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# UTILITY OF LOCALLY ISOLATED FOWL POX VIRUS DURING 2019 FOR POTENCY EVALUATION OF CURRENTLY USED VACCINES

By

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## ABSTRACT

This study was conducted to evaluate the efficacy of commercial Fowl Pox Virus (FPV) vaccines against re-emerged locally isolated strain late 2018, namely FWPVH/Egypt/2018. FPV was isolated from affected chickens in laying hen's farm in Qalubya governorate late 2018. Virus isolation and titration were carried out on collected nodular skin lesions, on the chorioallantoic membrane (CAM) of specific pathogen free (SPF) embryonated chicken eggs (ECE). Characteristic pox lesions of FPV were obtained with a virus titer of 6.0 $\log_{10}$ $EID_{50}$/ml at the $5^{th}$ virus passage.Further identification achieved by polymerase chain reaction (PCR) amplifying 578 bp and 1982 bp of P4b (*fpv167*) and *fpv140* genes of isolated FPV, respectively. Molecular characterization of the virus was obtained by nucleotide sequencing of the amplified P4b fragment. However, FWPVH/Egypt/2018 showed 100% nucleotide sequence identity to FPV forming a single cluster at subclade A1 with other FP and it shared only 90% nucleotide identity to pigeon pox viruses (PPV) which clustered in subclade A2. To evaluate the efficacy of commercial FPV and PPV vaccines to protect chickens against challenge with FWPVH/Egypt/2018, five groups of SPF chicks were vaccinated with fife available commercial market vaccines FPV (VSVRI), FPV (Diftosec-Merial), FPV poxin, FPV (Intervet) and, PPV (VSVRI). Percentage of take count for each group were 100, 95, 95, 95, and 85, respectively.Virus neutralization test (VNT) using collected serum samples of vaccinated chicks groups against FWPVH/Egypt/2018 revealed 3, 3, 2.75, 2.75, and 2.25 neutralization index (NI),respectively, showing high antigenic relationship between vaccinal FPV and isolated FPV but lower antigenic relationship to PPV Vaccine.

Protection percentages against challenge with FWPVH/Egypt/2018 were100. 95, 95, 95 and 85%, respectively,for used commercial vaccines.Protection was 0% in control non-vaccinated group. The present study reports the antigenic and genetic similarity between the tested commercial vaccines and the re-emerged locally isolated FPV strain (FWPVH/Egypt/2018) and the utility of the strain to be used as a challenge virus to evaluate commercial vaccines used against FPV.

**Keywords:**

Fowlpox virus, isolation, PCR, P4b, challenge test, vaccine.

## INTRODUCTION

Fowlpox (FP) is a poultry industry threatening disease causing high economic losses due to drop in egg production and high mortalities reaching up to 50%. The disease occurs in two forms, cutaneous form that was characterized by proliferative nodular lesions on the comb, wattle, eyelids and un-feathered parts, and diphtheritic form in which mucous lining of the upper respiratory tract (mouth, esophagus and trachea) oropharynyx and internal organs were covered by fibro-necrotic lesions **(Masola *et al.*, 2014)** .Nodular proliferative skin lesions (cutaneous form) cause major problems as lesions around the eyes and mouth will be ulcerated making the affected bird unable to eat or drink leading to dehydration and starvation ending by death **(Abdo *et al* ., 2017).** FP is a global epitheliotropic viral disease in different species of fowl that caused by Fowl pox virus (FPV),a double stranded DNA virus belonging to genus Avipoxvirus (APV)within subfamily Chordopoxvirinae andPoxviridae family**(Andraw, 2012).**

Classical laboratory diagnosis of FP virus (FPV) is achieved by virus isolation from cutaneous nodular or diphteric lesions. Virus isolation is routinely performed by inoculation on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE), and is characterized by the development of pinheaded pock lesions **(OIE,2018;Gilhare *et al.*, 2015).** Polymerase chain reaction (PCR) is an important tool for identification of an avian pox virus (APV) by amplification of P4b (core protein) and *fpv167* genesequences resulting in product sizes of 578 bp and 1800 bp, respectively **(Jarmin *et al.*, 2006; Luschow *et al.*, 2004)**. Based on the phylogenetic analysis of (*fpv140*), the orthologue of vaccinia virus H3L gene encoding the virion envelope protein p35, APVs are divided into three clades; clade A (FPV) with subclade A1 including FPV, subclade A2 including Piegon pox viruses (PPV), and clade

B Canary pox viruses (CNPV), and clade C (Psittacinepox virus) **(Offerman *et al.*, 2014; Manarolla *et al.*, 2010; Rampin *et al.* 2007).** Early in 2011, an increasing number of FPV new cases were recognized in commercial poultry farms in Egypt. Vaccination was adopted as a main strategy for control and prevention, especially in laying hens **(Abdallah and Hassanin, 2013).** Different types of vaccines are used for protection against FP in Egypt, including locally produced and imported vaccines either prepared on SPF-ECE or cell lines **(Susan and Christine 2014).** In addition, PPV vaccine, can be used in layer poultry farms as it is safe and it was not reported to cause decrease in egg production **(Christine and Nada, 2019).PPV vaccine is routinely used for more than half a century to prevent fowl pox in commercial poultry during endemic areas (Siddique *et al.*, 2011; OIE 2018).**

Viruses continue to reemerge in continuous episodes through a mechanism of socioecologic adaptaion of a virus strain or variant. Such adaptation leads to the increase of virus progeny and virulence. The change in the viral genomic structure denoted as mutations or reassortment of genome segments (in segmented viruses), also recombination, is considered the main mechanism leading to virus reemergence, and this genetic variation can be reflected on the antigenic makeup of the virus (**Domingo 2010; Kusters and Almond, 2008).**

To monitor these variation analysis at the genomic and antigenic levels of the reemerged viruse and check for the cross-neutralization between the available vaccines and such strain is necessary.

