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*Afr. J. Biomed. Res. Vol. 24 (May, 2021); 307 - 310*

*Research Article*

## **Pathology and Molecular Detection of Fowlpox Virus in Chickens in Ibadan, Nigeria.**

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### **ABSTRACT**

Fowlpox is known to develop rather insidiously in infected birds, producing distinct proliferative skin lesions on the unfeathered parts of the infected bird (i.e., wattles, comb, shanks, etc.) and sometimes on the mucosae of the oral cavity and the upper respiratory tract. Several diseases of birds such as infectious coryza, infectious laryngotracheitis, Mareks, and vitamin A deficiency can be confused with fowlpox, therefore there is need for an accurate and reliable method of diagnosis. A combination of histopathology, Polymerase Chain Reaction (PCR) and sequence analysis can be employed as diagnostic tools to confirm the presence of the disease. Consequently, fifty scab samples from suspected cases of fowlpox from backyard poultry in Ibadan, Nigeria were collected, and PCR, sequencing with sequence analysis were employed to detect and characterize the open reading frame 168 (39 kDa core protein) gene of the virus. Also, lesions from the eyelids were excised and histopathology was carried out. Thirty-one out of 50 samples (62%) tested positive for fowlpox based on PCR and sequence analysis. Histopathology revealed the presence of dermal hyperplasia and Bollinger bodies which are confirmatory of fowlpox. PCR amplification, sequence analysis of 39 kDa core protein gene and histopathology provide a reliable diagnosis of fowlpox.

**Keywords:** *Fowlpox virus, polymerase chain reaction, histopathology, diagnosis*

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*Received: July, 2020; Accepted: April, 2021*

### **INTRODUCTION**

Fowlpox is a highly contagious viral disease of birds which is similar to poxvirus infections in various mammalian species such as swinepox, monkeypox and sheep pox (van Riper and Forrester, 2007). Fowlpox is of major economic importance in the poultry industry, causing increase in bird mortality, drop in egg production, retarded growth, and sometimes blindness (Tripathy *et al.*, 2013; Sarker *et al.*, 2021). The disease has been reported in 232 in 23 orders of the about 9,000 avian species (Bolte *et al.*, 1999).

Fowlpox is mostly a disease of chickens and turkeys which spreads slowly, and is known to show 3 distinct clinical manifestations: the cutaneous, the diphtheritic and systemic form (Nakaruma *et al.*, 2006; Maranolla *et al.*, 2010). The cutaneous form (also known as dry pox) presents as proliferative lesions, which could be of varying sizes ranging from tiny nodules to roughly spherical wart-like elevations on the comb, wattle and other parts of the body not covered by feathers; and feather folliculitis in the feathered skin (Wagner *et al.*, 2008). The diphtheritic form (wet pox) is typified by the presence of slightly raised white opaque nodules on the mucosae. These nodules increase in dimension and coalesce to form a yellow diphtheritic membrane covering the mucosae of the oral cavity, oesophagus, larynx or trachea. Tracheal lesions must be differentiated from tracheal lesions caused by

the avian infectious laryngotracheitis virus (Tripathy *et al.*, 2013). The third form, systemic infection, is mainly caused by the virulent form of the virus and it is very rare (Tripathy *et al.*, 2013; Ogasawara *et al.*, 2016). Lesions are commonly visible on parts of the body not covered by feathers (i.e., shanks, feet, eyelids, base of the beak, comb and wattles (van Riper and Forrester, 2007).

Fowlpox has a worldwide occurrence and it is caused by Fowlpox virus belonging to the family *Poxviridae*, subfamily *Chordopoxvirinae* and genus *Avipoxvirus*. Poxviruses are large double-stranded DNA viruses and the genomes range in size from 135kb to 360 kb which consists of up to 328 predicted Open Reading Frames (ORFs). Avipoxvirus undergoes intracytoplasmic replication after successful infection of host (Skinner *et al.*, 2005) and cause skin lesions, especially on the unfeathered areas surrounding the eyes, nares, beak, and on the shanks (Skinner and Laidlaw, 2009; Catroxo *et al.*, 2009). Biting insects being implicated as mechanical vectors of the virus may explain the distribution of the lesions to parts of the body not covered by feathers. The pathology of the lesions is mainly dermal cell hyperplasia. Avipoxvirus infections are generally diagnosed using serology, virus isolation characterized by pock formation on embryonated chicken chorioallantoic membranes (CAMs), histopathology showing the characteristic eosinophilic

intracytoplasmic inclusion bodies Bollinger bodies, electron microscopy (Jarmin *et al.*, 2006) and PCR.

