Detection and molecular characterization of avipoxviruses isolated from different avian species in Egypt

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Abstract Six clinical cases of avipoxvirus (APV) infection were investigated and molecular biologically studied. The samples were collected from different domesticated birds reared in the Egyptian backyard management system and were propagated on the chorioallantoic membrane of embryonated chicken eggs. The virus isolation was confirmed via PCR amplification of fpv167 (P4b) gene locus. All the studied isolates were characterized as Fowlpox-like viruses based on the amplicon length of fpv140 gene locus. The phylogenetic analysis of fpv167 (P4b) gene clustered Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV strains within subclade A1. Furthermore, Elsharqyia_PGPV strain was clustered within subclade A2 (Turkeypox virus) and showed 100 % nucleic acid identity with the wood pigeon Indian which was isolated in 2009. On the other hand, when the fpv140 gene was used for the phylogenetic analysis, Elsharqyia_PGPV was clustered within subclade A4 (Pigeonpox virus) with the other PGPVs. This study is considered the first molecular record for APVs circulating in the Egyptian birds. Further studies in a larger scale need to be developed to have a better understanding about the molecular characterization of the Egyptian APV strains.

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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 [8]. It has many species such as Fowlpox virus (FWPV), Turkeypox virus (TKPV), Pigeonpox virus (PGPV), Canarypox virus (CNPV), Ostrichpox virus (OSPV), Penguinpox virus (PEPV), Falconpox virus (FLPV), and Sparrowpox virus (SRPV), and many others recently emerged poxviruses in different avian species. There are about 9,000 bird species; 232 species of them have been recorded to have acquired a natural poxvirus infection and to have various forms of poxvirus infection [4]. 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) [19]. APV infection is usually associated with a low mortality rate in chickens and turkeys; however, during some APV outbreaks, the mortality rate can reach 65–100 %, especially in the Canary [18, 19]. FWPV has a double stranded DNA genome which contains a central coding region surrounded by two identical inverted terminal repeat regions. The genome size is 288 kbp approximately and encodes 260 open reading frames, of which 101 exhibit similarity to genes of known function [1]. fpv167 is a conserved gene among APVs that encodes orthologs of vaccinia virus core protein P4b. Polymerase chain reaction amplification of the fpv167 specific 578-bp fragment was applied to diagnose

APV infection [10]. Lüschow et al. [12] found that *fpv*167 (P4b) PCR amplification in combination with restriction endonuclease analysis and sequencing are rapid and effective diagnostic tools for APVs. Although it has been a successful diagnostic marker, PCR amplification of the P4b gene could not distinguish between different APV strains. However, sequencing and phylogenetic analysis of *fpv*167 (P4b) nucleotide sequence discriminate between different APV strains and revealed divergence among the different viruses that can be consistently correlated to the host species [21]. *fpv*140, an ortholog of vaccinia virus strain Copenhagen H3L, was also used in PCR amplification of APVs and interestingly it was useful in the differentiation between Fowlpox-like and Canarypox-like viruses on the basis of their fragment size. However, the routine usage of this gene for APV molecular characterization has some limitations. Since PCR of *fpv*140 was carried out for 15 isolates in northern Italy, eventually only seven of them gave positive results [13, 16].

Based on the phylogenetic analysis of *fpv*167 (P4b), APVs are divided into three clades; clade A (FWPV), clade B (CNPV), and clade C (Psittacinepox virus) [11, 13]. Jarmin et al. suggested additional nomenclature for the phylogenetic groups; FWPV (subclade A1), TKPV (subclade A2), Falcon and Albastrosspox virus (subclade A3), CNPV (subclade B1), SLPV (subclade B2), and PSPV (clade C). Although according to the phylogenetic analysis of *fpv*167 (P4b) PGPV could not be used as a specific nomenclature as it can be clustered in either subclade A2 or B2 [11], it has a specific nomenclature and can be distinguished from the TKPV and SLPV in a separate cluster (subclade A4) based on the phylogenetic analysis of *fpv*140 (H3L) nucleotide sequence [11].

