Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 5, 799-806

Isolation and genetic diversity of fowlpox virus circulating in chicken flocks in Egypt

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ARTICLE INFO

Recieved: 02 January 2023

Accepted: 23 February 2024

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Keywords:

Electron microscopy
FPV
Isolation
PCR
Pox virus
Sequencing.

ABSTRACT

FowlPox virus (FPV) was detected in eight chickens suffering from a diphtheritic lesion on the oropharynx and trachea with nodular skin lesions around the unfeathered parts in two Egyptian governorates (El-Sharkia and Ismailia governorates) during summer 2023. A variety of serological and molecular methods were performed for identification and characterization of the virus. on specific-pathogen free (SPF) embryonated chicken eggs via chorio-allantoic membranes (CAM), the distinguishing focal pock lesions were detected on CAM. Concerning electron microscopy, FPV appeared as enveloped quadrangular brick shaped Avipoxvirions. The neutralizing antibodies level against FPV were detected in all eight samples. Serum neutralization test showed a neutralization index of ≥ 1.6 in all serum samples, meanwhile ELISA test displayed an S/P ratio of ≥ 1.5 in the affected chickens. Notably, two positive FPV samples were sequenced then submitted to the GenBank (Sharquia-1 and Ismilia-2 with accession numbers; OR920788-OR920789). The phylogenetic tree construction based on the fpv167- (P4b) gene of FPV revealed high nucleotide identity with Elsharqyia_FWPV1, Elsharqyia_FWPV2 and Fowlpox-AN5, FWPVN, FWPVD (Egyptian isolates) with nucleotide identity percentage 100%, 99%, 100%, 99%,99%; respectively. Likewise, FPV isolates were of low homology with VSVRI-Vac (vaccinal strain) with 88% similarity. In context, the local recent our strains can be applied in vaccine production for appropriate vaccination programs in Egypt.

Introduction

Avipoxviruses (APVs) are large, complex DNA viruses that belong to the *Chordopoxvirinae* subfamily in the of the family *Poxviridae* (Giotis and Skinner, 2019). APV has been documented in over 200 various bird species, involving domesticated and wild birds; resulting in significant economic losses (Hye *et al*., 2013). Moreover, APVs are transmitted either by direct contact of broken skin or indirectly through mosquitoes (Alley *et al*., 2010). Fowlpox virus (FPV) is a viral disease of poultry which was discovered in 1873 and is prevalent worldwide. The fowlpox infection reduces fowl productivity as it causes significant economic losses because of reduced growth rates, significantly drop in egg production, with elevated morbidity and mortalities as well as primarily in the diphtheritic form can reach to 50 - 100% (Molini *et al*., 2022). FPV infection is frequently slow to progress and manifests in cutaneous and diphtheritic form (Zhao *et al*., 2014). Cutaneous form (dry form) is very common as it is usually self-limiting; characterizing by wart-like nodules which appear around the eyes, combs, beak and non-feathered skin, accompanied by low mortality. Meanwhile, the diphtheritic form (wet pox) is characterized by proliferative lesions formation on the mucosal membranes of the mouth, digestive tract and respiratory tract of infected poultry farming especially young fowls, resulting in impaired breathing and feeding difficulties. Pathogenicity and clinical manifestations tend to differ among infected fowls with even the similar Avipoxvirus strain (Kabir *et al*., 2015; Masola *et al*., 2014). Vaccination is primary method of controlling fowlpox infection particularly in laying hens. Currently, in Egypt, new emerged fowlpox infection have reported in backyard-reared chicken then it spreads to commercial flocks since 2011 (Abdo *et al*., 2017; Lebdah *et al*., 2019).

FPV is large, double-stranded DNA (~300 kb genome) of two identical inverted terminal repetitions surrounding the central core coding area (Tripathy *et al*., 2000). Normally applied for comparative genetic identification, a core protein (75.2 kDa) which encoded by the avipox P4b (AP-P4b) protein gene. Additionally, among APVs, FPV167 gene of highly conserved region can give orthologs of P4b core protein of the vaccinia virus. Notably, there are various conventional methods applied for the detection of FPV infections as electron microscopy, histopathological examination, serologic methods and virus isolation. Lately, APVs diagnosis has been investigated by polymerase chain reaction (PCR) which is a rapid, efficient, and highly sensitive diagnostic method. To identify FPV infection, 578-bp fragment unique to FPV167 amplification by PCR was used for confirmatory diagnosis through specific primers (Ruiz-Martínez *et al*., 2016; Lecis *et al*., 2018).

To date, phylogenetic analysis has displayed three major clades of APVs, namely clade A (FPV-like), clade B (Canarypox virus) and clade C (Psittacinepox virus); then these clades have further been clustered into multiple subclades (Bányai *et al*., 2015; Joshi *et al*., 2019). Also, the sequencing of FPV167 (P4b) nucleotide sequence illustrated a divergence among most viruses, which might be reliably related to the host species (Gyuranecz *et al*., 2013). Many cases of infected fowls showed formation of proliferative lesions on the mucosal membranes of the mouth, digestive tract and respiratory tract of infected poultry. Considering these facts, the current study was undertaken to determine and isolate the causative agent via electron microscopy as well as various serological methods in a variety of Egyptian governorates. Furthermore, molecularly identify the P4b amplicon of FPV in 2023 using sequencing and phylogenetic analysis in order to develop and evaluate the local strain-based vaccination programs.

