

Pathology and Characterization Of Fowl Pox Virus Infection in A Turkey-Chicken Backyard Flock, Nigeria

Olatunde Babatunde Akanbi¹ , Emmanuel Tumininu Obishakin^{*2,3} , Amos Gambo Rimfa⁴, Olayinka Oluwafemi Asala⁵ , Adeyinka Jeremy Adedeji⁶ 

¹Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ilorin, Ilorin Nigeria

²Biotechnology Division, National Veterinary Research Institute, Vom, Nigeria

³Biomedical Research Center, Ghent University Global Campus, Songdo, Incheon, South Korea

⁴Central Diagnostic Laboratory, National Veterinary Research Institute, Vom, Nigeria

⁵Viral Vaccine Production Department, National Veterinary Research Institute, Vom, Nigeria

⁶Viral Research Department, National Veterinary Research Institute, Vom, Nigeria

E-mail: akanbi.ob@unilorin.edu.ng

ABSTRACT

Cutaneous scabs were seen on the nares and shanks in three grower turkeys in a mixed turkey-chicken flock in Langtang-north LGA of Plateau State, Nigeria. A chicken in the flock had diphtheritic membrane covering the mucous membranes of the oro-pharynx and the turkeys had cutaneous pox lesions on the nares and shank. Fowlpox virus infection was diagnosed by gross and histopathology and confirmed by isolation of the virus in chorioallantoic membrane of 9-12 weeks chicken embryonating eggs. The DNA of Fowl Pox Virus (FPV) was detected in the cutaneous scabs of the turkey and chicken using Polymerase Chain Reaction (PCR) which amplified a 578 base pairs fragment of the 4b core protein gene. Sequencing and phylogenetic analysis revealed that the fowlpox virus responsible for this infection clustered with the sublineage A1 genotype of clade A of fowlpox virus and shares 98% homology with the vaccine strain produced in Nigeria. The sequence has been deposited in the GenBank under the accession number MK435242. Our results confirmed the presence of FPV in the turkeys and suggest a predisposition by the chickens. We therefore suggest vaccination for small holder poultry to mitigate against mortality in turkeys and chickens and molecular epidemiology of fowlpox viruses in Nigeria to unravel viral evolution.

Key words: Fowlpox, pathology, molecular analysis, mixed species, poultry

INTRODUCTION

Fowlpox infection is known to be a slowly spreading and economically important disease of chickens and turkeys, as it can cause egg production losses and mortality, especially in commercial poultry (Tripathy and Reid, 2011). The causative agent being one of the avipoxvirus genus which contains closely related viruses that belong to the subfamily chordopoxvirinae and the family Poxviridae and affects vertebrates with no strict host specificity (Quinn et al., 2011). This genus contains avian viruses which includes fowlpox virus (FPV), known to infect fowls, turkey pox virus infecting turkeys and pigeon pox virus infecting pigeons. In chickens, fowlpox is known to affect all ages, sexes and breeds (Weli and Tryland, 2011) and mostly seen in poultry kept in free-range holdings (Bwala et al., 2015), although it is believed to be widespread in backyard and to some extent intensively reared poultry flocks in Nigeria (Adene and Fatumbi, 2004; Meseko et al., 2012). Diagnosis of the cutaneous and diphtheritic forms of FPV is commonly carried out by histopathological examination and virus isolation on embryonated chicken eggs. PCR based amplification of highly conserved region of the genome (Manaroll et al., 2010) and sequencing have been used for confirmatory diagnosis (Lee and Lee, 1997). Phylogenetic studies have shown that avian poxvirus isolates clustered into

three major clades: the *Fowlpox virus* (clade A), the *Canarypox virus* (clade B), and the *Psittacinepox virus* (clade C) with clades A and B having multiple subclades (Manaroll et al., 2010; Meseko et al., 2012; Banyai et al., 2015). This paper is aimed at reporting fowlpox virus infection in a mixed backyard semi-intensive turkey-chicken flock in Plateau State, Nigeria, and to determine the pathology and molecular characterization of fowlpox virus.

