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Research Article

Molecular Detection and Characterization of Fowl pox Virus in Cutaneous Pox in Turkeys

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ABSTRACT

Cutaneous pox is an insidious proliferative disease characterized by the lesions such as thick scabs, small nodules, wart-like masses on the skin of avian species. This disease can be confused with other diseases and conditions such avian papilloma, infraorbital sinusitis, infectious coryza and trauma. To detect the causative agent of the lesions, samples of suspected pox lesions were collected from sixteen turkey poults. Polymerase chain reaction (PCR) and sequencing analysis were employed to detect the causative agents responsible for the lesions. PCR was used to amplify FPV 167, FPV 140 and thymidine kinase genes of avipoxvirus. Eight (50%) out of the 18 samples tested positive using PCR. Sequence analysis based on multiple sequence alignment and phylogenetic tree reconstruction revealed the causative agent to be fowlpox virus. Also, amino acid substitutions P25S, S26P, G49E, V142I and V252M in FPV 167 gene, and T78K, I79I and A92G in thymidine kinase gene were revealed to clearly distinguish fowlpox virus from other avipoxviruses analyzed. This study demonstrated the importance of employment of molecular tools PCR and sequence analyses for precise identification and characterization of causative agents of infectious diseases.

Keywords: *Pox; avipoxvirus; fowlpox virus; polymerase chain reaction; sequence analysis*

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INTRODUCTION

Pox lesions in mammals and avian species range from thick scabs to nodules. Avian pox is caused by avipoxviruses. These are poxviruses that infect avian species and they include species such as fowlpox virus, turkeypox virus, canarypox virus, pegionpox virus, penguinpox virus ostrichpox virus, penguinpox virus, falconpox virus, sparrowpox virus etc (Buller et al., 2005). They belong to the family Poxviridae, subfamily Chordopoxvirinae and genus Avipoxvirus. Fowlpox is the most reported of all avipoxviruses and mainly responsible for pox in chickens (Sarker et al., 2021). Three types of pox have been reported namely cutaneous, diphtheritic and systemic (Atkinson et al., 2012). The cutaneous type usually results in low mortality rate and infected chickens most often recover unlike the diphtheritic type. In the diphtheritic type, lesions are usually found in the nasal passages, larynx or trachea that can lead to respiratory distress and death due asphyxiation. The systemic type occurs occasionally. Diagnostic techniques such as histopathology, virus isolation, electron microscopy and molecular techniques are applicable for detecting Avipoxvirus infections (Bolte et al., 1999). Molecular techniques provide an easy avenue to diagnose and characterize poxviruses. Furthermore, the relatedness of avipoxviruses obtained from a variety of avian species have been studied employing molecular detection and analysis of DNA sequences of the FPV 167 gene. The FPV 167 gene is the orthologue of vaccinia virus (VACV) A3L, canarypox virus (CNPV) 240 and turkeypox virus (TKPV) 122 gene that encodes P4b (Lee and Lee, 1997; Afonso et al., 2000; Manarolla, 2010). The P4b protein is a 75.2 kDa virion core protein which is highly conserved amongst all poxviruses (Binns et al., 1989). Based on phylogenetic analysis of the FPV167 clear majority of avipoxvirus isolates clustered into three major clades, namely clade A (fowlpox virus), clade B (canarypox virus) and clade C (psittacinepox virus) (Gyuranecz et al., 2013). Other genes that can be used to bolster the detection and characterization based on FPV167 gene include FPV140 and thymidine kinase gene (FPV 086) .

The former encoding a putative IMV envelope protein and it is equivalent to VACV strain Copenhagen H3L, CNPV186 and TKPV 104 (Jarmin et al., 2006; Carulei et al., 2009; Manarolla et al., 2010). The latter is a nonessential gene employed for foreign gene insertion and a convenient gene for selecting recombinant viruses and it is equivalent to VACV strain Copenhagen J2R, CNPV 113 and TKPV 060 (Banyai et al., 2015; Chervyakova et al., 2021). The amino acid sequences of the thymidine kinase genes have also been employed to characterize and assigned poxviruses into different evolutionary groups (Boyle et al., 1987; Amano et

al.,1995; Ueda et al.,1995). Poxviruses from various parts of the world have been characterized based on their genetic properties (Abdallah et al., 2013, Offerman et al., 2013; Mapaco et al., 2018; Arathy et al., 2010). However, fowlpox virus isolates in turkeys in Nigeria has not been characterized; as such in this study, we report molecular characterization of fowlpox virus from turkey poults in Nigeria employing PCR amplification and sequence analysis of FPV 167, FPV 140 and thymidine kinase genes.