ISSN: 2090-6277/2090-6269/

Materials and methods

Ethical approval

The study protocol and all animal experiments were carried out following regulations and guidelines for animal experimentation as approved by the Institutional Animal Care and Use Committee of animal health research institute, Agricultural Research Center under number, Egypt (ARC-AHRI-67-23).

Sample collection and preparation

During 2023, the affected poultry flocks (vaccinated and non-vaccinated, their age about 3-4 weeks in El-Sharkia and Ismailia governorates) which had a diphtheritic lesion on the oropharynx and trachea with nodular skin lesions around the eyes, mouth, and comb were sampled aseptically (Total number =50). From each flock, five birds were slaughtered then necropsied to investigate the pooled nodular lesions from available clinical cases. The flocks displayed lesions at ages of 3 and 4 weeks, with a 3 - 5% mortality rate and a 7 -10% morbidity rate. The lesions were minced using sterile blades from infected fowls then placed in 0.5 ml of sterile virus transport medium (VTM), which consisted of 1:1 volume of phosphate-buffered saline (PBS) and glycerol with streptomycin (1 mg/mL), penicillin (100 IU/mL) and antifungal additions for 1 hour at 37°C. Thereafter, the nodular lesions suspension was finely homogenized using sterile sand inside a sterile mortar and pestle. The tissue homogenates were then centrifuged at 12,000 x g for 15 minutes (min.) (OIE, 2018), this suspension was used for virus isolation and molecular characterization. Blood samples were collected from the affected fowls then the serum samples were preserved at -20oC till used in SNT and ELISA.

Virus isolation and propagation in Specific Pathogen Free-embryonated chicken eggs (SPF-ECE)

After centrifugation, 0.2 mL of the tissue homogenates supernatant was inoculated onto SPF-ECE via chorio-allantoic membrane (CAM) route of 10–12- day-old (kindly supplied from Quem Oshem production Farm, El-Fayoum, Egypt). The inoculated eggs were further incubated at 37◦C for 5–7 days with candling examination daily for presence of focal white pock lesions or characterized generalized thickening areas of the CAM (Gilhare *et al*., 2015; OIE, 2018).

Electron Microscopy

Electron microscopy was used for FPV detection through the electron microscopy unit at Assiut University, Egypt. The positive staining method of ultra-thin sections of CAM of SPF-ECE, inoculated with suspected nodules homogenates was achieved using the method described by Bozzola and Russell (1991).

Serum Neutralization Test

A reference FPV strain was kindly supplied by the Veterinary Quality Control Unit, Animal Health Research Institute (AHRI), Dokki, Egypt. Equivalent volume of ten-fold serial dilutions of reference FPV strain were added to the tested serum samples (n. = 8). Then, this mixture was incubated at 37◦C for 1 hour. Thereafter, the mixture (0.1 mL) was inoculated onto the SPF-ECE via CAM route of 10–12- day-old. The serum collected from healthy fowls considered as a negative control meanwhile, collected serum of inoculated rabbits with commercial FPV vaccine (Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo), was acted as a positive control. All inoculated eggs were further incubated at 37◦C for 7 days with candling daily. Furthermore, CAM was harvested then examined for presence of pock lesion, seven days' post inoculation (PI) (OIE, 2018; Morita, 1973). The virus titer (VT) and the serum virus titer (SVT) were estimated using the statistical method mentioned by Reed and Muench (1938). The neutralization index (NI) was subsequently estimated as this equation: NI = VT-SVT according to pilchard *et al*. (1962).

Indirect Enzyme Linked Immuno-Sorbent Assay (ELISA)

Indirect ELISA was done according to Williams and Scalarone (1987). Concisely, Nunc™MaxiSorp™ flat-bottom plates (96 well) were coated with FPV antigen (50 Ml), diluted in carbonate-bicarbonate buffer and incubated overnight at 4◦C. After there, the wash solution (0.29 M NaCl, 0.05 % Tween 20, 3x) was used to wash the coated wells. Blockage of nonspecific binding sites with 3% bovine serum albumin (BSA) in PBS for 1 hour at 37◦C were performed. Adding of test sera of diseased fowls (n = 8) in 45 µL of the dilution buffer to each well then, the plates were incubated at 37◦C for 2 hours. Following 3 times washing, the plates were incubated with a rabbit anti-chicken immunoglobulin (Ig) G conjugated with horse radish peroxidase (HRP) (Sigma, USA) in PBS containing 1% BSA for 1 hour at 37◦C. After 3 times washing of the plates, the attached antibodies were investigated with a TMB-peroxidase kit (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, Maryland, USA) following the manufacturer's instructions. The reaction ended using 2.5 M sulphuric acid. Finally, the absorbance at 450 nm was detected using BioTek ELX-800 ELISA plate reader (BioTek, USA). The sample to positive ratio (S/P) ratio was estimated as follows: S/P ratio = (OD (optical density) of sample − OD of negative control) / (OD of positive control − OD of negative control).

