Comparative molecular study on *Avian pox viruses*

Sherif .M. Ibrahim[*], Abd El-Razek. B. Abd El-Razek[*]. Hanan. M. El-Zahed Amal. A. Fatouh[*] and Ayatollah. I. Ibrahim[*]

[*]Veterinary Serum and Vaccine Research Institute (VSVRI), Central Laboratory For Evalution of Veterinry Biologics (CLEVB), El-Seka-Beda Street, Abbasia, 131, Cairo, Egypt

ABSTRACT

The current study was conducted to determine the similarities and differences between vaccinal and isolated strains of *Fowl pox virus* (FPV) and *Pigeon pox virus* (PPV) based on biological and molecular basis. Vaccinal and isolated strains were propagated on the chorioallentioc membrane of specific pathogen free (SPF) embroynated chicken egg (ECE) showing the characteristic pock lesions for each strain with titers reached 4.5 and 3.5 $\log_{10}$ EID_{50}/ml at 5^{th} passage for FPV and PPV isolates, respectively. The isolated strains were further identified using the specific hyper-immune sera for each virus by virus neutrization test (VNT). As the Neutrization index (NI) for PPV and FPV isolates were 2 and 2.75 when neutrized by FPV hyperimmune sera (HIS) while it was 2.5 and 2 when neutrized by PPV (HIS). DNA was extracted from the produced pock lesions and polymerase chain reaction amplifying 578bp fragment of P4b gene and 1800 pb of *fpv*140 for FPV and PPV isolates and vaccine strains. Phylogenetic analysis for the amplified P4p product revealed close similarity of the FPV isolate (FPLH) and fowl pox vaccine (FPVV) with the published sequences for FPV in the Genebank. Unexpectedly, PPV isolates (PPLA, PPLR) and PPV vaccine (PPVV) showed high similarity to the published FPVs-P4b sequence in gene bank and distributed under genogroup A1 in the phylogenetic tree, while the PPV isolate (PPLH) was classified under genogrop A2 with high identity to published PPVs-P4b sequence in gene bank. This study recommend preparation of PPV vaccine from the circulating local PPV isolate, PPLH, which characterized through this study, after confirming its immune response as a candidate vaccine.

INTRODUCTION

The *Poxviridae* family is subdivided into the *entomopoxvirinae* and *chordopoxvirinae* subfamilies. The genus avipoxvirus (APV) has the largest and the most divergent genome among the chordopoxvirus genera **(Gubser et al., 2004)**. Avian pox (AP) viruses have a double stranded DNA genome which contains a central coding region surrounded by two identical inverted terminal repeat regions. The genome size is 288 Kb approximately and encodes 260 open reading frames **(Afonso et al., 2000)**. APV includes many virus species such as Fowl pox virus (FPV), Pigeon pox virus (PPV) and Canary pox virus (CNPV) and many others poxviruses infecting different avian species. There are about 9,000 bird species; 232 species of them have been recorded to have acquired natural poxvirus infection and to have various forms of poxvirus infection (**Andraw .2012).**

Avian pox is a slowly spreading disease characterized by the development of discrete proliferative nodular skin lesions (cutaneous form) or fibrino-necrotic lesion in the mucous membrane of the upper respiratory tract (diphtheritic form) **(Tripathy and Reed, 2003)**. AP infection is usually associated with a low mortality rate in chickens and turkeys; however, during some AP outbreaks, the mortality rate can reach 65–100 %, especially in the Canary and pigeon **(Pawar et al., 2011)**. The conventional laboratory diagnosis of FPV is carried out by electron microscopy, virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE) and serologic methods as Virus neutrization test on ECE **(Islam et al., 2008).**

The 4b core protein gene (p4b) of AP encoding a protein with a molecular weight of 75.2 kDa, is usually chosen for comparative genetic analysis. On the other hand, amplification of the AP-p4b by PCR has often been used as a molecular tool for the detection of avian poxviruses **(Manarolla et al., 2010)**. Based on the phylogenetic analysis of (P4b), APV are divided into three clades; clade A (FWPV), clade B (CNPV), and clade C (Psittacinepox virus) **(Jarmin et al., 2006)**. Other genes, like the *fpv*167,

have been reported to be used in order to validate or improve the obtained findings. In addition, the *fpv*140 locus (*fpv*139, *fpv*140 and *fpv*141 genes), has also been used for identification of APV and the phylogenetic analyses based on *fpv*140, the orthologue of vaccinia virus H3L gene encoding the virion envelope protein p35, has provided an improved strain discrimination within some subclades **(Rampin et al. 2007; Offerman et al., 2014).**