MATERIALS AND METHODS

Sample collection and DNA extraction: Sixteen scab lesions from 16 turkey poults at the Teaching and Research Farm, University of Ibadan were harvested into 400 µl of virus transport medium. Thereafter, samples were homogenized with manual homogenizer. Total DNA was extracted from the homogenate using DNeasy Blood and Tissue kit (Qiagen®, Valencia, CA) according to the manufacturer's instructions. DNA was also extracted from a commonly used avipoxvirus vaccine.

Polymerase chain reaction and sequence analysis: Polymerase chain reaction (PCR) amplifications of FPV167, FPV140 and thymidine kinase genes were carried out using the following gene specific primers:
FPV167F:5'-AACGACCTATGCGTCTTCGT-3',
FPV167R:5'-CGTAGCCTATGGAATCCTGGT-3';
FPV140F:5'-TGGCGAGTCCTATGATAACAA-3',
FPV140R:5'-CCGGGTGACAAGAAACAAAT-3' and
FPVTKF5'-TCCGGTAAAACATCGGAG-3'
FPVTKR5'-AACGAAGCGTCGCAATAG-3'
which were designed with the Primer3 program (http://justbio.com). The reaction was carried out in 50 µl volume containing 5 µl of total DNA, of 0.2 µM of each primer, 25 µl of PCR master mix (10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl2, 50 units/ml of Taq DNA polymerase, 0.2 mM each dNTP, 5% glycerol, 0.08% IGEPAL®CA-630, 0.05% Tween®20, 0.024% Orange G, 0.0025% Xylene Cyanol FF) and 19 µl of nuclease free water. The GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) was used for amplification under the following conditions:94°C for 2 min for initial denaturation, 35 cycles of 95°C for 20 s, 50oC/53oC/52oC (FPV167/FPV140/thymidine kinase genes, respectively) for 30 s, 72°C for 1 min, and final extension at 72°C for 5 min. PCR amplicons were electrophoresed in a 2.0 % agarose gel, stained with SYBR, and visualized under UV light. The amplified DNA fragments were purified using GeneJET PCR Purification Kit (ThermoSCIENTIFIC®, Pittsburgh, PA).

Automated nucleotide sequencing was performed on an ABI 3130XL. Nucleotide sequences were viewed and edited with Chromas 2.6.6 (Technelysium, South Brisbane, Australia). Six partial FPV 167 gene sequences from six samples, three partial FPV140 gene sequences from three samples and a sequence from a fowlpox vaccine, and three partial thymidine kinase gene sequences from three samples and a sequence from a fowlpox vaccine from this study were compared with published avipoxvirus sequences deposited in the GenBank database using BLAST search (Altschul, 1990) via the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment of the partial FPV 167, FPV 140 and thymidine kinase gene sequences from this study and other avipoxviruses retrieved from the GenBank were carried out. Phylogenetic trees were constructed by the maximum likelihood method in MEGA version X (Tamura et al., 2011). Bootstrap re-sampling of the sequences was employed to test the robustness of the analysis. The bootstrap values were calculated based on 1000 replicates. Phylogenetic tree construction for FPV 167 involved six sequences from this study and 16 avipoxvirus retrieved from the GenBank. The sequences were the following: fowlpox virus (FWPV) (MN971579), FWPV (KF032407), FWPV (KF722859), turkeypox virus (TKPV) (NC028238), TKPV (KP728110), falconpox virus (FLPV) (AM050376), black-browed albatrosspox virus (ABPV) (AM050392), ostrichpox virus (OSPV) (AY530305), penguin (PEPV) (FJ948105), pigeonpox virus (PGPV) (DQ873811), macawpox virus (MCPV) (AM050382), parrot (PRPV) (AM050383), sparrow (SPPV) (AY530308), great tit (GTPV) (AY453173), black-billed magpie (BMPV) (KC018037) and canarypox (CNPV)(GQ487567). FPV 140 phylogenetic tree generation involved sequences from three samples and a vaccine from this study and 13 avipoxviruses sequences obtained from the GenBank. This included the following sequences: FWPV (MH734528), FWPV (MN708967), TKPV (KP728110), TKPV (NC_028238), flamingopox virus (FGPV) (MF678796), FLPV (AM050376), PEPV (KJ859677), CNPV (MG760432), CNPV (NC005309), CNPV (AY318871), FLPV (AM050376), ABPV (AM050392), PGPV (JX464827), PGPV (KJ801920). For the thymidine kinase gene phylogenetic construction, sequences from three samples and a fowlpox vaccine from this study and 11 avipoxvirus sequences obtained from the GenBank were used. The sequences were: FWPV (NC0021880), FWPV (AJ581527), FWPV (AF396867), FWPV (MH734528), FWPV (M16617), FGPV (MF678796), PEPV (KJ859677), TKPV (KP728110), TKPV (NC028238), CNPV (MG760432) and CNPV (NC005309). Monkeypox virus (MKPV) NTPase gene (KJ642619) was used as the outgroup for the three trees constructions. Multiple sequence alignments of the deduced amino acids of the FPV 167, FPV 140 and thymidine kinase gene sequences from this study and their corresponding Avipoxvirus gene sequences retrieved from the GenBank were also carried out.