Nucleic acid extraction and conventional PCR Amplification

DNA extraction from tissue samples (n = 6) was conducted using the QIAamp DNA mini kit (Qiagen Digital Insights, Germany, GmbH) according to manufacturer's instructions. Briefly, the amplification targeting P4b gene of 578 base pair (bp) (FPV167 gene) was achieved using EmeraldAmp MAX PCR Master Mix (Takara Bio Europe, Saint-Germain-en-Laye, France) with APV-specific primers set (p2FPF—5′CAGCAGGTGCTAAACAACAA3′ and p2FPR—5′ CGGTAGCTTAACGCCGAATA 3′) using ProFlex thermal cycler- PCR machine (Applied Biosystems, Foster City, CA, USA) at the thermal cycling conditions according to method described by Lee and Lee (1997). Agarose gel electrophoresis on 1.5 % agarose gel was applied to determine gene specific amplicons with a GeneRuler™ 1 kilo base DNA ladder (Thermo-Fisher scientific, USA) and 4 selected positive PCR products were chosen for sequence analysis.

Sequencing of P4b gene fragment and phylogenetic analysis

The PCR amplicons purification of P4b gene at 578 bp size among FPV selected isolates from tissue samples was performed by a QIAquick gel purification kit (Qiagen, Germany) according to the manufacturer's instructions. Subsequently, the purified amplicon sequencing was conducted by Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The homology among sequences was analyzed through alignment and compared with other references and vaccinal sequences deposited in GenBank using nucleotide Basic Local Alignment Search Tool (BLASTn) (Table 3). Multiple alignment and phylogenetic analysis were done using Bioedit and MEGA version 7 programs using a bootstrap of 1,000 replicates of the Clustal W alignment tool algorithm (Tamura *et al*., 2013) and the maximum likelihood tree method.

Results

Clinical and gross lesions

The diseased fowls of age 3-4 weeks in El-Sharkia and Ismailia gov-

ernorates have been exhibited diphtheritic lesions as raised yellowish patchy, necrotic, diphtheritic membrane covering the mucous membranes of oropharynx, trachea, larynx, syrinx, laryngeal papillae and at the glottis groove. Likewise, the nodular skin lesions displayed as variable in size around the eye region and non-feather areas (Fig. 1). The mentioned mortality rates were up to 5% besides 7-10% morbidity rate. The sick chickens were depressed, dull, with weight loss and declined egg production.

Virus isolation

All FPV samples displayed visible pock lesions in the inoculated CAM of SPF-ECE. Notably, typical focal whitish pock lesions with a noticeable thickening of CAM and fine grayish discoloration lesions were observed (Fig. 2). These pock lesions numbers and size have been increased by successive FPV serial passage.

Electron microscopic examination

The ultra-thin sections of examined CAM cells appeared as abundant oval and quadrangular brick-shaped FowlPox virus (Fig. 3a, b). Furthermore, mature FPV particles displayed as an electron-dense biconcave nucleocapsid core with two bodies laterally surrounded by the intermediate coat as well as an outer lipoprotein coat (red arrows in figure 3b). Meanwhile, immature virus particles were distinguished in the infected cells cytoplasm (white arrows, Fig. 3a).

Serum neutralization test

Neutralization test results were expressed as NI in which NI ≥ 1.0 was considered as positive results. The neutralizing antibodies level in positive serum samples were above 1.5 NI (Table 1). Additionally, NI values of examined healthy fowls varied between 0.5– 0.8 with an average of 0.65 ± 0.120 standard deviation (SD). Whereas NI of positive control serum accumulated from New Zealand rabbit inoculated with FPV vaccine strain was 2.6.

Table 1. Neutralization index of sera collected from diseased fowls and other healthy ones.

Sample number	NI of sera of infected chickens	NI of sera of healthy chickens
1	1.8	0.8
2	1.9	0.5
3	1.7	0.7
4	1.9	0.6
5	1.7	0.5
6	1.6	0.8
7	1.8	0.6
8	1.6	0.7
		Mean = 0.65 SD = 0.120

ELISA test

Serum samples showing S/P ratio ≥ 0.76 means a positive sample (Table 2). It was observed that all FPV positive serum samples produced S/P ratios ≥ 1.5, meanwhile S/P ratios of healthy chickens were ≤ 0.5.

Molecular identification

Amplification of P4b of positive FPV in six samples revealed an amplicon of 578 bp in size from both governorates in Egypt.

Table 2. ELISA values for serum samples of infected and healthy chickens.

Sample number	S/P* ratio of sera collected from diseased pigeons	S/P ratio of healthy pigeons
1	1.5	0.5
2	1.6	0.3
3	1.6	0.5
4	1.7	0.3
5	1.5	0.4
6	1.7	0.3
7	1.5	0.5
8	1.6	0.4
		Mean = 0.40 SD = 0.093

*S/P ratio: Sample to positive ratio; ** SD: times standard deviation.