The present study provide a report for the sequence analysis of the *fpv*167 (P4b) locus amplified from different used AP vaccines and isolates of FPV and PPV, to asses the diversity between local isolates and the used vaccines based on molecular basses.

MATERIAL AND METHODS

1-Virus strains

Four isolates of APV (PPLA, PPlH, PPLR, and FPLH), from different avian species, were used in this study. These viruses were isolated from backyard chickens and pigeons in some villages in Monofi governorate, Egypt. These birds were suffering from skin lesions on the comb, eyelids, beak, and wattles as well as the diphtheritic upper respiratory tract. The affected birds suffered from low or even no mortality except in case of pigeon; where a high level of mortality reaching 30% was observed. In addition to local isolates, commercially available live-vaccine strains against FPV and PPV were included in the study.

2-Viruses propagation and titration in SPF- ECE

Virus propagation was performed following the procedure mentioned by **OIE (2012)**. Briefly, tissue homogenates of collected crust samples for isolation of FPV and PPV from affected birds were prepared in Hanks balanced salt solution containing penicillin (100IU) and streptomycin (1mg) before centrifugation and collection of supernatants. The FPV (Budate strain) and PPV (Hungrian strain) vaccine strains were inoculated in 12 day old SPF-embryonated chicken eggs (ECE). Both of inoculated and non-inoculated (negative control) eggs were incubated at 37^{o}C, observed and candled for 5 days post inoculation (PI). Thereafter, inoculated eggs were opened and chorio-allantoic membranes (CAMs) were cut to collect pock lesions areas before pooling and further virus passage in ECE. To titrate the propagated virus, tenfold dilution of the CAMs homogenates were inoculated into 5 SPF-ECE for each dilution and pock lesions detected after 4-5 days post-inoculation before titer calculation using statistical method described by **Reed and Meunch (1938).**

3-Virus neutralization test

To confirm that the propagated viruses belong to avian pox viruses, the neutralization test was applied on isolated viruses on SPF-ECE using specific hyper immune serum against FPV and PPV, according to **(OIE, 2012)**, then the virus titer (VT) and the serum virus titer (SVT) were calculated by the statistical method described by **Reed and Meunch (1938).** The neutralization index (NI) was subsequently calculated as follows: NI = VT-SVT.

4-DNA extraction and PCR amplification

DNA was extracted from clarified suspension of collected pock lesions produced by vaccinal and isolate strains using GF-1 tissue DNA extraction kit (Vivantis, Malaysia) following manufacturer instructions. To amplify fragments of the p4b and FP140 genes, two primer pairs **(Jarmin et al., 2006)** were used (table 1).

PCR amplification was performed in 50µl reaction volumes and contained 7ul of DNA template, 100uM of each deoxynucleoside triphosphate (Thermo Scientific, USA), 2.5 units of Dream *Taq* Green DNA polymerase (Thermosentific, USA) 5µl of amplification buffer and 20 pmole of each primer. The PCR amplification was carried out using GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, USA) and the cycling conditions for each gene are illustrated in tables (2) and (3). PCR amplicons were analyzed by running 10 µl of PCR reaction in 1% agarose gel stained with Ethidium Bromide (0.5ug/ml). Thereafter, gels were photographed under UV illumination using gel documentation and analysis system supplied with starlight express MX516 16-bit CCD camera and AAp-M5 software. The amplification pattern of each virus was determined according to molecular size of the amplified products.

