. In addition, the surface epithelium of the ventriculus, vascular endothelium in the lamina propria of proventriculus and intestine, occasionally within epithelial cells stained positive for AIV antigen. There was a positive correlation between gross and histological evidence of lesions such as edema, necrosis and hemorrhage with varying degrees of Immunohistochemical staining i.e severity of pathological lesions caused by the virus correlates with abundance of Immunohistochemical detection.

### Molecular Analysis

All experiments to amplify parts of or the whole HA gene by conventional RT-PCR assays were not successful. The real-time PCR assay with the probe specific for the HA cleavage site of clade 2.2 viruses revealed weak positive signals for two samples (A/07 and B/07) not sufficient for direct sequencing,but was sequenced after cloning into the bacterial vector pGEM-T Easy (Promega). The deduced HA cleavage

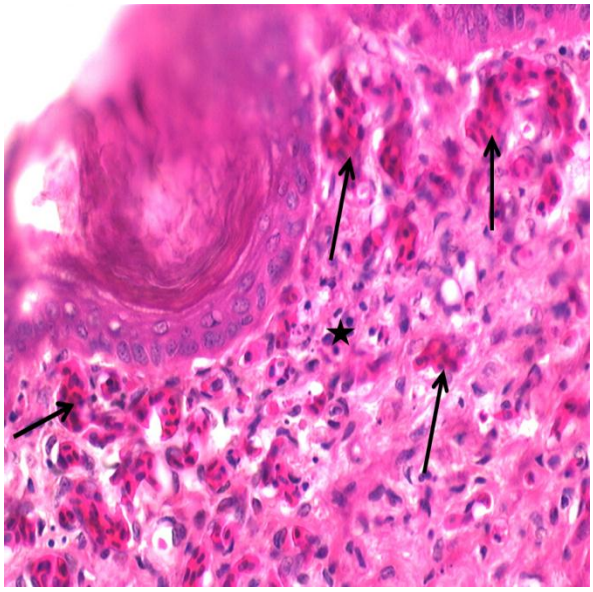

**Figure Ia**: chicken, comb A/07: congestion of dermal capillaries (arrows) with polymorphonuclear cellular (star) infiltration into the superficial dermis of the comb H&E X400

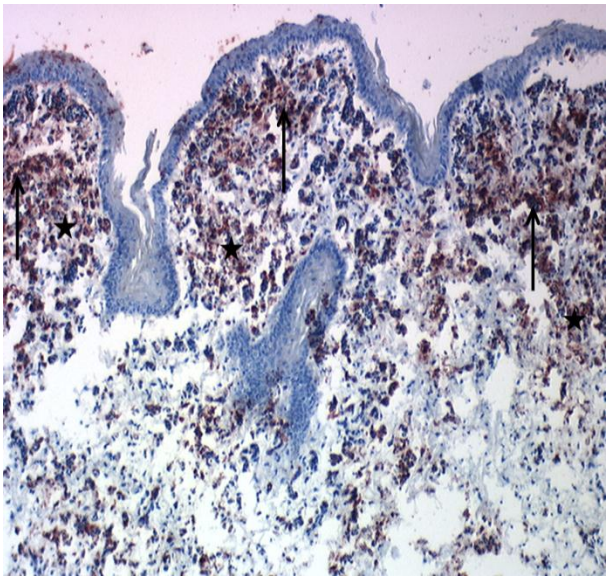

**Figure Ib**: chicken, comb A/07: Immunopositive staining of capillary endothelial lining of blood vessel (arrows) and dermal connective tissue (star) of the comb ABC staining method X400

site amino acid sequences is consistent with those of clade 2.2. Viruses: PQGERRRKKR*GLF. In contrast, matrix gene specific rRT-PCR assays of seven samples revealed threshold cycle (CT) values between 24. 5 and 38. 4. CT values of the H5 HA2 rRT-PCR were lower (28 to 39) and allowed purification and sequencing of the PCR products of four samples (A/07, B/07, D06 and I/06). Sequencing data covering 154 nucleotides (nt) (1531-1684) generated by this rRT-PCR was obtained. The homology between sequences from outbreaks A/07, D/06 and I/06 were 100%, whereas the fourth sample from outbreak B/07 differed at 4 nucleotides (97,4% homology). Three nucleotides difference were silent mutations whereas the fourth mutation led to an amino acid exchange (544 Ala → Thr).