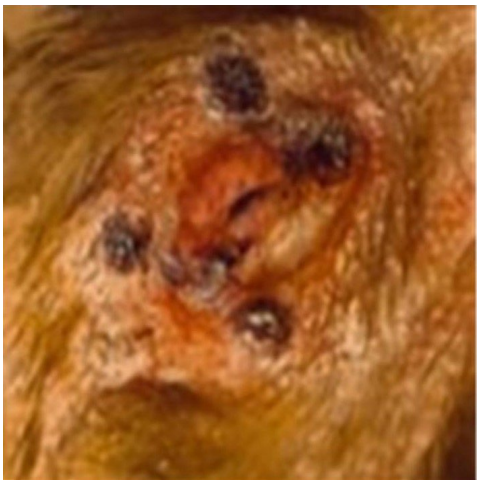

Fig. 1. Wart-like nodular skin lesions on head, peak, comb, and non-feathered areas of pox virus infected fowls, typical for FPV infection.

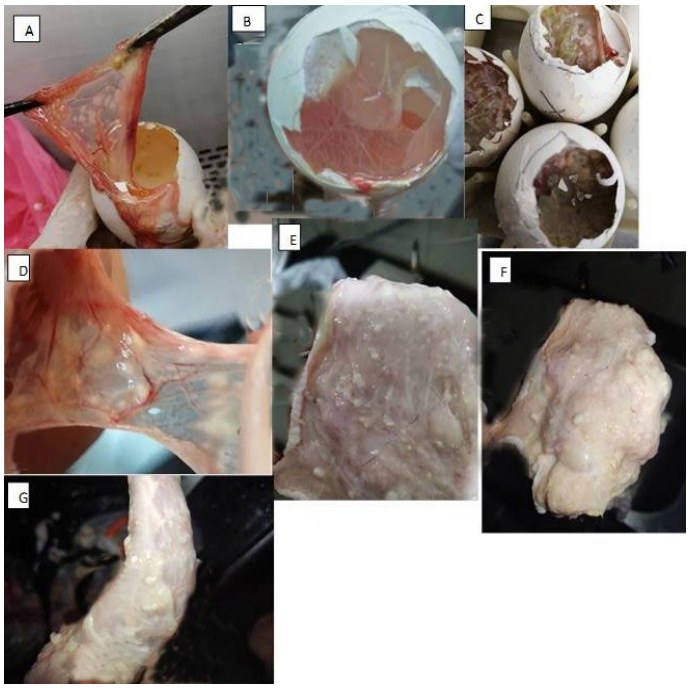

Fig. 2. (A-D): Typical pock lesions appeared as opaque, edematous, whitish, thickened CAM, and variable size nodular shaped pock lesions induced by FPV isolates on CAM of SPF-ECE. (E-F): Trachea mucosa and (G) mucous membranes of oropharynx of chicken have been showed a diphtheritic form of FPV.

Sequencing analysis of fpv167 (P4b) Gene

Sequencing of fpv167-578-bp was revealed two isolates which were named Sharquia-1 and Ismilia-2 with accession numbers OR920788 and

OR920789, respectively. Phylogenetic analysis was performed using sequences from 46 FPV strains of 16 different bird species deposited in Genbank with their accession number (Table 3). Notably, there were three domain clades in the phylogenetic tree of APVs: clade A (Fowlpox like viruses), which are classified into three subclades A1, A2, and A3, clade B (Canarypox-like viruses), and clade C (Psittacine pox-like viruses). Our study clarified that all examined isolates were clustered in subclade A1 (Figure 4). The phylogenetic trees construction based on the fpv167 gene sequences analysis showed that all the obtained Egyptian FPV isolates (Sharquia-1 and Ismilia-2) (Figure 4) are genetically related to Elsharqyia_FWPV1, Elsharqyia_FWPV2 and Fowlpox-AN5, which all are Egyptian isolates, with nucleotide identity percentage 100% and on the amino acid level were with 100%, 99%, 100%; respectively (Table 4). Moreover, our isolates are also genetically related to FWPVN, FWPVD, Fowlpox-AN4 (Egyptian isolates) with nucleotide identity percentage 99%, and on the amino acid level was with 99%, 99%, 97%; respectively. Meanwhile, FPV

Table 3. Various reference strains of Avipox viruses sequences used in phylogenetic analysis.