Table (1): Avianpox specific primers sequence

Primer	Orientation	Sequence	Specificity
M2925	Forward	5'- CACAGGTGCTAAACAACAA -3'	P4b gene
M2926	Reverse	5'- CGGTAGCTTAACGCCGAATA -3'	P4b gene
M2904	Forward	5-GAAGTAGAGTTATCGGTTC-3	FP140 gene
M2912	Reverse	5-GGTGATCCATTTCCATTTC-3	FP140 gene

Table (2): Cycling protocol for amplification of P4b gene

Steps	Temp	Time	No of cycles
Initial denaturation	**95°C**	**5 min**	**One cycle**
Denaturation	**95°C**	**30 sec**	**35 cycles**
Annealing	**50°C**	**30 sec**	
Extension	**72°C**	**35 sec**	
Final extension	**72°C**	**7 min**	**One cycle**
Preservation	**4°C**	∞	

Table (3): Cycling protocol for amplification of FP140 gene

Steps	Temp	Time	No of cycles
Initial denaturation	**95°C**	**5 min**	**One cycle**
Denaturation	**95°C**	**30 sec**	**35 cycles**
Annealing	**45°C**	**30 sec**	
Extension	**72°C**	**2 min**	
Final extension	**72°C**	**7 min**	**One cycle**
Preservation	**4°C**	∞	

5-Gel Purification and sequencing of PCR products:

Gel purification of the 578 bp product, obtained from FPV and PPV isolates and vaccines was performed using the QIAquick® Spin kit (Qiagen, Germany) following manufacturer instruction. The purified product was forwarded to MACROGEN company laboratories (Korea) for sequencing where chain terminator single strand sequencing PCR using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI) was then applied, using in different tubes the M2925 and M2926 forward or reverse primers to analyze the complete sequence of the 578 bp amplified fragment. Following purification and concentration, the PCR product was then placed in an ABI Prism 3130 xI Genetic Analyzer (ABI, USA) and data were analyzed for local similarities with GenBank published sequences using the Basic Local Alignment Search Tool (BLAST) before further analyses using Bioedit® and MEGA5® software packages.

RESULTS

Virus isolation, propagation and titration in SPF- ECE

Skin lesions of suspected birds (photo. 1) were collected and processed as mentioned before to isolate, propagate, and titrate pox viruses on SPF–ECE. Pock lesions were detected at the 3rd passage in case of FPV (photo. 2) where thickening of the CAM with the presence of small sized (~1mm diameter), distinctly scattered nodular lesions of greenish white color were detected. Notabely, these lesions were increasing in number and size with serial passaging in ECE. On the other hand, pock lesion appeared in SPF eggs inoculated with PPV at the 5th passage (photo.3) with moderate thickening in the CAM with few yellowish white colored and large sized pock lesions. The virus titer for both FPV and PPV reached 4.5 and 3.5 $\log_{10}$ EID_{50}/ml at passage number 5, respectively.

Virus neutralization test on SPF- ECE

Alpha procedure of neutralization test was carried on the isolated viruses in addition to canary pox virus isolated before **(Abd El-Razek et al., 2012)** on SPF-ECE using standard anti-fowl and anti-pigeon pox hyper immune sera. While a higher cross-neutralization index for the Fowlpox virus hyper-immune (FPHIS) serum with Canary pox vius than observed with pigeon pox virus, both FPHIS and PPHIS have the same neutralizing capacity with PPV and FPV, respectively as shown in table (4).

Polymerase chain reaction (PCR) for P4b and *fpv* 140 genes of FPV and PPV isolates and vaccines strains.

Polymerase chain reaction was applied on the DNA extracted from propagated FPV and PPV isolates and vaccine strains using P4b and *fpv*140 genes forward and reverse specific primers (Jarmine et al., 2006). PCR amplification of the tested samples reveled the specific PCR amplicons of 578 bp and 1800 bp, approximate sizes, for P4b and *fpv* 140 genes, as shown in figures 5 and 6, repectively.

Fig. 1: skin pox lesion in pigeon: A case of pigeon showing characterizing lesions of poxvirus infection appeared as crusts around the eye lids and nostrils. These were scraped and forwarded to virus isolation.