MATERIALS AND METHODS

Ethics statement

All methods were carried out in accordance with relevant case record guidelines, regulations and poultry diagnostic protocols as approved by the Central Diagnostic Laboratory of the National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Case report of a fowlpox virus infection in turkey-chicken backyard flock

A mixed turkey-chicken flock was reared in PilGani, a semi-arid area of Langtang-north LGA of Plateau State, Nigeria consisting of three 8-weeks old grower indigenous black turkeys and an adult indigenous domestic hen reared in a mixed backyard semi-intensive turkey-chicken flock that consist of other two domestic adult hens and five adult turkeys were observed to have cutaneous scabs on

their nares, around their eyes and shanks. The affected chicken had a diphtheritic lesion on the oropharynx and trachea while the remaining two adult hens were moribund but showed no lesion. Also, the five adult turkeys were active and showed no lesion. A tentative diagnosis of poxvirus infection was made pending further laboratory investigation. This mixed turkey-chicken flock was reared in *PilGani*, a semi-arid area of Langtang-north LGA of Plateau State, Nigeria. There was history of initial infection of the chickens in the flock in early 2015 in which all the young and some adult chickens died before the eleven turkey growers were hatched in October 2015, from the dreick of which 8 died and 3 were recovering from the infection but with resolving lesion as of 9th January 2016. The case was recorded under the case name: CD⁻ 029- A/2016. The mortality rate in the affected turkey flock was 72% (8 out of 11) while it was eventually 100% in the chickens.

Gross and Histopathology

Cutaneous scabs seen on the nares and shank of the turkeys were removed alongside skin biopsy. In addition, necropsy was performed on the moribund chicken which eventually died and the tissues including oropharyngeal tissue and trachea were removed and fixed in 10% buffered formalin. The lesions were physically examined thoroughly before processing for histopathology. After fixation, the

tissues were embedded in paraffin, sectioned at 5 µm, mounted on clean glass slides, and stained with hematoxylin and eosin (H&E) stains for histopathologic examination using low and high-powered field of Carl Zeiss Axio Imager A1 binocular microscope, as previously described (Akanbi et al., 2021). Fresh tissues were also preserved in phosphate buffer saline at pH 7.4 for virus isolation and dry scab was stored for DNA extraction and molecular analysis using PCR and gene sequencing. The samples were stored at the Central Diagnostic Laboratory of the National Veterinary Research Institute, Vom, Nigeria.

Virus Isolation

For virus isolation, suspensions (1:10) in sterile phosphate buffered saline of scab's sample was made. The suspension was clarified by centrifugation at 1500 rpm for 15 min at room temperature and supernatant was treated with a mixture of penicillin Sandoz® (10,000IU/ml of supernatant) and streptomycin (10,000µg/ml of supernatant) from for 45 min at 37°C. One vial of freeze-dried fowlpox vaccine (Kabette strain) was reconstituted in 1ml PBS as positive control. All the field and vaccine (0.2 ml) sample were inoculated onto the chorioallantoic membrane (CAM) of minimal-disease-free 10-day-old chicken embryos as described before (Cunningham et al., 1960). CAM were harvested and examined for pock

lesion, five days post inoculation (PI). Subsequent passage was required for adaptation of the virus in CAM.