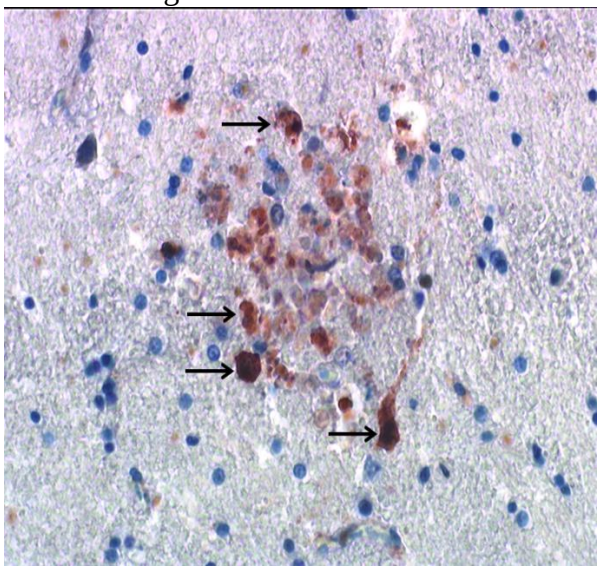

**Figure II**: chicken, cerebrum A/07: Immunopositive staining of neurons (arrows) in the cerebrum correlates area of severe neuronal necrosis H&E X400

## DISCUSSION

Formalin-fixed paraffin embedded tissue samples included in this study demonstrated varying degree of lesions consistent with acute infections of HPAI-H5N1 and showed AI specific virus antigen in certain tissues of some of the cases using IHC assay. In these tissues, IHC immunoreactivity varied from strongly positive comb and wattle to moderately positive lung and spleen, proving tissue localization of the viral antigens. Therefore, seven out of ten RNA

samples tested positive by rRT-PCR assays targeting the genes for the viral matrix protein and four revealed positive for the haemagglutinin gene (HA2 part). Two out of four (A/07 and D/06, with CT-values in the HA2-PCR of about 30) showed weak positive results using the real-time PCR assay specific for the HA cleavage site of clade 2.2 viruses. The remaining five M specific rRT-PCR positive samples (B/07, C/06, E/06, F/06, and I/06) were too weak for successful detection using the cleavage site rRT-PCR which is known to have a reduced sensitivity compared to other two rRT-PCR assays (Hofmann et al., 2007). When the rRT-PCR results were compared with the IHC using serial sections, the 3 rRT-PCR negative RNA samples from outbreaks C/06, E/06, F/07 had at least 4 tissue immunoreactivity by IHC (Table I), this was attributed to the probably absence of strongly immunoreactive tissues in the sections used. In contrast to the promising results of the M and HA2 rRT-PCR assays, all experiments to amplify the RNA samples by conventional RT-PCR were not successful. This might be due to the higher product sizes aimed in these assays and/or reaction conditions which were not optimized for FFPE tissues (Godfrey *et al*., 2000).

The rRT-PCR results were further confirmed by sequencing of four HA2 rRT-PCR positive samples (A/07, B/07, D/06 and I/06). The result of the analysis was in agreement to previous studies which showed co-circulation of different strains in Nigeria (Ducatez *et al*., 2006). Beside the nucleotide and amino acid comparison of the four sequences, BLAST search might suggest that the viruses belong to different sub-clades although this should be verified by longer sequences. Formalin-fixed paraffin-embedded tissue samples have been used on molecular level especially for gene expression analyses in neoplasm (Bibikova *et al*., 2004). Few studies were published about virus RNA detection from FFPE materials (Abdueva *et al*., 2010, Ludyga *et al*., 2012). Due to the degradation of RNA during the formalin fixation process, the efficiency of RT-PCR assays is often clearly reduced compared to assays with RNA prepared from fresh tissues (Masuda *et al*., 1999). However, real-time RT-PCR assays with their increased sensitivity and aiming at products of small size can compensate this disadvantage (McKinney *et al*., 2009).

In summary, this study has shown the suitability of archival FFPE tissues from HPAIV infected chickens in the detection of H5N1 viral antigen by IHC, extraction of RNA, and generation of short fragment sequences. It was also partially possible to classify the viruses into sub-clade 2.2. via HA cleavage site- specific RT-PCR. This study also provided the opportunity to correlate retrieval of proteins for IHC and extraction of nucleic acids from archival HPAIV H5N1 paraffin embedded tissue sections.

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