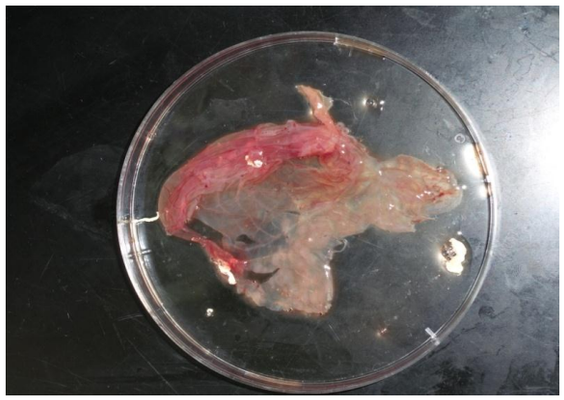

Fig. 2:pock lesion of fowl pox virus on CAM of ECE: A large number of Small sized, greenish white nodular lesions forming a thickening of the CAM, were detected at the 3rd passage of ECE infected with FPV.

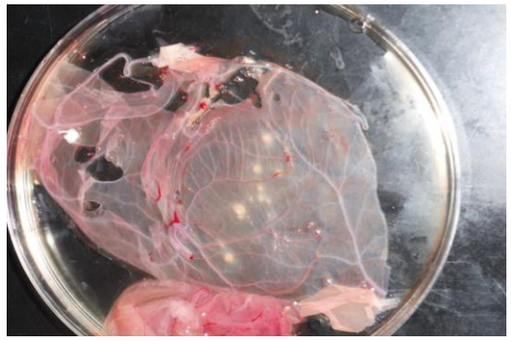

Fig. 3: pock lesion of pigeon pox virus on CAM of ECE: A few number of widely scattered white colored large sized nodular lesions induced by PPV were detected at the 5th passage of inoculated ECE.

Table (4): Results of VNT for different avian pox virus isolates on SPF-ECE

Isolated virus	Virus titer	VST with FPHIS	VST with PPHIS	NI with FPHIS	NI with PHIS
	Titer expressed as log_{10} EID_{50}/ml				
Fowl pox virus isolated from Canary **(Abd El-Razek et al., 2012)**	4	1.5	2.25	2.5	1.75
FPV isolate	4.5	1.75	2.5	2.75	2.0
PPV isolate	3.5	1.5	1	2.0	2.5

PPHIS= pigeon pox hyper immune serum FPHIS= fowl pox hyper immune serum
VST= virus serum titer VT= virus titer

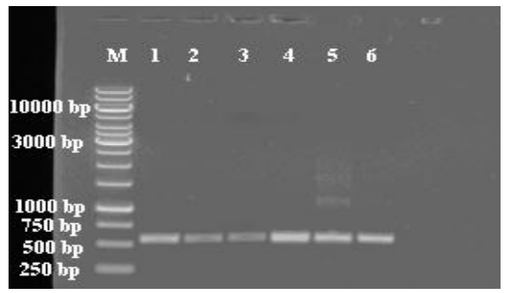

photo (5): PCR amplified products for p4b gene M- generuler 1kb DNA ladder 250 to 10.000 bp. Lane 1-PPV vaccine. Lane 2- PPLH 3rd passage. Lane 3- PPLR local isolate. Lane 4- PPLH. Lane 5- FPV vaccine. Lane6- FPV local isolate 3rd passage .

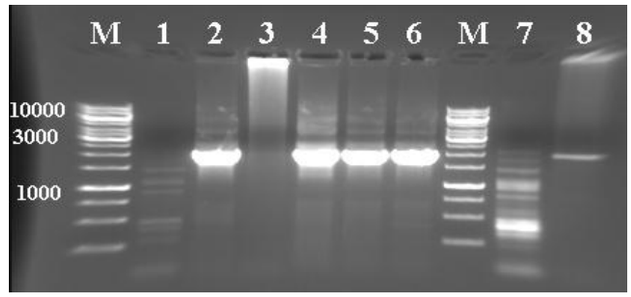

Photo (6): PCR of *fpv*140 gene. PPLR, PPLH, FPV, and FPLH in lanes 2, 4, 5, and 6, respectively, gave clear sharp bands of 1800 bp approximate size as compared with GeneRuler 1kb (M). PPLA gave a very faint band of 1800 bp size (lane 3) and PPV showed multiple non-specific bands of different sizes (lane 1) when amplified with 48C annealing temperature. PCR repetition using 45 C for annealing, revealed a sharper and more clear band for PPLA (lane 8), but for PPV (lane 7), the same non-specific amplicons were obtained.