The main objective of the present study is to characterize a recently emerging FPV isolated from a layer farm in Egypt and to compare the efficacy of different commercial vaccines to protect chickens against infection with such isolate.

## MATERIAL AND METHODS

**Ethical approval and area of work:**

Sampling, challenge, and examination procedures were approved by the Institutional Animal Ethics Committee of the veterinary serum and vaccine research institute (VSVRI).

**Virus isolation in SPF embryonated chicken eggs (ECE):**

Virus isolation was carried out at late 2018 from a layer hen's farm in Qalubya governorate, in Egypt, where layer chickens were suffering from extensive nodular skin lesions on peak and wattele. Virus isolation and propagation was performed following the standard procedure of **OIE (2018)**. Briefly, tissue homogenate of the pooled nodular wart-like lesions in peak and

wattles of chickens were excised with sterile scalpel and homogenized with sterile sand and PBS containing penicillin and streptomycin at a concentration of 1000 IU and 1000 µg/ml, and clarified before inoculation onto Chorio-allantoic membrane (CAM) of twelve-day-old SPF ECE.CAMs with pock lesions were collected and preserved at -70$^{o}$C, before usage for re-passaging and titration.

**Polymerase Chain Reaction (PCR), sequencing and phylogenetic analysis of P4b gene fragment:**

DNA extraction was performed on clarified CAM pock lesions homogenates using the PureLink$^{TM}$ viral RNA/DNA mini kit (Invitrogen, USA) following manufacturer instructions. For amplification of P4b gene fragment, two primer pairs (Table 1) were used to characterize the local isolate, as a fowl pox virus as described before by Huw Lee and Hwa Lee (1997)**,** and flanking 578 bp of P4b gene (fpv167) and 1982 bp spanning the 3' rd of fpv 139, fpv 140 and 5' end of fpv141. Primers are supplied by Metabion (Germany).

**Table (1):** Primers used for detection of fowl pox virus.

| Primer name | 5'-Primer sequence-3' | Location in FWPV genome | Product length |
|---|---|---|---|
| **M2925** | **CAGCAGGTGCTAAACAACAA** | ***fpv*167 (P4b) gene nt. 459-478** | **578 bp** |
| **M2926** | **CGGTAGCTTAACGCCGAATA** | **complementary to nt. 1016-1035 in *fpv*167 (P4b) gene** | |
| **M2904** | **GAAGTAGAGTTACGGTTC** | ***fpv139* gene nt. 171290-171311** | **1982 bp** |
| **M2912** | **GGTGATCCATTTCCATTTC** | **Complementary to nt. 173254-173272 in *fpv141* gene** | |

Two fragments of the P4b gene of 578 bp and 1982 bp sizes were amplified in 50 µl volume Polymerase chain reactions (PCR), each composed of 5 µl of 10x Dreamtaq Green buffer, 1 µl 10 mM dNTPs mix, 5 units of Dream *Taq* polymerase (Thermo Fischer scientific, USA), 30 pmole of each primer (Table 1), and nuclease free water to 50 µl volume. PCR conditions were as follows: strand separation at 95°C for 5 min, followed by 35 cycles of 95°C for 15 seconds, 50°C for M2925/M2926 annealing or 46°C for M2904/M2912 primers annealing for 15 seconds, and 72°C for 35 seconds (M2925/M2926) and for two minutes (M2904/M2912). Finally, there was seven minutes at 72°C for further strand extension. Then, amplified PCR

product was resolved by electrophoresis on 1.5% agarose gel in parallel with a GeneRuler$^{TM}$ 1 kilo base DNA ladder- ready to use (Thermo-Fisher scientific, USA). Before sequencing, PCR amplicon of P4b gene of 578 bp size was purified using QIAquick gel purification kit (QIAGEN, Germany), and DNA concentration was determined using Qubit 4 fluorometer and DNA broad range (BR) quantification kit (Thermo scientific, USA), thereafter purified amplicon was submitted to GATC company (Germany) for sequencing. The similarity of the sequences was evaluated through alignment and comparison with sequences from GenBank using nucleotide Basic Local Alignment Search Tool (BLASTn). Homology and phylogenetic analysis were performed using MEGA-X and BioEdit software packages.