DNA extraction and conventional PCR detection of FPV from tissues and Fowl Pox Vaccine

Skin scabs from the lesions and trachea tissues were sent to the Biotechnology laboratory of the National Veterinary Research Institute (NVRI), Vom for Polymerase chain reaction confirmation of Avipox infection. From the samples, DNA was extracted using ZR™ DNA MiniPrep extraction kit (Zymo research USA) according to the manufacturer's instructions with slight modifications. Briefly, 0.2 grams of the scabs and tissues were homogenized in 200µl of PBS with sterile sand in a ceramic mortar with pestle. 200µl of PBS reconstituted lyophilized, live egg adapted fowl pox vaccine (Beaudette strain) produced by NVRI vom was used as positive control while PBS was used as negative control. Sequel to homogenization, 200µl of the mixture was transferred into clean 1.5mL eppendorf tube. 800µl of Lysis buffer containing 0.5%(v/v) of B-Mecaptoethanol was added to the mixture in the tubes. The mixture was vortexed for 10 minutes. The tube was centrifuged at 14,000rpm for 1 minute. The supernatant was transferred into a 2ml Zymo- collection tube and 1,200 µl of DNA Binding Buffer was added to the filtrate in the collection tube. 800 µl

of the resulting mixture was transferred to a Zymo-Spin II Column in a collection tube and centrifuged at 8000 rpm for 1 minute. 200 µl DNA pre-wash buffer was added to the Zymo-Spin II Column in a new collection Tube and centrifuged at 8000 rpm for 1 minute. Subsequently, 500 µl Fecal DNA wash buffer was added to the Zymo-Spin II Column in a new collection tube and centrifuged at full speed for 1 minute. The column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNase/RNase-Free water was added. The tube was centrifuged at 14000 rpm for 30 seconds to elute the DNA. The eluted DNA was used for PCR amplification.

FPV specific PCR Oligonucleotide primers and PCR Amplification

A pair of primers targeting the 4b gene sequence of FPV was used. The sequences are codenamed as: P1, 5'-CAGCAGGTGCTAAACAACAA- 3' and P2, 5'-CGGTAGCTTAACGCCGAATA-3' (8). The primers produce PCR amplicons of 578 base pairs as expected band size. PCR was carried out in a 1X master mix composition of *Taq* 2X Master Mix (New England Biolabs™, UK) containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTPs. The mix was carried out in a 25µl total volume per sample. The following thermal cycling profile was used: Initial denaturation at 94°C for 2 minutes, followed by 35 cycles of

denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, final extension was at 72°C for 2 minutes. DNA amplification was performed using a GeneAmp 9700™ PCR thermal cycler (Applied Biosystems, USA). 1.5 % w/v of agarose gel was prepared using Tris- borate-EDTA (TBE) buffer. 10µl of ethidium bromide was added and poured into the casting tray with combs placed at each end and allowed to polymerize. The tray was gently placed in TBE electrophoretic tank. Each sample was placed in separate sample wells and O' Gene Ruler™ (Sigma Aldrich, Germany) (100bps DNA ladder) was loaded at the beginning and end of each well. After loading the samples in each well, the electrophoresis tank was connected to the power source and run at 140 volts, 400Amperes for 30 minutes. This was placed in a UV transilluminator (Biorad gel imaging doc™, USA). The gel was viewed in a computer screen connected to the UV transilluminator through the Biorad gel imaging doc™ software.

Partial sequencing of 4b gene of FPV

DNA amplicons from the positive samples and from the vaccine strain (positive control) were sent to Macrogen Inc. (South Korea) for purification and sequencing of 578bp partial 4b core protein gene. The same primer pairs were used for both amplification and sequencing.

Sequence analysis and Phylogenetic analysis

The generated partial nucleotide sequences from the 578bp PCR products were obtained and first analyzed for Basic Local Alignment Search Tool (BLAST) search program of the National Center for Biotechnology Information (NCBI) website against the whole GenBank data base of nucleotide sequences. Subsequently, the sequences were aligned with 578bp fragment of the partial 4b gene sequences from the GenBank. The sequences of two Nigerian strains, two commercial fowlpox vaccine strains from Europe, one Turkeypox strain from Germany, one Sparrowpox strain from Germany, one Turkeypox from Italy and one Pigeonpox from the United Kingdom were downloaded from the GenBank. These fowlpox isolates all belong to subclade A1 and A2 genotypes of the A clade of avian poxvirus isolates (Meseko et al., 2012). The alignment was done using Clustal W multiple alignment method on Bioeditsoftware package version 7.2.5.0. Phylogenetic and molecular evolutionary analyses of nucleotide sequences were performed using MEGA version 7 (Kumar et al., 2016). Phylogenetic tree was constructed by the maximum likelihood method using bootstrap analysis with 1000 replicates.