Alignment and phylogenetic analysis of *fpv*167 (P4b) gene

The nucleotides sequence alignment of 578 nucleotides (truncated to have the same length) of the FPV and PPV isolates and vaccine strains amplicons, was performed using MEGA6 software package. The obtained sequences were aligned with the published APVs-P4b nucleotides sequences, including CNPV, of which were used as reference strains in sequence analysis.

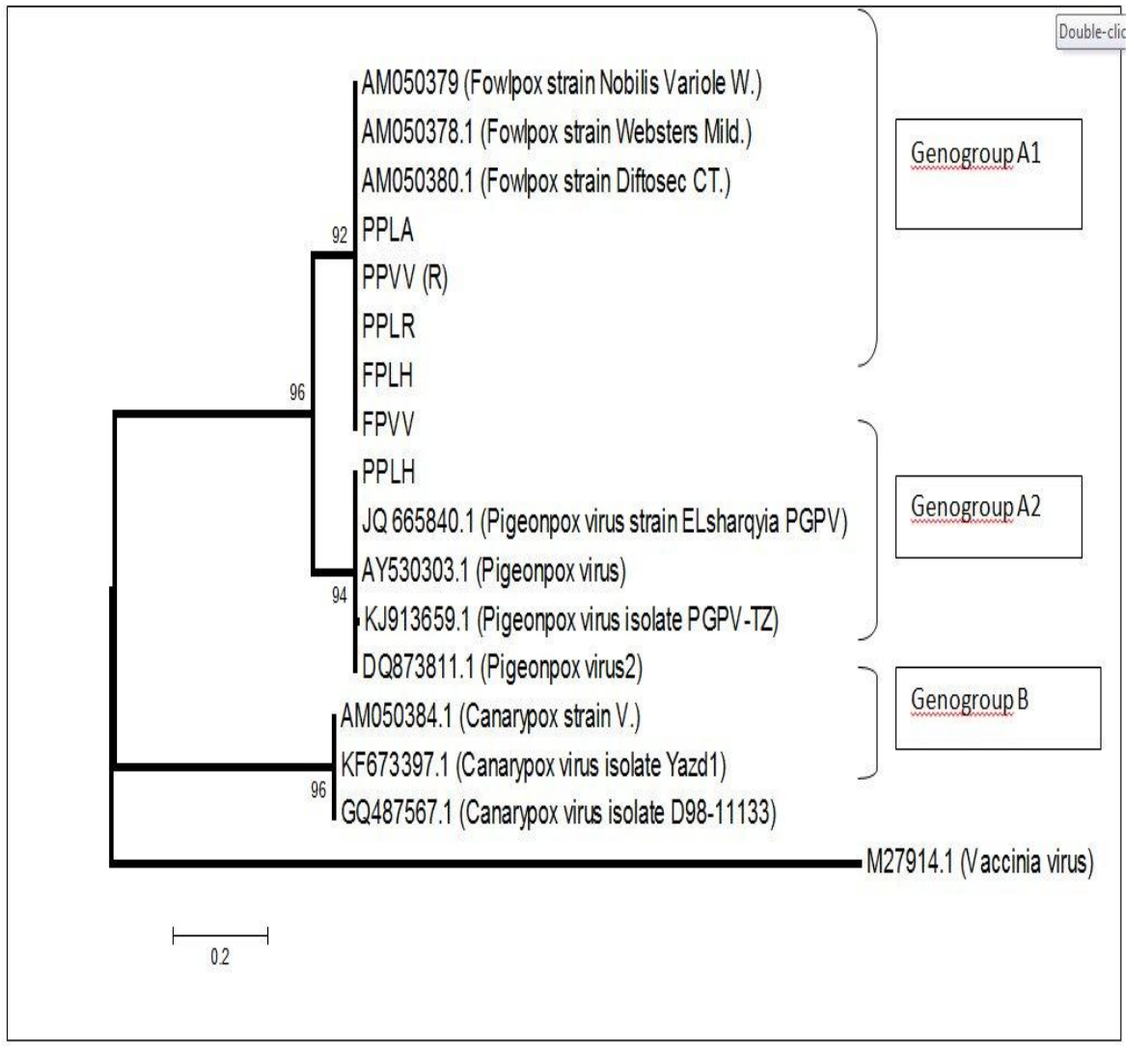

Fig. 7: Phylogenetic tree of P4b gene sequences. Nucleotide sequences of the obtained amplicons were aligned with a panel of FPV, PPV, and CNPV sequences obtained from the genebank with genebank accession numbers written at each branch. Sequences were distributed into two major clades A and B. While clade A included two clusters forming genoroups A1 and A2, one cluster formed clade B. As expected, local isolates and vaccine strains of FPV were clustered in genogroup A1 with other FPV strains from genebank, while two isolates and one vaccinal strain of PPV namely PPLA, PPLR, and PPVV were clustered at the same genogroup. Genogroup A2 formed of a cluster of PPV including the local isolate PPLH. CNPV were clustered forming genogroup B and vaccinioa virus sequence formed an out group in this analysis.