**Table (2):** Data of *Avipox viruses* sequences compared to FWPVH/Egypt/2018 (GenBank accession number: MW147745)

| Virus name | Host | Nature | GenBank Accession No. |
|---|---|---|---|
| **FPV_Diftosec CT (Merial)_FWPVD** | **Chicken** | **Commercial vaccine** | **AM050380** |
| **FPV_NobilisVariole W (Intervet)_FWPVN** | **Chicken** | **Commercial vaccine** | **AM050379** |
| **FPV_Mild (Websters; Fort Dodge)_ FWPVM** | **Chicken** | **Commercial vaccine** | **AM050378** |
| **FPV VSVRI** | **Chicken** | **Clinical isolate, Egypt** | **MN708968** |
| **FPV_Sharkia_2017/VSVRI** | **Chicken** | **Clinical isolate, Sharqyiah, Egypt** | **MN542415** |
| **FPV_PM/Sharkia2017/VSVRI** | **Chicken** | **Clinical isolate, Sharqyia,h Egypt** | **MH035836** |
| **PPV_PGPV1** | **Pigeon** | **Clinical isolate, Tamilnadu, India** | **MH365477** |
| **PPV_ Elsharqyiah_PGPV** | **Pigeon** | **Clinical isolate, Sharqyiah, Egypt** | **JQ665840** |
| **PPV_Avipox virus CVL_Peekham** | **Pigeon** | **Clinical isolate, UK** | **AM050385** |
| **PPV_PPLH** | **Pigeon** | **Clinical isolate, Egypt** | **MN892361** |
| **CNPV_712** | **Serinus Canaria** | **Clinical isolate, Brazil** | **KX863707** |
| **CNPV_CVL_1445/97/33** | **Canary** | **Clinical isolate, UK** | **AM050375** |
| **CNPV_Duphar; Fort Dodge strain V** | **Canary** | **Commercial vaccine** | **AM050384** |

**Vaccines and vaccination:**

Commercial FPV vaccines collected from market (FPVV dittoes- Merial ), (FPVV- poxine) and (FPVV - Intervet) in addition to locally produced (FPVV - and PPVV - VSVRI), were used to vaccinate 5 groups of SPF chicks via wing web according to **OIE (2018)** by piercing the wing web with a needle dipped in the vaccine. The vaccinated chicks were observed for 14 days after vaccination and the vaccine takes formation in the vaccinated birds was recorded.

One hundred and twenty SPF chickens of 45 days old, obtained from Quom Osheem SPF chicken farm, Fayoum governorate, Egypt, were used in this study and divided into 6 groups (Twenty chickens per group) as follows: group (1): vaccinated with FPVV-VSVRI; group (2): vaccinated with FPVV dittoes- Merial; Group (3): vaccinated with (FPVV- poxine); group (4): vaccinated with FPV-V Intervet; group (5): vaccinated with PPVV-VSVRI; group (6): was kept as control non-vaccinated chickens. Efficacy of commercial fowl pox vaccines to protect chickens against infection with fowl pox isolate-FWPVH/Egypt/2018, was evaluated by challenge of all vaccinated and non-vaccinated chickens groups using the isolated virulent local FPV strain according to OIE (2018). The challenge virus dose was 6 log10 egg infective dose fifty (EID50) inoculated via wing web route at the wing opposite to site of vaccination. Chickens were observed daily for 14 days and the deaths and the number of surviving chickens that showed clinical signs of disease (cutaneous pock lesions in comb, wattle and other un-feathered areas of the skin and/or diphtheritic lesions in mucous membrane of the oropharyngeal mucosa) were recorded. Serum samples were collected from all chickens after 21 days post vaccination and before challenge for virus neutralization test.

**Virus Neutrization Test (VNT):**

The collected serum samples from vaccinated chicks were suspended to conduct VNT on SPF ECE by using the isolated FPV. Equal volumes, of ten-fold serial dilutions of the local isolate, FWPVH/Egypt/2018 strain, were added to tested serum samples (n=5) or to sterile PBS, to determine the virus pock-forming titer (virus control). Before incubation at 37°C for 1 hour followed by inoculation of 0.1 ml of the mixture onto the CAM of 12 days old SPF chicken embryos. The negative control was serum collected from negative control unvaccinated chicken group (group 6). Seven days post inoculation, CAMs of live embryos were harvested and examined for the presence of pock lesions (Morita, 1973). The virus titer (VT) and the serum virus titer (SVT) were calculated using the statistical method described by Reed and

Muench, (1938) to determine the 50% pock forming infective dose end point (ID50). The neutralization index (NI) is the antilogarithm of the difference of the ID50 of virus control (VT) minus the ID50 of the serum-virus mixture (SVT) (NI = VT-SVT) according to **Pilchard *et al.*, (1962).**

## RESULTS

**Isolation and titration of FWPV in SPF-ECE:**

CAMs of inoculated eggs showed white small sized pock pinheaded lesions at higher virus dilutions and thickening of the CAMs of inoculated eggs at lower virus dilutions Fig. (1).