Most molecular detections and characterizations of fowl poxviruses are usually based on ORF FWPV167 virion core protein P4b (4b) gene, a vaccinia virus A3L gene homolog (Boulanger *et al.*, 1998; Lecis *et al.*, 2017). However, herein, we employed PCR amplification of the immunodominant, 39,000-molecular weight core protein (39K protein) gene of fowlpox virus, a vaccinia A4L gene homolog (Singh *et al.*, 2003) and histopathology to confirm fowlpox outbreaks in chickens in Ibadan, Nigeria.

## MATERIALS AND METHODS

**Sample collection and DNA extraction:** Scabs from fifty chickens from backyard poultry in Ibadan, Nigeria tentatively diagnosed as fowlpox were collected into virus transport medium (1X Hank's balanced salt solution, 1% bovine serum albumin, 15 µg/ml amphotericin B, 100 unit/ml penicillin G and 50 µg/ml streptomycin, pH 7.4) and stored at -20°C until tested. Samples were homogenized in virus transport medium. Thereafter, the total DNA extraction was done by employing the DNeasy DNA extraction kit (Qiagen®, Valencia, CA) according to the instructions provided by the manufacturer.

**Polymerase chain reaction and sequence analysis:** Polymerase chain reaction amplification of the partial 39K core gene was carried out using these primer-pairs: 39K.1 :5'-CAGGAATTCGCTGAGAACTCCACA-3', 39K.2: 5'-TTCCAGCTCGAGTTAAGGAATAATAGC-3' (Singh *et al.*, 2003]. A 50 µl reaction volume constituted with 5 µl of the extracted DNA, 0.1 µM of each of the stated primers, 25 µl volume of PCR master-mix (DreamTaq buffer, 0.4 mM of each deoxyribonucleotide triphosphate and 4 mM MgCl<sub>2</sub>) and 18 µl nuclease-free water. Amplification was carried out under these conditions: 2 min of initial denaturation at 95°C; 35 cycles of 30 s of denaturation at 95°C; 30 s of annealing at 52°C; 1 min of extension at 72°C; and 5 min of final extension,

using the GeneAmp PCR system 9700. Agarose gel electrophoresis was carried out on the PCR amplicons using a 2% preparation of agarose powder in Tris Acetate EDTA buffer, SYBR Green used for DNA-staining, and viewed under ultraviolet light. DNA purification was carried out on 5 of the PCR amplicons using the GeneJET PCR Purification kit (ThermoSCIENTIFIC®, Pittsburgh, PA). Thereafter, nucleotide sequencing was carried out with ABI 3130XL sequencing machine. Nucleotide sequences were viewed and edited with Chromas 2.6.6 (Technelysium, South Brisbane, Australia) and deposited in the GenBank. These nucleotide sequences were compared with other avipoxvirus 39k gene sequences obtained from the GenBank database by means of a BLAST search (Altschul *et al.*, 1990) conducted on the National Center of Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignment of the Nigerian partial 39k gene sequences and sequences retrieved from the GenBank (Table 1) was carried out using the BioEdit 7.0.5.3 computer software package using Clustal W algorithm (Hall *et al.*, 1999; Thompson *et al.*, 1994). A maximum likelihood phylogenetic tree, with bootstrapping at 1000 replicates, was constructed using the MEGA 7.2.2 computer software package (Tamura *et al.*, 2011).

**Histopathology:** Fixation of scab samples (from infected eyelids) was achieved by immersion in 10% neutral buffered formalin solution for a 24 h period. The fixed samples were dehydrated by immersion in increasing concentrations of ethanol, ending in absolute (100%) ethanol, to effect dehydration. The ethanol was cleared from the dehydrated samples with xylene, and then infiltrated with molten paraffin. The samples were then embedded in paraffin, trimmed and sectioned to 4 µm thickness using Microm HM 360 microtome (Microm Laborgeräte GmbH, Walldorf, Germany). The sectioned samples were dewaxed, placed on glass slides, and stained by hematoxylin and eosin (H&E) method.

**Table 1.**

**Details of avipoxviruses strains retrieved from the GenBank.** Nine avipoxvirus strains 39k gene nucleotide sequences were obtained from the GenBank with fowlpox virus p4b gene sequence as the outgroup.