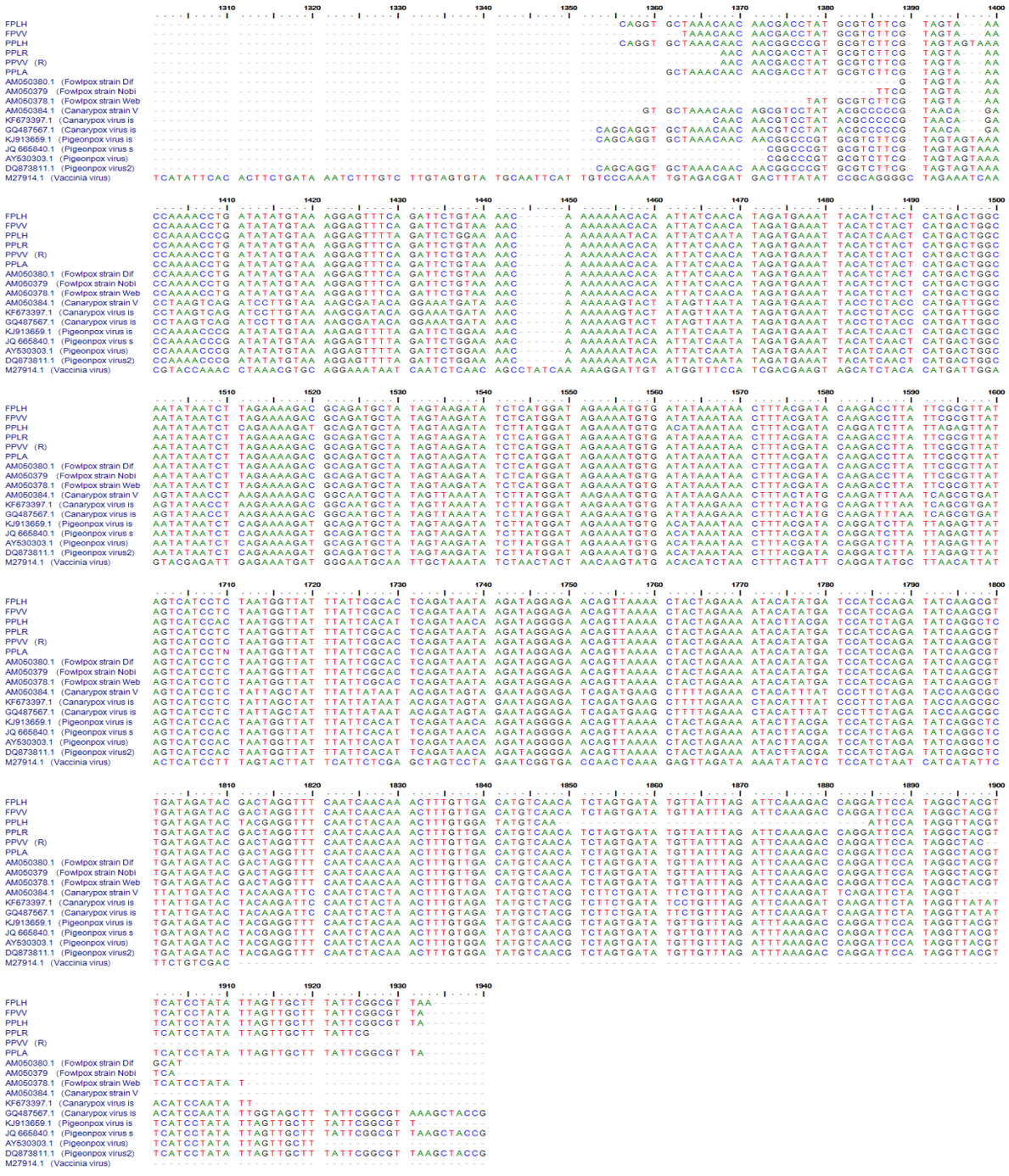

Fig.9 Nucleotide sequence alignment of P4b amplicons. Alignment of the 578 bp amplicons obtained from PCR reactions using M2925 and M2926 primers revealed different mismatches of the PPLA and PPLR isolated from pigeon and PPV vaccine with other pigeon pox viruses sequences. These mismatches could be detected as nucleotides deletion at positions numbered 1296 to 1298 and point mutations in positions 1409, 1429, 1432, 1511, 1520, 1562, 1586, 1709, 1726, 1739, 1814, 1826, and 1837. PPLA, PPLR, PPVV, FPLH and FPVV sequences were highly matching with FPV sequences. PPLH showed a complete matching with pigeon pox viruses with gene bank accession numbers: KJ913659,JQ665840, AY530303 and DQ873811 at these positions. A deletion mutation was observed at positions 1849-1884 in PPLH.

DISCUSSION

Avian pox is a well-recognized and widely distributed infectious disease in birds caused by a family of viruses collectively known as *Poxviridae* **(Young and Vander; 2008)**. The disease has two forms, the dry or cutaneous form with high prevalence but less severity and the diphtheritic form **(Elias et al., 2014).**

At the present study, the isolated viruses were propagated and titrated on CAM showing the characteristic pock lesions for pox viruses. Notably, FPV pock lesions were more smaller in size and larger in number than that of PPV which were larger in size and less in number with some hemorrhage as described before by **Kristy et al. (2013), Masola et al. (2014)** and **Abdallah and Olla, (2013)**. In addition, **Sivasothy (1997)** described that mother pock lesions produced by the isolates from pigeon and turkey in comparison with isolates from chicken were produced as scattered pock lesions, which come in accordance with the data obtained here for PPV.