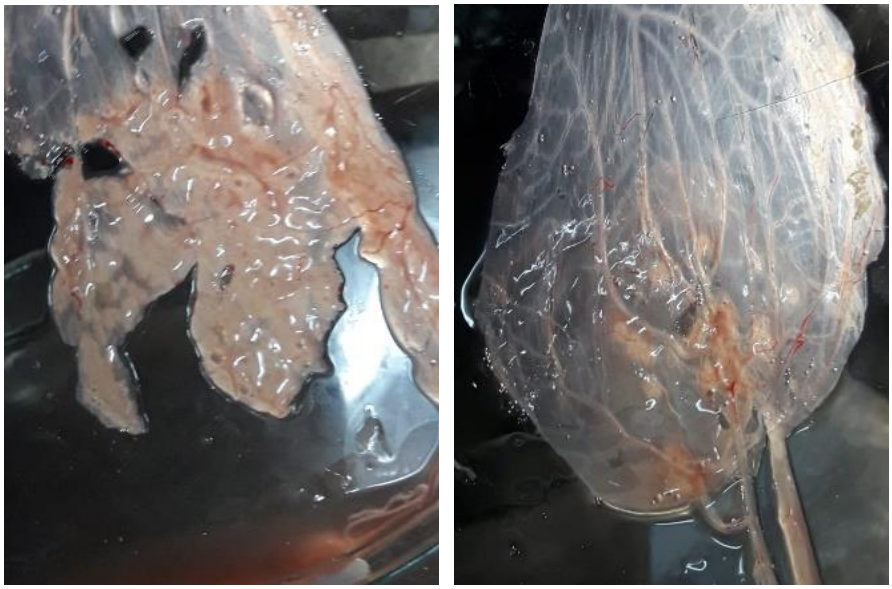

**Fig. (1): CAMs of SPF eggs inoculated with FPVH isolate.** $10^{-2}$ virus dilution (left) induced condensed white pock lesions and thickening of CAM and $10^{-4}$ virus dilution induced scattered pock lesions at site of inoculation.

Titration of FPVH revealed a virus titer of 2.25, 3.5, 5.0, and 6 log10 EID50/ml at the 2nd, 3rd, 4th, and the 5th passage, respectively.

**Counts after vaccination of SPF chickens:**

Birds vaccinated with different commercial vaccine formulations were observed for 7-10 days for the evidence of "takes" formation. A 'take' consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination (OIE, 2018). Table (4) shows that, the percentages of takes counts ten days post vaccination was 100% , 95, 95, 95 and 0% in chicken groups vaccinated with (FPVV - VSVRI), (FPVV - Diftosec-Merial), (FPVV – poxin), (FPVV - Intervet), and (PPVV - VSVRI), respectively.

**Table (4):** Takes count after vaccination of SPF chickens.

| Vaccine used | Chicks group | No. of chickens/group | Number of birds showing takes | | | | Percentage of reaction (Takes) |
|---|---|---|---|---|---|---|---|
| | | | 7 days post vaccination | | 10 days post vaccination | | |
| | | | + | - | + | - | |
| FPV (VSVRI) | group (1) | 20 | 12 | 8 | 8 | 0 | 100% |
| FPV (Diftosec-Merial) | group (2) | 20 | 13 | 7 | 6 | 1 | 95% |
| poxine | group (3) | 20 | 11 | 9 | 8 | 1 | 95% |
| FPV (Intervet) | group (4) | 20 | 10 | 10 | 9 | 1 | 95% |
| PPV (VSVRI) | group (5) | 20 | 0 | 20 | 0 | 20 | 0% |

**(+) = birds showed takes, (-) = birds did not show takes.**

**Virus neutralization test on SPF- ECE:**

Virus neutralization test was carried out on the isolated virus using sera collected from vaccinated chickens with commercial market vaccines on SPF-ECE and neutralization index (NI) was calculated as shown in (Table 5). NI values of chickens groups vaccinated with FPV vaccines FPVV (VSVRI), FPVV (Diftosec- Merial), FPVV poxin, FPVV (Intervet) were 3, 3 2.75and 2.75, respectively, while it was 2.25 for chicks vaccinated with PPV vaccine (PPVV-VSVRI).

**Table (5):** VNT for different commercial vaccines used against fowl pox.

| Chicken groups | FWPVH/Egypt/2018 | VT | VST with serum collected from vaccinated birds | NI |
|---|---|---|---|---|
| | Vaccine used | Titer expressed as $log^{10}$ $EID_{50}$/ml | | |
| Group (1) | FPV (VSVRI) | 6.0 | 3.0 | 3.0 |
| Group (2) | FPV (Diftosec- Merial) | 6.0 | 3.0 | 3.0 |
| Group (3) | poxine | 6.0 | 3.25 | 2.75 |
| Group (4) | FPV (Intervet) | 6.0 | 3.25 | 2.75 |
| Group (5) | PPV (VSVRI) | 6.0 | 4.25 | 2.25 |

**VST= virus serum titer, VT= virus titer, NI= VT-VST. NB: NI > 1.5 considered positive result.**

**Challenge test of vaccinated chicken using FWPVH as a challenge virus:**

The results of challenge test using locally isolated FPV (FWPVH/Egypt/2018) after three weeks of chickens' vaccinated with different avian pox vaccines available in the market, are shown in (Table 6). All chickens groups vaccinated with commercial FPV vaccines had a protection percent of 95-100% for FPV vaccines, 85% protection for PPV vaccines, and 0% protection in control non-vaccinated chicken group.