RESULTS

PCR and sequence analyses: Fragments of FPV167, FPV 140 and thymidine kinase genes were amplified from the extracted DNA. Of the 16 scabs tested, 8 (50%) were positive for poxvirus. The PCR products were purified and six of them were sequenced for FPV167, and three and a FWPV vaccine for FPV140 and three and a FWPV vaccine for thymidine kinase genes. BLAST search showed the sequences to be fowlpox viruses. The sequences were designated as NGA_IbT1_FWPV, NGA_IbT2_FWPV, NGA_IbT3_FWPV, NGA_IbT4_FWPV, NGA_IbT5_FWPV and NGA_IbT6_FWPV, and the vaccine sequences as FWPV_Vacc. The sequences have been deposited at the GenBank.

Sequence analysis of FPV 167, FPV 140 and thymidine kinase genes: Multiple sequence alignments of the deduced amino acids of the FPV 167, FPV 140 and thymidine kinase sequences from this study and avipoxviruses sequences retrieved from the GenBank were carried out. Sequences from this study were identical to other FWPV sequences in the GenBank. However, they differ from other avipoxviruses such as TKPV, FLPV, PRPV, CNPV and SPPV (Fig. 1). FPV 167 multiple sequence alignment revealed positions 25-26 PS in FWPV is substituted by SP (substitutions P25S and S26P) in other avipoxviruses sequences analyzed. Other substitutions include G49E, V142I and V252M (Fig. 1, panel A) This substitution distinguishes FWPV from other avipoxviruses analyzed. FPV 140 multiple sequence alignment only revealed substitutions at positions 104-180 which can be used to differentiate TKPV from other avipoxviruses analyzed (Fig. 1., panel B). In the thymidine kinase multiple sequence alignment, the following substitutions were observed to distinguish FWPV from avipoxviruses analyzed: T78K, I79I and A92G (Fig. 1., panel C).