Virus	Strain	Host	Country	Accension number	Abbreviation
Fowlpox virus	HP-438	Chickens	Germany	AJ581527	FWPV DEU
Fowlpox virus	16069	Chickens	USA	AF198100	FWPV USA
Fowlpox virus	588/AD/2002	Chickens	India	DQ102368	FWPV IND
Fowlpox virus	FWVP-MN00	Merriam's Turkey	USA	MH709125	FWPV USA125
Penguinpox virus	PSan92	Penguin	South Africa	KJ859677	PEPV
Pigeonpox virus	FeP2	Pigeon	South Africa	NC_024447	PGPV
Cacarypox virus	ATCC VR111	Canary	U.S.A	NC_005309	CNPV
Flamingopox virus	FGPVKD09	Flamingo	South Africa	MF678796	FGPV
Shearwater virus	SWPV-2	Shearwater	Australia	KX857215	SWPV
Fowlpox virus	FWPV-MN00.1	Chicken	USA	MH709125	FWPV-P4b

**RESULTS**

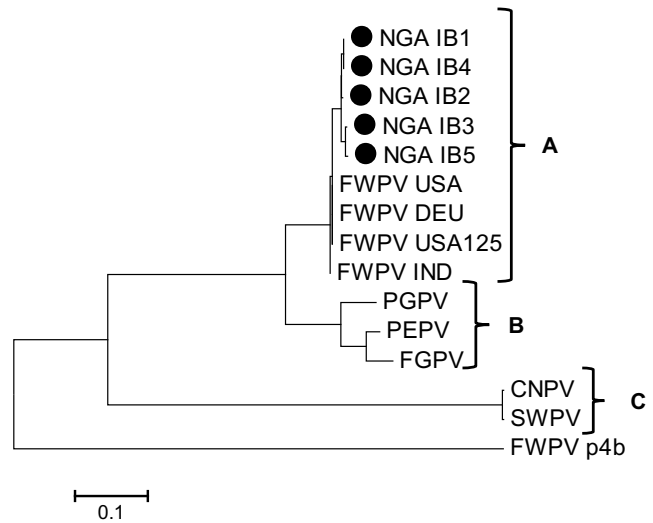
**Polymerase chain reaction and sequence analysis:** Scabs from fifty chickens from a backyard poultry in Ibadan were collected and total DNA was extracted. Partial sequences of 39k genes were amplified from the extracted DNA. Thirty-one out of the 50 samples (62%) were positive for the 39k gene. BLAST search revealed the sequences to be fowlpox virus. Five 39K genes sequences designated NGA-IB1, NGA-IB2, NGA-IB3, NGA-IB4 and NGA-IB5 have been deposited at the GenBank with accession numbers MN652556-MN652560. A maximum likelihood tree was constructed using nine partial 39k gene sequences from avipoxviruses reported world-wide including five Nigerian fowlpox viruses from this study. All the 14 viral sequences phylogenetically distributed into three clades A, B and C (Fig. 1). Phylogenetic analysis using maximum likelihood method also showed the Nigerian FWPV clustering very closely with fowlpox virus (FWPV) from USA, Germany and India. Penguinpox virus (PEPV), pigeonpox virus (PGPV) and flamingopox virus (FGPV) cluster separately as B while canarypox virus (CNPV) and shearwaterpox virus (SWPV) cluster as C.

**Histopathology:** Sections of the eyelid revealed marked acanthosis, cutaneous ulcerations and exaggeration of the epidermal projections (Fig. 2). Varying sized circumscribed lesions were evident. Overlying the surface epidermis in some sections was a thick layer of necrotic debris. The dermis was moderately infiltrated with heterophils and a few macrophages and lymphocytes (Fig. 3). The keratinocytes were markedly swollen, vacuolated and necrotic. Many of the keratinocytes had very large eosinophilic intracytoplasmic inclusions (Bollinger bodies) (Fig. 4).