Strain name	Abbreviation	Host	Accession number
Sharquia-1-FPV	Sharquia-1-FPV	Chicken	OR920788
Ismilia-2-FPV	Ismilia-2-FPV	Chicken	OR920789
Fowlpox_Mild (Websters; Fort Dodge) _	FWPVM	Chicken	AM050378
Nobilis Variole W	FWPVN	Chicken	AM050379
Fowlpoxvirus_Diftosec CT (Merial)	FWPVD	Chicken	AM050380
Pigeonpox_ Elsharqyia	PGPV	Pigeon	JQ665840
Pigeonpox _PPVNV1	PPVNV1	Pigeon	MW602950
Pigeonpox _PGPV1	PGPV1	Pigeon	MH365477
Pigeonpox_FZRP9C	FZRP9C	Pigeon	MF102270
Pigeonpox_ PiPVIR18	PiPVIR18	Pigeon	MG787227
Pigeonpox_PPLH	PPLH	Pigeon	MN892361
950 24/3/77	PGPV950	Pigeon	AM050386
B7	FPVB7	Chicken	AY453177
India_2006	PGPVIndia_2006	Pigeon	DQ873811
India_08_2009	PGPVIndia_08_2009	Pigeon	HM481409
San92	PEPV	Penguin	FJ948105
GB 724/01-20	OSPV	Ostrich	AY530305
Fort Dodge	CNPV	Canary	AM050384
1445/97/33	CNPV1445	Canary	AM050375
Canarypox virus_712	CNPV712	Canary	KX863707
1381/96	FLPV1381	Falcon	AM050376
GB362-02	FLPV36202	Falcon	AY530306
APIII	AGPV	Agapornis	AY530311
353/87	ABPV	Black-browed albatross	AM050392
GTPV-256	GTPV-256	Great tit	AY453174
GTPV-A310	GTPVA310	Great tit	AY453173
9037 31/5/66/23	SRPV23	Sparrow	AM050390
1305/86	MCPV	Macaw	AM050382
27	SLPV	Starling	AM050391
364/89	PRPVCVL364_89	Parrot	AM050383
2009/India/06	SRPV06	Sparrow	HM481407
GB 320/02	SRPV32002	Sparrow	AY530308
10/12/1998	TKPV98	Turkey	AM050388
2/11/1966	TKPV66	Turkey	AM050387
2008/India/05 FWPV2008_	FWPV2008_India_05 Silver	pheasant	HM481406
TP-2	PGPVTP-2	Pigeon	AY530303
Sharkia2017/VSVRI	Sharkia2017/VSVRI	Chicken	MN542415
PM/Sharkia2017/ VSVRI	PM/Sharkia2017/ VSVRI	Chicken	MH035836
Vaccine VSVRI vaccine	VSVRI-Vac	Chicken	MN708968
FWPVH/Egypt/2018	FWPVH/Egypt/2018	Chicken	MW147745
Elsharqyia_FWPV2	Elsharqyia_FWPV2	Chicken	JX464819
Elsharqyia_FWPV1	Elsharqyia_FWPV1	Chicken	JQ665838
Fowlpox-AN1	Fowlpox-AN1	Chicken	OP429101
Fowlpox-AN4	Fowlpox-AN4	Chicken	OP429104
Fowlpox-AN5	Fowlpox-AN5	Chicken	OP429105
Fowlpox	CD-029-A2016	Turkey	MK435242

Table 4. Nucleotide and amino acid identities of the fpv167 (P4b) gene sequence of our FPV isolates compared to other references and vaccinal strains.

Sequence ID	FWPVM-AM050378	FWPVN-AM050379	FWPVD-AM050380	FWPVH/Egypt/2018-MW147745	Elsharqyia_FW-PV2-JX464819	Elsharqyia_FW-PV1-JQ665838	Fowlpox-AN1-OP429101	Fowlpox-AN4-OP429104	Fowlpox-AN5-OP429105	Sharkia2017/VSVRI-MN542415	PM/Sharkia2017/VSVRI-MH035836	VSVRI-Vac-MN708968	FPVB7-AY453177	Sharquia-1-FPV	Ismilia-2-FPV	
Nucleotide identity %																
1-FWPVM-AM050378	ID	99%	98%	97%	98%	98%	87%	87%	87%	93%	95%	93%	68%	79%	78%	1
2-FWPVN-AM050379	99%	ID	99%	96%	97%	97%	86%	86%	86%	94%	94%	94%	67%	79%	79%	2
3.FWPVD-AM050380	98%	99%	ID	96%	96%	96%	85%	85%	85%	95%	94%	95%	67%	80%	79%	3
4.FWPVH/Egypt/2018-MW147745	97%	96%	95%	ID	100%	100%	84%	84%	84%	90%	98%	90%	66%	76%	75%	4
5.Elsharqyia_FWPV2-JX464819	98%	96%	96%	99%	ID	100%	85%	85%	85%	91%	97%	91%	66%	77%	75%	5
6.Elsharqyia_FWPV1-JQ665838	98%	96%	96%	99%	100%	ID	85%	85%	85%	91%	97%	91%	66%	77%	75%	6
7.Fowlpox-AN1-OP429101	85%	84%	84%	83%	84%	84%	ID	99%	99%	85%	82%	85%	77%	69%	68%	7
8.Fowlpox-AN4-OP429104	85%	84%	84%	83%	84%	84%	97%	ID	100%	85%	82%	85%	77%	70%	68%	8
9.Fowlpox-AN5-OP429105	85%	84%	84%	83%	84%	84%	97%	100%	ID	85%	82%	85%	77%	70%	68%	9
10.Sharkia2017/VSVRI-MN542415	92%	93%	94%	90%	90%	90%	84%	84%	84%	ID	88%	100%	67%	81%	80%	10
11.PM/Sharkia2017/ VSVRI-MH035836	95%	94%	93%	98%	98%	98%	81%	81%	81%	88%	ID	88%	64%	74%	73%	11
12.VSVRI-Vac-MN708968	92%	93%	94%	90%	90%	90%	84%	84%	84%	100%	88%	ID	67%	81%	80%	12
13.FPVB7-AY453177	74%	73%	72%	71%	72%	72%	83%	83%	83%	72%	70%	72%	ID	54%	53%	13
14.Sharquia-1-FPV	73%	73%	74%	71%	71%	71%	64%	64%	64%	76%	69%	76%	55%	ID	92%	14
15.Ismilia-2-FPV	71%	71%	72%	68%	68%	68%	61%	61%	61%	73%	66%	73%	53%	87%	ID	15
Amino acid identity %																