**Table (6):** Protection of vaccinated and control chicks against virulent locally isolated FPVH.

| Challenge Time post vaccination | Chicken groups | Used vaccine | No. of challenged Chickens/ group | No. of birds showing lesion post challenge | | | Protection percent (%) |
|---|---|---|---|---|---|---|---|
| | | | | 5dpc* | 7dpc | 10dpc | |
| 3 weeks | group (1) | FPV (VSVRI) | 20 | 0 | 0 | 1 | 100% |
| | group (2) | FPV (Diftosec-Merial) | 20 | 0 | 0 | 1 | 95% |
| | group (3) | poxine | 20 | 0 | 1 | 0 | 95% |
| | group (4) | FPV (Intervet) | 20 | 5 | 1 | 0 | 95% |
| | group (5) | PPV (VSVRI) | 20 | 0 | 1 | 1 | 85% |
| | group (6) | Unvaccinated | 20 | 3 | 13 | 7 | 0% |

*dpc= days post challenge.

**Amplification, sequencing and phylogenetic analysis of P4b gene fragments:**

Two fragments of 578 bp and 1982 bp sizes of *fpv*167 (P4b) and *fpv139* genes, respectively, were amplified from fowl pox vaccine and FPVH local isolate Fig. (2) using primers set mentioned in (Table 1). The obtained nucleotide sequence of the 578 bp fragment of the virus isolate FWPVH/Egypt/2018 P4b gene was deposited in gene bank with accession number MW147745. Comparison of truncated 465 bp fragment deduced amino acids sequence with those of published sequences for *Fowl*, *Piegon*, and *Canary Poxviruses* in GenBank (Table 2) was performed using identity matrix (Table 3) and BioEdit software package revealing 100% identity of the local isolated virus FWPVH/Egypt/2018 with nucleotide sequences of other FPVs. Deduced amino acids (aa) alignment was performed using BioEdit software Fig. (3) Showing complete similarity between FWPVH/Egypt/2018 and other FPVs and a single

mismatch with PPVs. CNPVs showed many aa mismatches with FWPVH/Egypt/2018. Phylogenetic tree was conducted using MEGAX software package Fig. (4).

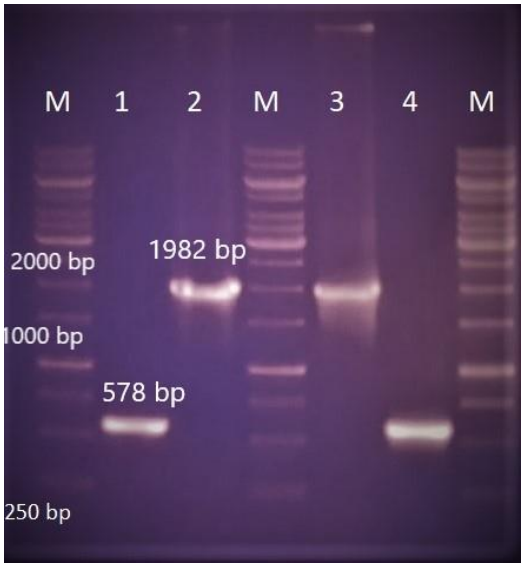


**Fig. (2):** Amplification of *fpv*167 (P4b) and *fpv139* genes fragments of FPVH isolate. Lanes 1 and 2 show amplification of two fragments of 578 bp and 1982 bp of *fpv*167 (P4b) and *fpv139* genes, from vaccine strain. Lanes 3 and 4 represent amplification of the same fragments with same sizes (578 bp and 1982 bp) from the locally isolated fowl pox virus strain, FPVH. Lane M represents O'GeneRuler 1kb DNA ladder.

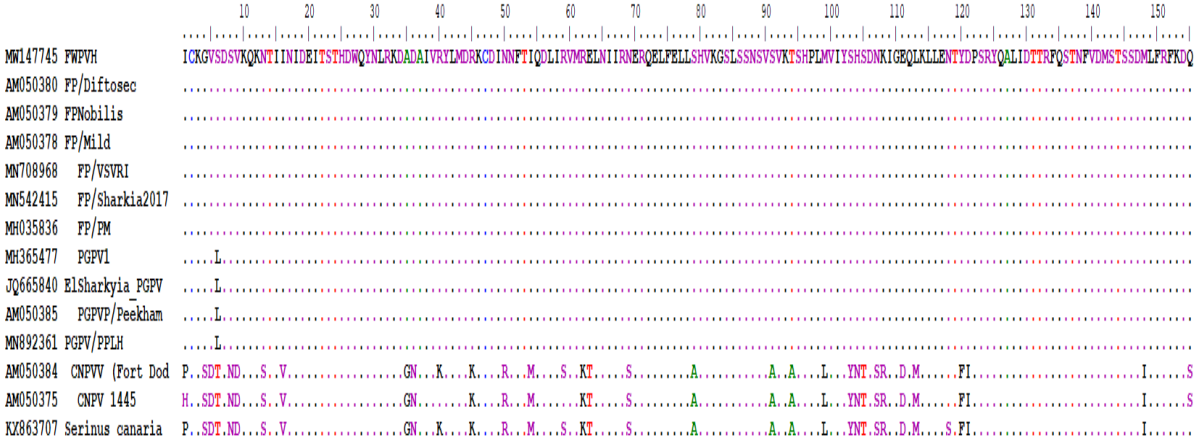


**Fig. (3):** Deduced amino acids alignment of P4b fragment. Amino acids sequence (155 amino acids) deduced from 465 bp fragment truncated from the amplified P4b fragment (578 bp), representing FWPVH/Egypt/2018 and 13 *Avipox* viruses's sequences derived from GenBank database (Table4). Identical amino acids with FWPVH/Egypt/2018 are represented with dots (.) and colored characters represent mismatches. Amino acids alignment was performed using MEGAX and BioEdit software packages.