Accession number:

The sequence of the isolate obtained in this study has been deposited in the GenBank under the accession number MK435242 and available at <https://www.ncbi.nlm.nih.gov/nuccore/MK435242>

RESULTS

Gross and histopathological examination:

The three surviving grower turkeys from the 11 poults hatched showed pale to yellow scabs which were oftentimes discolored at the periorbital and nasal regions. These scabs are multifocal and are about 0.5-1cm in diameter (Figure I a&b). Occasionally, solitary scabs were found on the face and ocular areas and the lesion is characterized by papule formation which thickens and coalesced

to form large dark brown scabs which often occludes the nares (Figure I b). In the poxvirus infected chicken that showed the diphtheritic lesion, there was an extensive raised yellowish patchy, necrotic, diphtheritic membrane which covers the mucous membranes of the oro-pharynx which can be seen when the lower beak is reflected (Figure I c) during necropsy. This raised yellowish patchy, necrotic, and diphtheritic membrane, also covers around the ridge of the choana, the larynx, the syrinx and laryngeal papillae and at the groove of the glottis. Histopathology of this diphtheritic membrane and the oropharynx revealed hyperplasia of the stratified squamous cells exhibiting acanthosis with severe inflammatory exudation of heterophils, macrophages and lymphocytes (Figure Id).

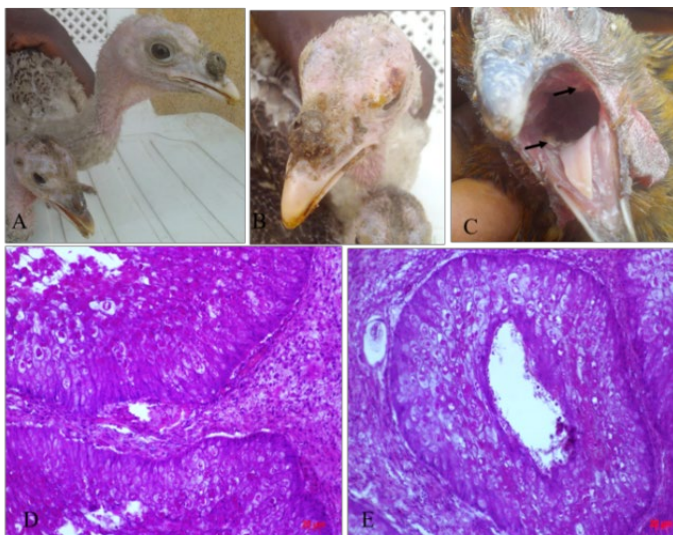


Figure 1. Gross and histopathologic lesion of Fowlpox in Turkeys and Chicken: A&B, turkey, 2-months old with large perinasal and small periorbital scab, C) Chicken, oropharynx 2 years old, diphtheritic membrane (arrows), D) Epithelial keratinocytes showing acanthosis and intracellular edema, and within the surrounding dermal tissue is a large, diffuse infiltration of mononuclear cells admixed with edema fluid. Hematoxylin and eosin (H&E) stain, E) Feather follicular epithelium showing medium to large eosinophilic intracytoplasmic inclusion bodies within keratinocytes (H&E) stain.

Also, there were several small to medium sized eosinophilic intracytoplasmic inclusion, identified as Bollinger bodies (Figure 1 d & e).

Virological analysis:

The identity of the pathogen was verified, pocks on the CAMs were subjected to microscopic examination.

Molecular detection of Fowlpox virus

The amplification of a fragment of 4b gene of FPV was confirmed by the presence of a 578bp band in the gel electrophoresis result. This was seen in the turkey scab lesion sample and in the fowl pox vaccine samples, but the fragment was not observed in the trachea-oropharynx tissue sample and the PBS negative control sample (Figure 2).