isolates were distinctly apparat from FPVB7 was sharing 67% nucleotide similarity (Table 4). Likewise, FPV isolates were of low homology with VSVRI-Vac (vaccinal strain) with 88% similarity. Amino acid sequencing analysis of fpv167(P4b) gene of our two isolates revealed 87% similarity to each other, meanwhile 92% similarity based on nucleotide identity level. In particular, the mutation analysis fpv167-gene in this study displayed that the Sharquia-1 and Ismilia-2 acquired various mutations in comparable to FWPVD reference strain (Figure 5).

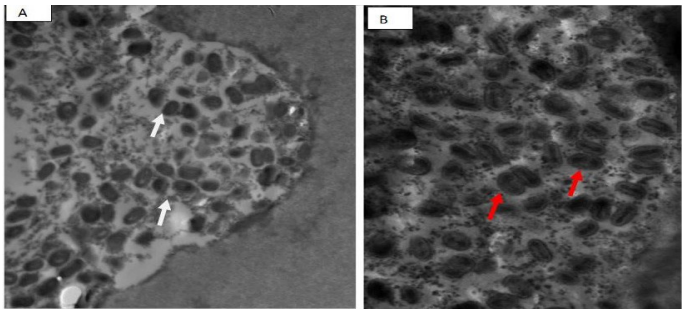

Fig. 3. Electron micrograph of ultra-thin section of FPV infected CAM, showing (A) abundant oval, brick-shaped FPV particles as immature virus particles in cells are resembled with (white arrow) meanwhile, (B) Mature virus particles which are brick-shaped (red arrow), with an electron-dense biconcave nucleocapsid core, an intermediate coat enclosing two bodies laterally and an outer lipoprotein coat.

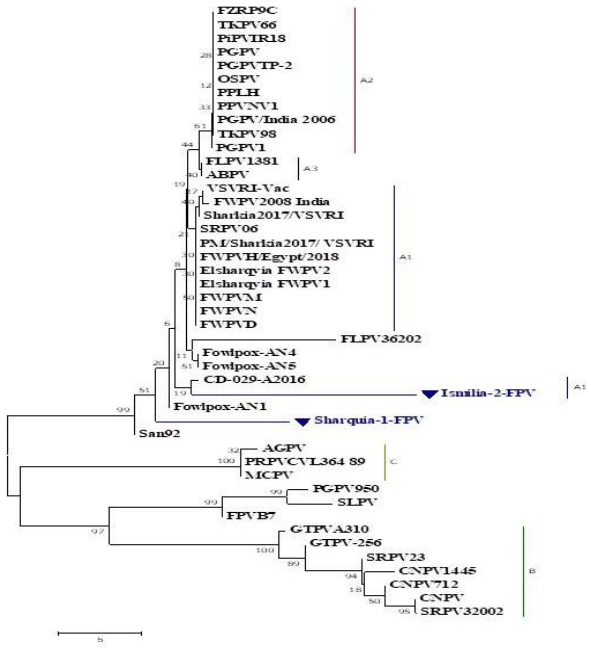

Fig. 4. Phylogenetic tree based on fpv167 (P4b) gene sequences alignment of FPV with other reference sequences. The phylogenetic analysis of FPV-P4b gene reveling that our FPV isolates clustered in subclade A1 (Sharquia-1 and Ismilia-2 with accession numbers OR920788 and OR920789) with other Egyptian strains cluster in the same subclade. The FPV two isolates in our study are indicated by a blue triangle. The tree was designed by the neighbor-joining method with 1,000 bootstrap replicates, using MEGA 7.0 software.