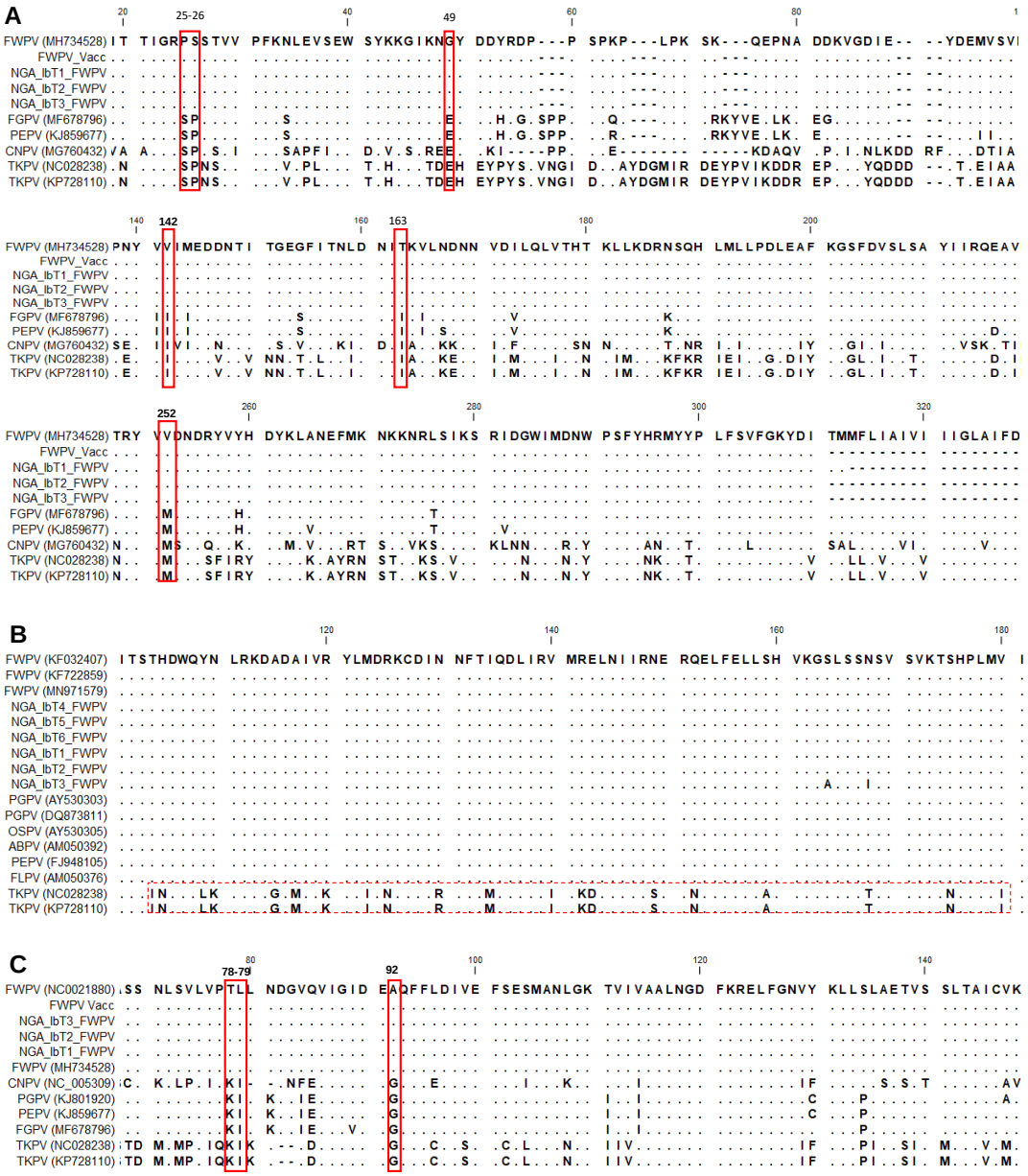

Figure 1.
Multiple sequence alignment of the deduced amino acid residues of partial FPV 167, FPV 140 and thymidine kinase genes from this study and other avipoxviruses sequences retrieved from the GenBank. A. Sequences here analyzed are represented by the sequence of samples NGA_IbT1_FWPV, NGA_IbT2_FWPV, NGA_IbT3_FWPV, NGA_IbT4_FWPV, NGA_IbT5_FWPV, NGA_IbT6_FWPV and other avipoxviruses. Amino acid residues at positions 25-26, 49, 142 and 163 distinguish fowlpox virus from other avipoxviruses analyzed. **B**. Sequences analyzed for the FPV 140 protein include NGA_IbT1_FWPV, NGA_IbT2_FWPV, NGA_IbT3_FWPV and other avipoxviruses obtained from the GenBank. The dotted box shows sequences that are unique to turkeypox virus in the FPV 140 protein. **C.** Amino acid residues analyzed are NGA_IbT1_FWPV, NGA_IbT2_FWPV, NGA_IbT3_FWPV and FWPV_Vacc. Amino acid residues in the boxes distinguish fowlpox virus from other avipoxviruses in the thymidine kinase protein

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Maximum likelihood trees were constructed using the FPV 167, FPV 140 and thymidine kinase sequences from this study and their corresponding avipoxvirus sequences previously retrieved from the GenBank. In FPV 167 phylogenetic tree, all the 31 avipoxvirus sequences were phylogenetically distributed into the three major clades of avipoxviruses fowlpox virus (clade A), canarypox virus (clade B), and psittacinepox virus (clade C). Phylogenetic analysis using maximum likelihood method also showed the FWPV sequences from this study clustering in the A1 subclade of fowlpox virus (clade A1) along with other FWPV sequences retrieved from the GenBank (Fig. 2).