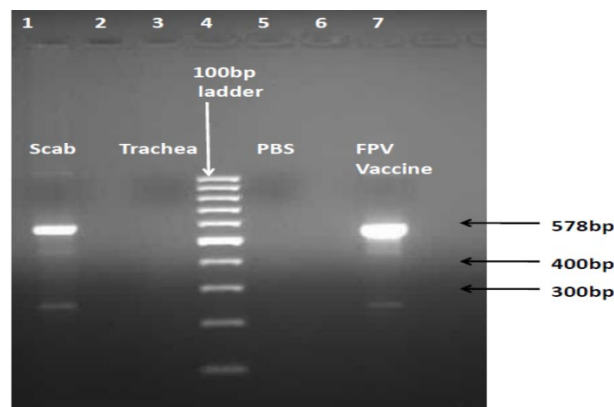


Figure 2. Electrophoresis gel image of the Amplification of DNA from FPV suspected turkey scab. Lanes: 1- Scab, 3- Trachea, 4-100bp ladder, 5-PBS (negative control), 7-FPV vaccine (positive control).

BLAST and phylogenetic analysis:

The DNA amplicons from the PCR of the positive samples were sequenced and the sequences were analyzed. The result of BLAST revealed that the sequence from sample has 98% homology with the vaccine strain from NVRI, Vom, (Joannis et al., 2008) while it shares 99% similarities with some avipoxvirus sequence samples from the GenBank. The BLAST analysis of

vaccine strain used as positive control also shares 100% homology both with all the sequences of Nigerian isolates and with the sequence of NVRI, Vom vaccine strain in the GenBank. The sequence of the isolate obtained in this study has been deposited in the GenBank under the accession number MK435242. The topology of the phylogeny shows all the Nigerian fowlpox strains are grouped together in

a monophyletic group with A1 subclade vaccine and field strains from the GenBank. The monophyletic group further bifurcates into two with sample clearly segregating alone. As expected,

the, subclade A2 (Turkeypox from Italy and one Pigeonpox from the United Kingdom) isolates are grouped together in another monophyletic group (Figure 3).