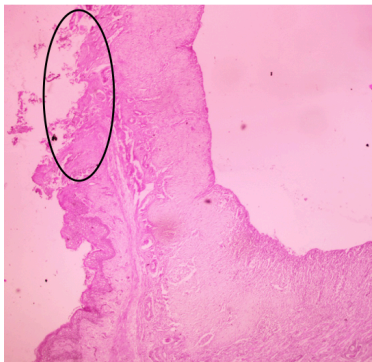
**DISCUSSION**

Fowlpox is routinely diagnosed based on clinical signs and gross pathological lesions of nodular lesions on the skin. This

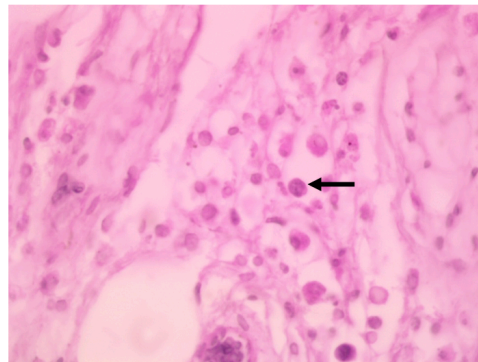
is usually confused with other avian pathologies such as infectious coryza, avian infectious laryngotracheitis, Mareks disease and vitamin A deficiency. In this present study, we employed PCR, sequence analysis and histopathology to confirm an outbreak of fowlpox in backyard chickens in Nigeria. Besides histopathology, molecular detection of the causative avipoxvirus using PCR and sequence analysis is imperative because multiple avipoxvirus can infect a single avian species (Maranolla *et al.*, 2010; Offerman *et al.*, 2013; Gyuranecz *et al.*, 2013 and Hess *et al.*, 2011).



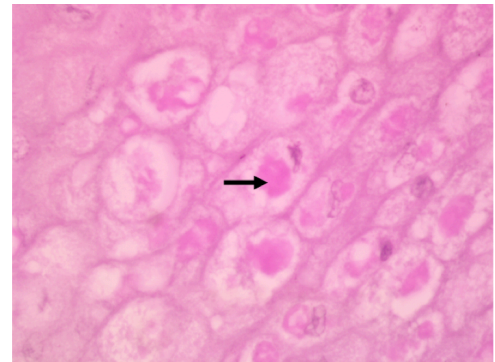
**Figure 1.** Genetic evolutionary analysis of fowlpox virus based on 39 kDa core protein gene nucleotide sequences. Phylogenetic relationship inferred by multiple alignments of 596-bp nucleotide sequence of 39 kDa core protein gene from nine avipoxvirus strains with the p4b gene of the fowlpox virus as the outgroup. The phylogenetic tree was constructed by maximum likelihood method with bootstrapping values at 1000 replicates. Labels A, B, and C designate the avipoxvirus clusters. The scale bar designates the nucleotide substitutions per site. See Table 1 for virus abbreviations.



**Plate 1.** Photomicrograph of avian eyelid showing necrosis and ulceration of skin epithelium (circle). H&E X160



**Plate 2.** Photomicrograph of eyelid submucosa showing moderate infiltration with heterophils and macrophages (arrowed). H&E X 1000.



**Plate 3.** Photomicrograph of a section of fowlpox infected eyelid. The keratinocytes show numerous degenerating, spongiotic and necrotic epithelial cells. Black arrow points to an eosinophilic intracytoplasmic inclusion (Bollinger body). H&E X1000.

Furthermore, avipoxviruses are diverse and as such, finding PCR primers that are specific to the whole genus or a particular species within the genus is challenging (Offerman *et al.*, 2013). As such, most molecular detection of fowlpox is usually based on PCR amplification of the 4b gene, however, we employed the amplification of the immunodominant 39K gene of FWPV and found it to be as useful as the 4b gene in classifying avipoxvirus into clades. This gene is a good target for PCR amplification and the amplicons generated are amenable for Sanger sequencing, as verified by this study, and also corroborated by other workers (Singh *et al.*, 2003 and Maranolla *et al.*, 2010). The 39k gene is clearly effective in diagnosing suspected fowlpox cases. However, because most molecular characterization of avipoxviruses is usually based on 4b gene, we observed that very few 39k gene sequences are available in the GenBank unlike the 4b gene. This is responsible for the short size of the phylogenetic tree in this study.

The histopathological lesions of swollen, vacuolated and necrotic keratinocytes and very large Bollinger bodies reported in this study have also been documented by other workers in other avipoxvirus infections (Boulanger *et al.*, 2002; Offerman *et al.*, 2013; Saitok *et al.*, 2019). Therefore, the PCR results, sequence analysis and observation of histological lesions characteristic of avipoxvirus infections led us to arrive at a precise diagnosis of fowlpox in the chickens. Our approach at arriving at the confirmatory diagnosis of fowlpox in chickens may also be applicable to other viral diseases.

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