Regarding virus titer, titers of the isolated FPV were higher than those of PPV reaching 4 $\log_{10}EID_{50}$/ml at the 5th passage while the titer PPV it was 3.5 $\log_{10}EID_{50}$/ml at the 5th passage which come in accordance with the results obtained by **Susan et al. (2014)** where Giza isolate 2012 had a titer of 4.5 $\log_{10}EID_{50}$/ml at the 3rd passage on ECE. Confirming to the obtained data, **Sivasothy (1997)** mentioned that the isolates from pigeon and turkey gave lower titer in comparison with other isolates from chickens on (CAM).

To serologically characterize the isolated viruses, VNT was performed in ECE where reduction of the titer of the previously isolated fowl pox virus from canary (**Abd El-Razek et al., 2012**) by 2.5 and 1.75 when FPV and PPV hyper immune serum were used, respectively, indicating a high serological and antigenic relation ship between fowl pox viruses isolated from canary or chickens than PPV. These results come in accordance with **Abd El-Razek and Ayatollah (2013) and Burnet and Lusk (1936)** where both canary and fowl pox virus-inactivating antibodies can be demonstrated in fowl-pox hyper immune sera and in the canary virus hyper immune sera. Both sera were active against both viruses in each case the homologous virus was inactivated to a slightly greater degree than the heterologous one. The results of VNT for both FPV and PPV isolates using hyperimmune sera prepared against both PPV and FPV showed reduction in VT by higher degree in case of using homologues hyper immune sera reaching 2,75 and 2.5 in FPV and PPV, respectively, than the using of heterologes hyper immune sera reaching 2.0 and 2.0 in FPV and PPV, respectively, confirming that the antigenic relationship between the isolated FPV and PPV is not as high as in case of CNPV and FPV. Similarly, **Sumaya. (2005)** detected a high immunological relationship between CNPV and FPV than FPV and PPV.