**Table (7):** Nucleotide sequence identity matrix. The percentage of identical nucleotides between 14 nucleotide sequences are shown. Analysis was conducted using the BioEdit software package.

| No | Genebank Accession No/Virus name | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | **MW147745/FWPVH/Egypt/2018** | ID* | 100 | 100 | 100 | 100 | 100 | 100 | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **2** | **AM050380/FP/Diftosec-CT(Merial)** | | ID | 100 | 100 | 100 | 100 | 100 | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **3** | **AM050379/FP/Nobilis/VarioleW-Intervet** | | | ID | 100 | 100 | 100 | 100 | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **4** | **AM050378/FP/Mild(Fort Dodge)** | | | | ID | 100 | 100 | 100 | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **5** | **MN708968/FP/VSVRI** | | | | | ID | 100 | 100 | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **6** | **MN542415/FP/Sharkia2017/VSVRI** | | | | | | ID | 100 | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **7** | **MH035836/FP/PM/Sharkia2017/VSVRI** | | | | | | | ID | 90 | 90 | 90 | 90 | 74.4 | 75.9 | 74.2 |
| **8** | **MH365477/PGPV1** | | | | | | | | ID | 100 | 100 | 100 | 74 | 74.6 | 74.2 |
| **9** | **JQ665840/PGPV/ElSharkyia** | | | | | | | | | ID | 100 | 100 | 74 | 74.6 | 74.2 |
| **10** | **AM050385/PGPVP/Peekham** | | | | | | | | | | ID | 100 | 74 | 74.6 | 74.2 |
| **11** | **MN892361/Pigeonpox virus/PPLH** | | | | | | | | | | | ID | 74 | 74.6 | 74.2 |
| **12** | **AM050384/CNPVV(Fort Dodge)** | | | | | | | | | | | | ID | 98 | 93 |
| **13** | **AM050375/CNPV 1445** | | | | | | | | | | | | | ID | 92 |
| **14** | **KX863707/Serinus canaries** | | | | | | | | | | | | | | ID |