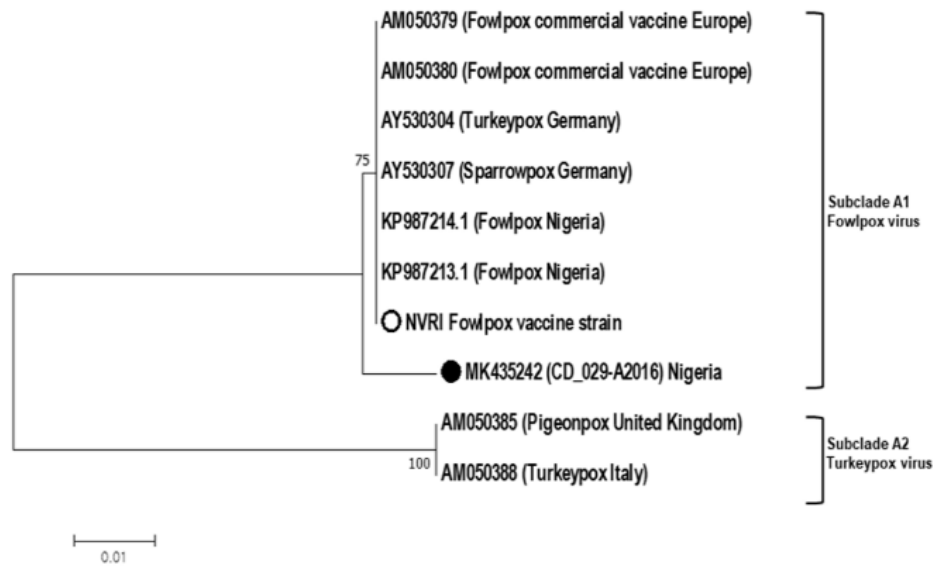


Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method: Phylogenetic tree of the partial 4b gene sequences of fowlpox virus strains in our study performed on MEGA 7 using the Maximum Likelihood method based on the Kimura 2-parameter model. The reliability of the tree was assessed by 1000 bootstrap replications. Reference sequences were retrieved from the Gen- Bank database, their sources and accession numbers are as shown. The sequences that were obtained in this study are indicated by shaded and unshaded circles. The NVRI vaccine sample used as positive control is indicated by an un-shaded circle (○) while the scab sample isolate (MK435242) is indicated by a black shaded (●) circle. The clade and subclade classifications are indicated by the right braces. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

DISCUSSION

The infection with fowlpox virus of a mixed turkey-chicken semi-intensive backyard flock at different ages as

presented in this case had fatal outcome in both species with 27% (3 out of 11) turkey growers surviving. Our result confirmed the presence of FPV in the turkeys and suggests a predisposition

by the chickens. Although the part of the trachea sample taken for PCR did not show gross lesion and fowlpox virus was not detected, the oropharynx in this 2 years old chicken which showed the gross lesion of the diphtheritic form of fowlpox also revealed characteristic histopathologic lesion consistent with fowlpox infection. Infection with FPV manifested different forms of the disease in this mixed turkey-chicken flock despite the presence and isolation of a fowlpox virus in the flock. The varied manifestation of fowlpox virus infection in these turkeys, and the chicken may be attributable to differences in age and species responses to the virus. Also, the chicken being an aged bird may be immunosuppressed, as there was a history of previous infection and lesion of poxvirus in the flock early 2015. The disease led to the death of the chicken, as the respiratory lesion resulted in gasping before death and oral lesion caused inappetence in the chicken. The infection was characterized mainly by morbidity and high mortality rate. The bifurcation of the scab samples reflects that although it groups together with the A1 subclade strains from Nigeria, it stands alone as a distinct field strain. It is also worthy of note that the newly sequenced Baudette vaccine strain is 100% similar to the sequence of the same strain which had been deposited in the GenBank. This clearly shows that the positive PCR scab sample sent for sequencing was not

contaminated with the vaccine strain used as our PCR positive control. The origin of the scab strain could be as a result of the mutation of the vaccine strain based on its 98% homology with the vaccine strain and also because the vaccine strain has been in use since the early 1970's. The sample CD 029-a/2016 could also have originated from a completely different source. Given that the other Nigerian field strain sequences in the GenBank are 100% similar to the vaccine strain (Joannis et al., 2008) and sample CD 029-A/2016 is 98% similar to the same vaccine strain, it is hence logical to favor the hypothesis that the scab strain described here emerged from a different source. Although, fowlpox cases have been reported in Nigeria before (Meseko et al., 2017) but in-depth analysis of the genetic makeup of responsible viruses for the infections have not been done to accompany the reports. In the same vein, phylogenetic analyses have been reported for some Nigerian field strains, such also lack full clinical histories. Our report here combines both the clinicopathologic and molecular investigations of an outbreak. In view of our findings, we recommend that farmers of small holder flock should be encouraged to vaccinate against fowl pox in susceptible turkey and chicken flocks as this has been proven to militate against mortality in turkeys (Odoya et al., 2006). In addition, a full genome sequencing of fowlpox isolates and a

larger molecular epidemiology study of fowlpox viruses in Nigeria will shed more light on viral evolution of fowlpox viruses in Nigeria and this requires an urgent attention.

CONCLUSIONS

This work confirms Fowlpox virus infection in this flock.

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Author contributions

Concept: OBA; Design of the work: OBA, ETO; Data Acquisition: OBA, ETO, OOA, AGR, AJA; Wrote the paper: OBA, ETO, OOA, AGR, AJA

Conflict of interest statement: The authors declare that there is no conflict of interest.

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