Without knowing more about the avian pox viruses, it is difficult to assess the threat as most of our knowledge concerning the avian pox viruses is based on clinical isolates from diseased birds. The infectious history of such isolates is rarely clear. It is important to characterizer these viruses at the molecular level to help identifying novel threats facing birds, whether captive, farmed or free-living, in response to natural or man-made changes to their habitats and avian contacts.

PCR amplification of the P4b (fpv167) gene revealed the highly conserved 578-bp APV-specific DNA fragment for all FPV and PPV isolates and vaccines. Based on the length of the amplicons, while the PCR of *fpv*140 gene for PPV and FPV isolates and vaccines locus revealed 1,800-bp PCR product as mentioned before **Jarmin et al., (2006), Manarolla et al., (2010) and Offerman et al. (2014).**

Sequencing of the P4b-578-bp DNA fragment was performed on FPV and PPV isolates and vaccines followed by Basic Local Alignment search Tool (BLAST) and phylogenetic analysis, revealed that fowl and piegon viruses belonged to the major clade A, while CNPV formed the major clade B. Notably, FPV (isolate and vaccine), two isolates of PPV (PPLA and PPLR) and vaccine strain (PPVV) were clustered with FPV at subclade A1, but pigeon pox virus isolate PPLH clustered with pigeon pox virus strains (accession numbers JQ665840 [El-Sharqya strain], AY530303, KJ913659, and

DQ873811) at subclade A2. This distribution comes in accordance with the data obtained from the BLAST analysis where PPLH showed a close identity of 99% with pigeon pox sequences in the gene bank (JQ665840- El-Sharqya strain). Contrarily, PPLA and PPLR strains isolated from pigeon cases in addition to the vaccine strain were widely related to pigeon pox sequences with 89% identity but closely related to fowl pox virus sequences with 97% identity. These data indicates that these pigeons were infected with a fowl pox virus. This is not surprising as viruses in *Poxviridae*, including avipoxviruses often cross species barriers **Weli et al.,(2004)** and the close contact between chickens and pigeons especially in open local markets directs us to the local concern that FPV of domestic birds could spread to pigeons. Changing of the host range was also contributed to significant recombination continues to occur among the avipox viruses strains in the endemic birds, which could generate new forms with possible differences in pathogencity and host range **(Thiel et al., 2005)**. Similar results were obtained by **Susan et al. (2014)** where indicated the high suspectability of pigeons to FPV infection where FPV (Giza isolate) gave pathogenic takes of 75% in pigeons.

In parallel, **Jarmin et al. (2006)** and **Luschow et al. (2004)** mentioned that there was a problem with the host species-based approach to the taxonomy of avipox viruses as sequences of isolates taken from particular species can be found in different subclades and as an example, the phylogenetic analysis of PGPV (PGPVTP2), placing it in a cluster with OSPV while sequence of a PGPV isolate (B7) clustered with CNPV like viruses in clade B, It is therefore apparent that viruses from clades A and B can infect and cause disease in pigeons.

The phylofenic analysis for fowl pox virus and vaccines comes in accordance with **Abdallah and Olla (2013), Masola et al. (2014) and Bithi et al. (2013)** as they proved that Tanzanian and Bengala FPV isolates and vaccine were 99.65 – 100% identical to each other and 99 – 100% identical to several published sequences of FPV isolates from various countries in different continents of the world, including Europe and Asia. Phylogenetic analysis revealed that all Tanzanian isolates belonged to clade A, subclade A1.

In conclusion, sequence analysis of the P4b of FPV isolate and vaccine, PPV isolates (PPLA and PPLR) and PPVV revealed that they belong to (genogroup A1= fowl pox) while PPV isolate (PPLH) belong to (genogroup A2 = pigeon pox) with the gene bank published sequences for pigeons.

RECOMMENDATION

Further molecular and immunological investigation on the strain confirmed to be pigeon pox virus (PPLH) to facilitate the production of PPV vaccine from the locally isolated strain (PPLH).

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