**ID*= Identical sequence.**

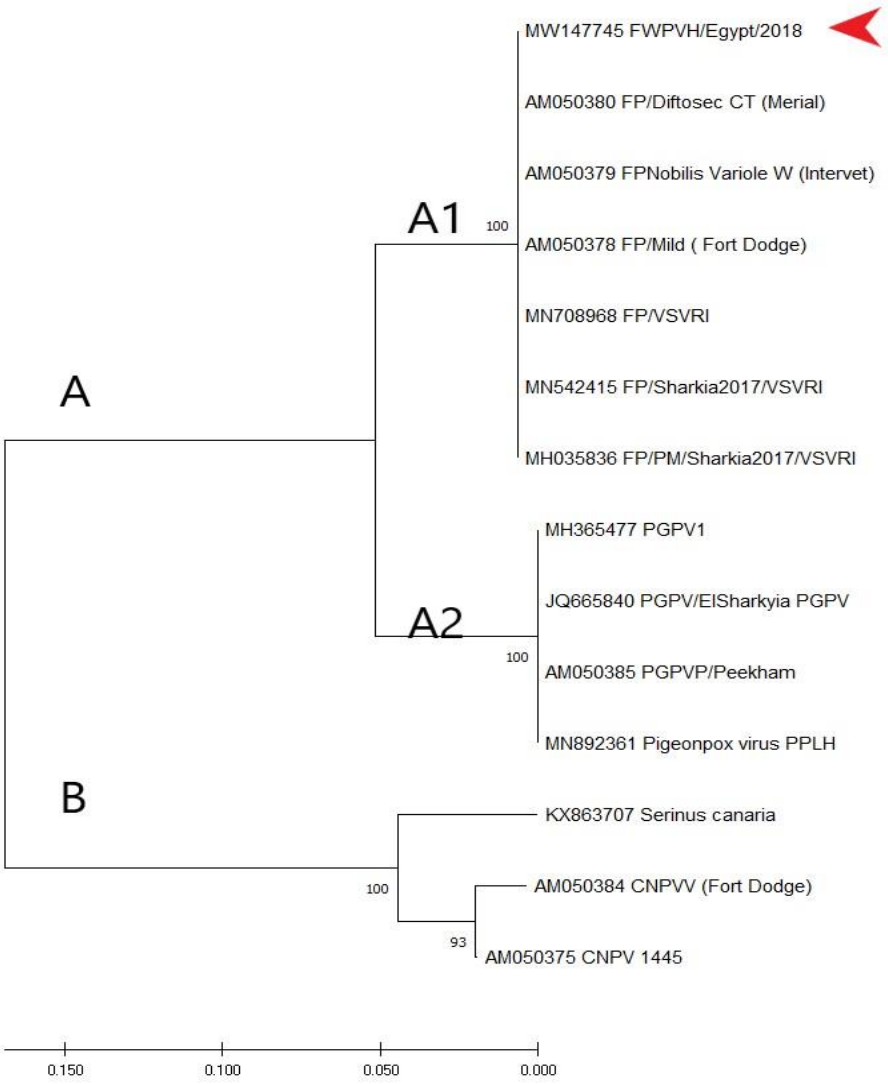


**Fig. (4):** Phylogenetic analysis of FWPVH and other *Avipoxviruses* based on P4b gene fragment nucleotide sequence. The phylogenetic relationship of *Fowl poxvirus* isolate, namely FWPVH/Egypt/2018 and nucleotide sequences of 13 APVs species including; fowl, pigeon, and canary *poxviruses* was inferred using the Neighbor-Joining method, based on their nucleotide sequences obtained from GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The unrooted tree represents clades A and B with subclades A1 and A2. FWPVH/Egypt/2018 isolate is pointed with red arrow at subclade A1. The evolutionary distances were computed using the Maximum Composite Likelihood method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted using MEGAX software.

## DISCUSSION

Remerging fowl pox (FP) viruses could show differences in antigenic properties from circulating viruses and new FPV phenotypes may break through immune responses developed by currently used vaccines. This study was conducted to identify the genetic and antigenic correlation between a recently isolated FPV and the currently used vaccine strains.

At the present study, a recently isolated FP virus from a layer farm was subjected to molecular characterization before usage to evaluate the efficacy of candidate commercial vaccines to protect chikens against infection with this re-emerging FPV.Virus propagation and titration of the isolated FPV strain on CAM of SPF-ECE showed characteristic pock lesions of FPV as white small sized pinheaded pock lesions which came in accordance with those previously obtained **(Abdallah and Hassanin, 2013; Masola *et al.*, 2014; Sherif *et al.*, 2015)** where they described FPV pocks as white small sized dots on CAM which are smaller in size and more abundant than pock lesions of other avipox viruses (APVs). Titer of the propagated FPV reached 6.0 $\log_{10}$ $EID_{50}$/ml at the $5^{th}$ passage as demonstrated in (Table 3) as more propagation is required for more virus titer as reported previously by **Susan *et al.* (2014)** who obtained a FPV Giza isolate, 2012 with a titer of 4.5 $\log_{10}$ $EID_{50}$/ml after three passages on SPF- ECE.

Breaking the rule of host species specificity was reported for APVs based on virus virulence and host susceptibility to be infected with candidate virus,

Ability of different Avipox viruses (APVs) to skip trans-species borders was proven depending on virus virulence and host susceptibility to infection **(Manarolla *et al*., 2010).** As an example, pigeon pox virus can infect both pigeons and chickens **(Kirmse, 1969; Sumaya, 2005**). Therefore, molecular characterization of the virus isolated at this study to explore its identity was a must. Although APVs have a large genome sized about 288-300 kilo base (kb), three genomic loci were identified for discrimination between different species of AVPs based on their nucleotide sequence. A 578 bp fragment of the pan-APVs *fpv*167 (P4b), a 1982 bp fragment of *fpv*140 (The orthologous of vaccinia virus H3L), and *fpv94* (DNA polymerase) of 1058 bp size **(Gyuranecz *et al*., 2013; Offerman *et al*., 2013; Afonso *et al*., 2000; Jarmin *et al*., 2006)**.

At this study, two loci, the *fpv*167 (P4b) and the *fpv*140 (H3L), were amplified from the genome of the isolated virus, namely FWPVH/Egypt/2018, producing the 578 and 1982

(~1800 bp) approximate sizes respectively Fig. (2) came in accordance with previous reports **(Jarmin *et al*., 2006)**.Although, amplicons sizes obtained for PCR of P4b or H3L loci do not constitute a unique character for FPV as P4b is APVs pan-genus conserved locus producing 600 bp approximate size (578bp) fragment in PCR and H3L produces a 1982 bp (~1800 bp) PCR fragment for clade A (fowl, turkey, albatross, pigeon, ostrich, sparrow and falcon *poxviruses*) viruses **(Binns *et al*., 1989; Jarmin *et al*., 2006)**. The PCR results indicate that, the isolated virus belongs to clade A viruses but not other clades of APVs**.** To confirm the notion that, the isolated virus at this study is a fowlpox virus, sequence analysis of the amplified P4b genome fragment was conducted and compared to sequences published in the GenBank using the nucleotide Basic Local Alignment Search Tool (BLASTn) which allocated FWPVH/Egypt/2018 with *Fowl poxviruses*. Amino acids (155 a.a.) sequence deduced from a truncated 465 (out of 578) nucleotides was aligned in Fig.(3)and phylogenetic analysis Fig. (4) was conducted in parallel to 13 other APV strains sequences derived from GenBank see (Table 2) for accession numbers). FWPVH/Egypt/2018 (GenBank accession no. MW147745) was clustered with previously annotated FPV forming subclade A1 in the consensus tree with 100% sequence identity. In addition, it shared only 90% nucleotide identity to subclade A2 (PGPV) comprising pigeon pox virus strains. The lowest nucleotide's identity (74-75%) referred to clade B (Canarypox viruses) Fig. (4), (Table 7). These results come in accordance with genomic P4b fragment identity of 91% between FPV and PGPV as detected by **Luschow *et al*., 2004** and 90.7% identity as detected by **Jarmin *et al*., (2006) and Lebdah *et al*., (2019)**.