In the FPV 140 phylogenetic tree, FWPV from this study also clustered with other FWPV sequences obtained from the GenBank but in the subclade A2 (Fig. 3).

Figure 2: Phylogenetic analysis of avipoxvirus based on FPV167 (P4b) gene nucleotide sequences. Phylogenetic tree was constructed via multiple alignments of 500-bp nucleotide sequence of P4b gene from 24 avipoxvirus strains. The tree was analyzed by maximum likelihood analysis with bootstrapping (1000). Avipopxvirus clades and subclades are labeled. (Dotted) avipoxviruses obtained from this study. Monkeypox virus NTPase gene served as the outgroup. Bar, 0.20 nucleotide substitutions per site. Fowlpox virus sequences from this study have black circles.

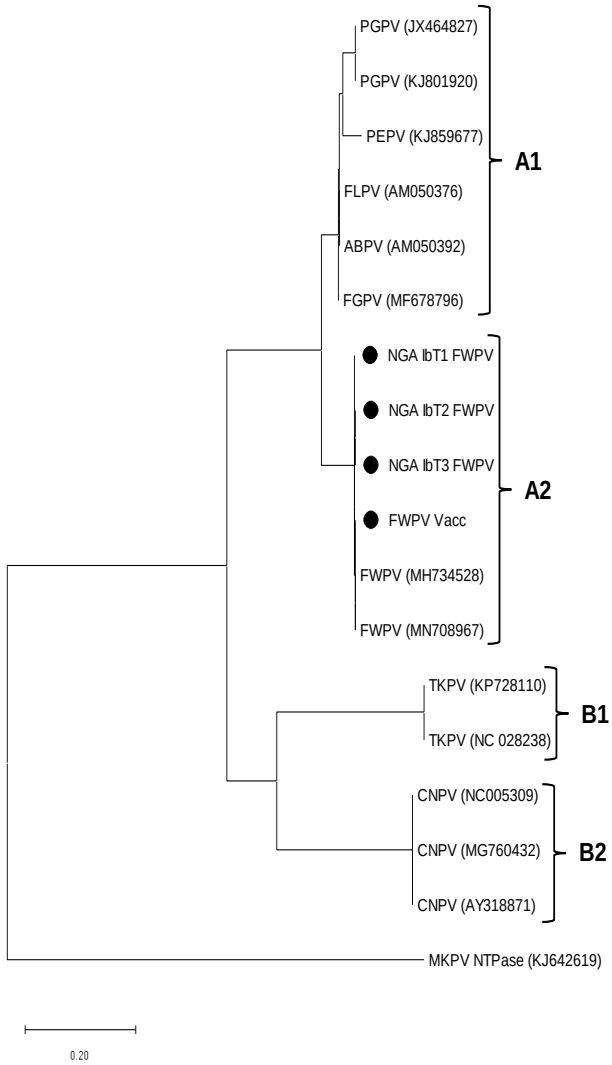

Figure 3:
Phylogenetic analysis of avipoxvirus based on fpv140 gene nucleotide sequences. Phylogenetic tree was constructed via multiple alignments of 900 bp nucleotide sequence of membrane protein gene from 18 avipoxvirus species. The tree was analyzed by maximum likelihood analysis with bootstrapping (1000). Avipopxvirus clades and subclades are labeled. (Dotted) avipoxviruses obtained from this study. Monkeypox virus NTPase gene served as the outgroup. Bar, 0.20 nucleotide substitutions per site. Fowlpox virus sequences from this study have black circles.

FWPV from this study also clustered with FWPV sequences retrieved from the GenBank in the subclade A1 in the thymidine kinase gene phylogenetic tree (Fig. 4).