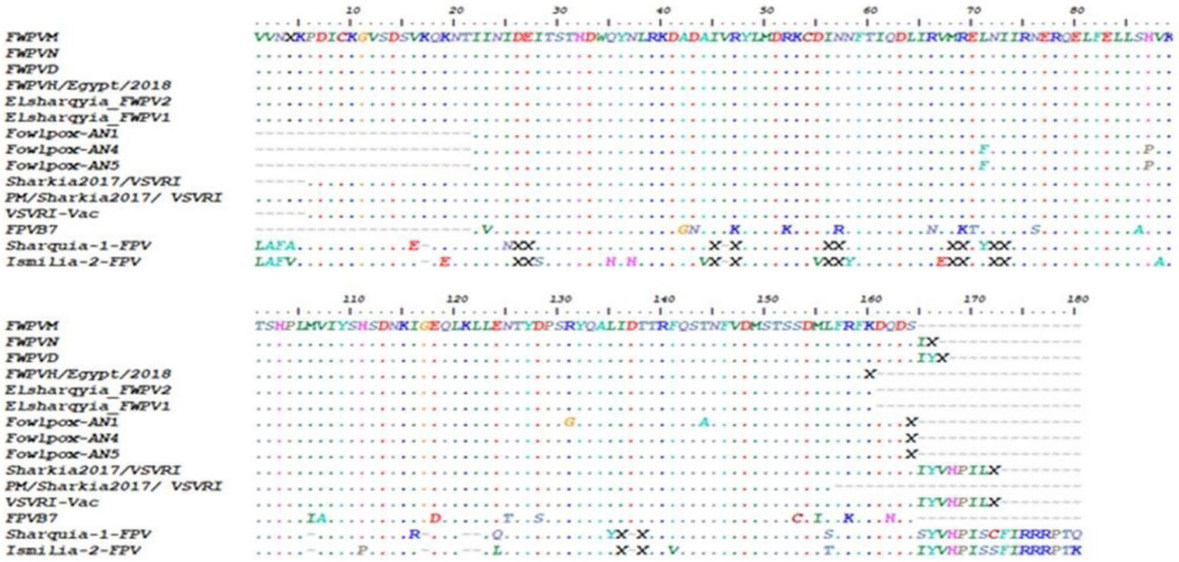

Fig. 5. The Amino acid alignment analysis of fpv167 (P4b) fragment of FPV with comparisons for the two FPV isolates and other reference strains. Sharquia-1 and Ismilia-2 acquired various mutations compared with FWPVD reference strain. The letters indicate amino acid substitutions. The dots (.) indicate identical amino acids, dashes (−) indicate gaps produced in the alignment using MEGAX and BioEdit software packages.

Discussion

The Genus Avipoxvirus is a contagious slow spreading viral disease commonly noticed in chicken flocks and other avian species and commercial poultries as pigeons, turkey, canary, quail, falcon are susceptible. Notably, the probability of FPV is extremely high in case of proliferative skin in non-feathered skin parts (dry pox) with oral and tracheal lesions diphtheritic lesions (Tripathy and Reed, 2003). Despite advances in FPV vaccination programs, the backyard and commercial poultry industries are still exposed to the infection (Abdallah and Hassanin, 2013). Thus, we should follow up the FPV evolution in Egypt and their compatibility with different available commercially used vaccine strains using various laboratory technique.

Virus isolation on CAM of SPF-ECE, electron microscopy, serological histopathological methods are conventional diagnostic methods of Avipoxvirus infection with time consuming and less sensitivity. Moreover, further molecular investigation via PCR and sequencing approaches was used to identify and characterize the fowlpox virus (OIE, 2018; Durairajan *et al*., 2022). In this study, high poultry production bird flocks at two governorates (El-Sharkia and Ismailia) showed a diphtheritic lesion with nodular skin lesions with a 3-5% recorded mortality rate and a 10-15% morbidity rate. These findings were consistent with a previous study by Yehia *et al.* (2023) and Akanbi *et al.* (2022). Interestingly, FPV infection susceptibility differ according to host species and age as an elevated FPV infection incidence were observed because of arthropods including mosquitoes were abundant in poultry flocks during summer period (Lawson *et al*., 2012; Samuel *et al*., 2018).

According to our findings, characteristic CPE of FPV was observed after 72 hours on SPF-ECE inoculation via CAM route as yellow nodular shaped focal pock lesions. These results come in line with the CPE findings of Yehia *et al*. (2023) and Gilhare *et al.* (2015). In context, (OIE, 2018) mentioned that the CPE of FPV as focal opaque pock lesions with a CAM general thickening. In addition, the CAM infected with FPV isolated from Egypt displayed similar results with a notable variable thickness degree

ranged from mild to severe (Lebdah *et al*., 2019). Concerning electron microscopy, ultra-thin sections of CAM positive tissue samples displayed an enveloped brick shaped virion. Furthermore, the immature virus particles were oval shaped without the dumbbell-shape lateral bodies distinguishing for matured virus particles. These results are similar with the reported results of Deern *et al*. (1997); Tripathy and Reed (2003). Neutralization test was achieved using a standard FPV antigen to determine neutralizing antibodies against FPV in infected fowls' sera. The pock reduction of diseased fowls' sera developed a NI ≥ 1.6. Whereas healthy fowls' sera revealed a NI < 1. These findings come in accordance with previous reports for FPV (Sumaya, 2005; Alehegen *et al*., 2014). To confirm the concept that FPV is the disease etiology in affected chickens, a serological examination of anti-FPV antibodies in sera has been done via indirect ELISA assay. S/P ratio of positive serum samples was ≥ 0.76. Despite all positive samples revealed an S/P ratio ≥ 1.5, which was meant a strong positive for FPV, meanwhile S/P ratios of healthy chickens were ≤ 0.5, demonstrating that a greatly specialized diagnostic threshold was applied for ELISA results evaluation (Wright *et al*., 1993). These subsequent results agree with Iritani and Sawaguchi (1994) and Aboul Soud *et al*. (2020). However, the ELISA test can detect whole FPV-reactive antibodies in diseased fowls, involving those antibodies which are unable of neutralization, all positive samples in ELISA were evidenced positive in SNT, establishing a high relationship between ELISA and SNT test.