Determination of the antigenic relationship between FWPVH/Egypt/2018 and the commercial vaccines used against FPV infection was conducted through VNT on SPF-ECE using sera collected from vaccinated chicks and FWPVH. VNT results (Table 5) showed that values of NI for chicks groups vaccinated with FPV vaccines are close to each other ranging from 3 - 2.75 NI and higher than the groups vaccinated with PPV vaccine (2.25 NI). This cross-neutralization and protection can be explained on the basis of genetic identity between FPV and PPV where a 90% similarity of P4b nucleotides sequences was obtained (Table 7). In addition, PPV originated from the same genetic ancestor with FPV and clustered at clade A with FPV but in subclade A2 Fig. (4). Similar results were obtained by **Sherif *et al*., (2015) and Sumaya (2005)** who reported the cross antigenicity between FPV and PPV by using heterologous and homologous serum neutralization where homologous neutralization was

higher than heterologous one by 0.5 NI value.

For evaluation of commercial vaccines used against FPV, 5 groups (20 SPF chicks for each group) were vaccinated with five different live vaccines. **OIE (2018)** recommended the examination of vaccinated chicks till the 10$^{th}$ day post vaccination (dpv) to record takes development (swelling of skin at site of vaccination) as an evidence for successful vaccination. Takes count percentage at the 10$^{th}$ dpv was 100, 95, 95, 95 and 90% in vaccinated chicken groups with (FPVV - VSVRI), (FPVV - Diftosec-Merial), (FPVV - poxin), (FPVV - Intervet), and (PPV - VSVRI), respectively. Similar results were obtained by **Ayatollah *et al.*, (2014)** and **Susan and Christine (2014)** who reported 96% takes count for FPV vaccine under field condition. Takes appeared in all chicks vaccinated with different FPV commercial vaccines in a ratio ranged from 90% to 100% at the 7$^{th}$ and the 10$^{th}$ dpv. It was noteworthy that takes were absent in groups vaccinated with PPV vaccine. Pigeon pox is a disease of pigeon and chickens represent a non-species host for PPV. Accordingly, attenuated vaccine may produce no or mild infection in chicken. Supporting to this notion, PPV vaccine have been routinely used for more than half a century to prevent fowl pox in commercial poultry in endemic areas **(Siddique *et al.*, 2011).**

Challenge test is the corner stone for live FPV vaccines evaluation. To evaluate the capacity of commercial vaccines to protect chickens against infection with the re-emerging FPV, FWPVH/Egypt/2018 and all vaccinated and non-vaccinated groups of chicks were challenged with the isolated FPV. Challenge test results are illustrated in (Table 6) that shows all chickenss groups vaccinated with FPV vaccines revealed protection percentage of 95% which is in accordance with the aforementioned close genetic and antigenic realtion beteen re-remerged FPV and tested commercial vaccines. Similar results were obtained by **Bahamn *et al.*, (2019) and Susan and Christine (2014)** who reported that commercial FP vaccines protected 90-100% of chickens challenged with local FP isolate.

The protection percentage with PPV vaccine was 85% against the challenge with FWPVH/Egypt/2018 isolate, while non-vaccinated control group showed 100% sever lesions of FPV. These results are in accordance with the obtained results of genetic similarity and identity (90%) between FPV strains in subclade A1 and PPV strains in subclade A2 and in accordance, also, with the results of VNT of sera collected from vaccinated chicks with PPV vaccine against the isolated FPV on SPF –ECE as the value of NI was 2.25. Similar results

were discussed by **Siccardi (1975)**, **Hy-Line (2019) and Christine and Nada (2019)** who reported the use of PPV vaccine against FPV infection in poultry farms due to cross antigenicity and protection between both viruses in case of using PPV vaccine against FPV infection with reduction in the post vaccinal side effect (reaction) in vaccinated chicks which stimulates a broader spectrum immune response for optimum protection especially when used alternatively or in combination with FPV vaccine.

In conclusion, molecular characterization of the recently re-emerged FPV strain, namely FWPVH/Egypt/2018, revealed 100% identity to FPVs but only 90% identity to PPV, based on P4b genomic fragment. Antigenic homology between FWPVH/Egypt/2018 and tested commercial vaccine strains was confirmed by achieving a high VNI indicating that FP and PP vaccines elicited humoral antibody response that neutralized FPV isolate. All tested FP vaccines protected chickens against infection with FWPVH/Egypt/2018 with 90% and 85% survival rates for chickens vaccinated with FPV and PPV vaccines, respectively. In addition, this study highlighted the utility of FWPVH/Egypt/2018 strain to be used as a challenge virus for evaluation of commercial vaccines used against FPV infection.

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