DISCUSSION

Cutaneous pox is usually characterized by insidious proliferative epithelial lesions that are common to all avipoxviral infections. As such, it might be difficult to identify the causative avipoxvirus species by gross and histopathological examination of lesions. In this study, molecular diagnostic tools PCR and sequencing analysis were employed to incriminate FWPV instead of TKPV as the

causative agent of cutaneous pox in turkeys. Poxviruses are capable of cross-species transmission as Hess et al. (2011) have also reported an outbreak of cutaneous pox in turkeys caused by FWPV.

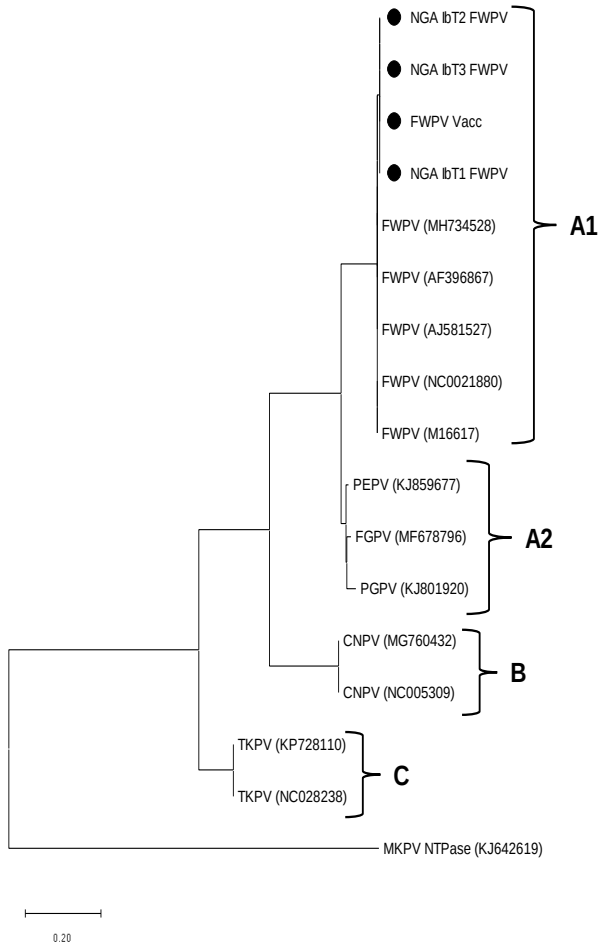

Figure 4:
Phylogenetic analysis of avipoxvirus based on thymidine kinase gene nucleotide sequences. Phylogenetic tree was constructed via multiple alignments of 450 bp nucleotide sequence of thymidine kinase gene from 15 avipoxvirus strains. The tree was analyzed by maximum likelihood analysis with bootstrapping (1000). Avipopxvirus clades and subclades are labeled. (Dotted) avipoxviruses obtained from this study. Monkeypox virus NTPase gene was used as the outgroup. Bar, 0.20 nucleotide substitutions per site. Fowlpox virus sequences from this study have black circles.

This is not surprising because most poxviruses possess a wide host range, for instance cowpox virus is capable of infecting horses, cats, dogs, rodents, primates etc., whereas others such as VACV is highly host-specific because it causes disease only in humans (Baker and Murcia, 2014). Furthermore, PCR and sequence analysis employed in this study also revealed key substitutions in FPV 167 and thymidine kinase genes and that can be used to differentiate FWPV from other avipoxviruses analyzed. Although, these genes have been described as being conserved in poxviruses (Jarmin et al., 2006; Carulei et al., 2009) but with detailed analyses, this study revealed amino acid susbstitutions P25S, S26P, G49E, V142I and V252M in FPV 167 gene and T78K, I79I and A92G in thymidine kinase gene that clearly distinguished other avipoxviruses TKPV, PGPV, PEPV and CNPV from FWPV. FPV 140 appears to be more conserved than FPV and thymidine kinase gene because of the absence of substitution distinguishing FWPV from other avipoxviruses analyzed. However, substitutions differentiating TKPV from other avipoxviruses analyzed were observed. Phylogenetic tree analyses for the three genes revealed clades and subclades which differentiates all the avipoxviruses analyzed. This study has highlighted a promising approach which can be harnessed to differentiate causative poxviruses from pox lesions. However, analysis of the complete genome of poxviruses from pox lesions will be a more excellent approach at distinguishing poxviruses.

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