In spite of evidence of virus growth in SPF-ECE, PCR being the most appropriate and accurate method to detect FPV supplying epidemiological data of various isolates prevalent in diseased flocks. Analysis of PCR assay of P4b of positive FPV six samples with specific primers revealed an amplicon of 578 bp in size. These molecular findings are similar with Rahman *et al*. (2019); Akanbi *et al*. (2022) and Yehia *et al*. (2023). Globally, phylogenetic analysis and molecular differentiation between APVs is dependent upon three genomic conserved loci. Firstly, the fpv167 (P4b) of 578 bp size which exist in all poxviruses. Secondly, the orthologous of vaccinia virus H3L (fpv140) locus comprises 1800 bp size. Thirdly, locus of DNA polymerase gene (fpv94) of 1058 bp. In both diagnostics and phylogenetic analysis of FPV, it contains a large genome size ranged from 288–300 kilo bp with 260 open reading frames (ORF) and a highly conserved P4b gene which act as a specific pan-gene marker Gyuranecz *et al*., 2013; Offerman *et al*. 2013). Regarding sequencing and phylogenetic analysis of P4b gene (fpv167), APV classified into 3 main clades: clade A, clade B, and clade C. In particular, subclades A1 (fowlpox virus), A2 (Turkey pox virus), A3, and A4 further cluster clade A, while subclades B1 (Canarypox virus) and B2 (Starling pox virus) were discriminated Manarolla *et al*. (2010). Interestingly, all the selected isolates in our study categorized into A1, sub clade A1 (FWPVs) with the commercial vaccines as vaccine-VSVRI. Our recorded findings are in agreement with Lebdah *et al*. (2019).

To investigate the genetic evolution of the fpv167 (P4b) amplicon in our FPV isolates, they were sequenced, systematically analyzed using nucleotide Basic Local Alignment Search Tool (BLASTn) then compared to other FPV reference sequences. The genetic characteristics of the reported strains (Sharquia-1 and Ismilia-2) showed the highest genetically related to Elsharqyia_FWPV1, Elsharqyia_FWPV2, Fowlpox-AN5 with nucleotide identity percentage 100%. Additionally, our isolates were sharing similarity to FWPVN, FWPVD, Fowlpox-AN4 with nucleotide identity percentage 99%. Our reported findings are nearly agreed with Aboul Soud *et al*. (2020) and Molini *et al*. (2022) who reported a 100% nucleotide similarity on the level of P4b gene confirming its conserved features. In contrast, our isolates were distinctly apparat from FPVB7 with 67% similarity that may indicate these isolates might be combined with different sources and ancestors. Concerning the FPV isolates identity, they revealed 87% similarity to each other on amino acid identity level, and 92% similarity based on nucleotide identity level. In view of our findings, we suggested that our isolates could have originated from different source correlated with environmental variations. Currently, the likeness of our Egyptian isolates comparable to vaccinal strain was 88% homology. According to Amino acid alignment analysis of fpv167 fragment, Our Sharquia-1 and Ismilia-2 isolates have been acquired different mutations compared with FWPVD reference strain that decreased the identity percentage with the vaccine strains. These current results weren't similar to reports of Aboul Soud *et al*. (2020) who mentioned that all three sequences from Sharkia2017/VSVRI isolate, either obtained from skin nodule or pock lesion or commercial FPV vaccine, revealed 100% identity based on the nucleotide level. Besides, Yehia *et al*. (2023) reported that the isolates (fowlpox-AN1, fowlpoxAN4, and fowlpox-AN5) had 98.6% similarity with the vaccine strains (vaccine-VSVRI). These subsequent findings approve that our circulating FPV isolates are antigenically different from vaccinal strains; demonstrating that the used vaccination strategy may be insufficient to provide protection, or chicken flocks may expose to other infections throughout vaccination. Hence, the advancement of cross-reactive vaccines against currently FPV isolates is urgently warranted. Consequently, the available vaccination was constructed from specific APV strains, but positive cases were detected on vaccinated chicken farms, this situation was mentioned also by Lebdah *et al*. (2019). Finally, the vaccination efficiency and the utilized vaccine updating needs further investigation through a comprehensive whole genome sequencing of analyzed viruses. Subsequently, the financial losses caused by APV in the local chicken industry can be reduced.

Conclusion

The above-mentioned data approved FPV isolation from clinically infected fowls using various diagnostic assays involving virus isolation, EM, SNT, ELISA, PCR and sequence analysis. FPV consider a threat to the economic situation of local poultry industry in Egypt as the available vaccine isn't a long-term solution. According to phylogenetic analysis, all the APV studied strains are correlated to fowlpox virus (subclade A1). Our Sharquia-1 and Ismilia-2 isolates have acquired different mutations. Likewise, further investigation is required for complete genome sequencing and updates the vaccine efficacy. In context, the recent our isolates can be used in vaccine production for appropriate vaccination programs in Egypt.

Acknowledgments

The authors would like to express their deep appreciation to the Faculty of Veterinary Medicine, New Valley University, Veterinary Serum and Vaccine Research Institute and Reference laboratory for quality control on poultry, Animal Health Institute, Egypt for their collaboration, and support during all procedures of this experimental research.

Conflict of interest

The authors declare that they have no known competing financial and non-financial interests or even personal relationships that could have appeared to influence the work reported in this paper.

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