Many studies have been designed to analyze different APVs from different geographic areas and study their phylogenetic relationship with each other. One such example, in northern Italy, sequencing analysis of 15 isolates revealed that most of the isolates belonged to either clade A or clade B, whereas only one isolate from Japanese quail was classified and clustered with clade C [11, 13]. Another example, the majority of New Zealand APV isolates belonged to A1 subclade; however, an isolate from a wood pigeon (kereru) belonged to subclade A3 [9]. In India, APV infection has been reported in different wild and domesticated birds [3, 14, 15]. The authors explained the recurring of FWPV infection in the backyard Indian chickens due to the integration of a near full-length reticuloendotheliosis provirus in the FWPV genome which leads to state of immunosuppression [3].

In Egypt, there are many reports discussing the isolation and the epidemiological studies of APVs [2, 6, 7, 17]. So far and up till now, there are no available data about the molecular characterization of APVs in Egypt and their phylogenetic relationship with each other and the other published strains in the database. In the present study, we provide the first report for the sequence analysis of the *fpv*167 (P4b) and *fpv*140 loci amplified from different APVs. Six isolates were recovered from different domesticated backyard bird species in the Elsharqyia province, 2011.

Results

Virus isolation

Virus isolation was performed on embryonated chicken eggs (ECE) obtained from non-vaccinated chicken flock. Pock lesions were evident on CAM after either the first (Elsharqyia_TKPV, Elsharqyia_PGPV, Elsharqyia_FWPV2, Elsharqyia_FWPV3, and Elsharqyia_FWPV4 isolates) or second (Elsharqyia_FWPV1) passage. Except Elsharqyia_PGPV, morphologically, the pocks were similar for most of the samples with grayish-white discoloration, compact shape, and marked thickening of the CAM. Elsharqyia_PGPV was isolated from the pigeon clinical sample and the pocks have a characteristic morphological shape with yellowish discoloration, nodular shape, and moderate thickening of the CAM.

Histopathology

Histopathologic examination of the cutaneous lesions revealed ballooning degeneration of the keratinocytes having large eosinophilic intracytoplasmic inclusions with central pale zone (Bollinger bodies). The virus replication evidences were observed in the CAM consisted of hyperplasia of the epithelium with cellular edema (hydropic degeneration) and Bollinger bodies (Fig. 1).

PCR and sequencing

PCR was performed for vaccinal strain (FWPFPV9) and skin lesions as well as pock lesions from CAMs. Two PCRs were performed with two sets of primers for the detection of APV-specific DNA (P4b-578 bp) and then discrimination between Fowlpox-like viruses and Canarypox-like viruses (*fpv*140 gene). In the first PCR, avian Poxvirus-specific 578-bp fragment was detected from the vaccinal strain (FWPVFPV9) and all the tested skin & CAM samples (Fig. 2a). All the amplicons from the second PCR were clustered as FWPV-specific with 1,800 bp (Fig. 2b). Sequencing of *fpv*167-578-bp and *fpv*140-1,800-bp fragments were carried out for six isolates which were
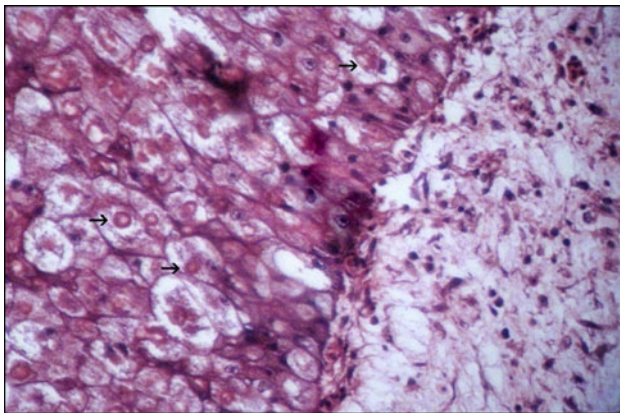
Fig. 1 Histopathology of APV lesions. Histopathology of CAM of ECE inoculated with APV isolate (Elsharqyia_PGPV) which was collected from Elsharqyia province in 2011 showing cellular edema (hydropic degeneration) with eosinophilic intracytoplasmic Bollinger bodies (indicated by *arrows*)
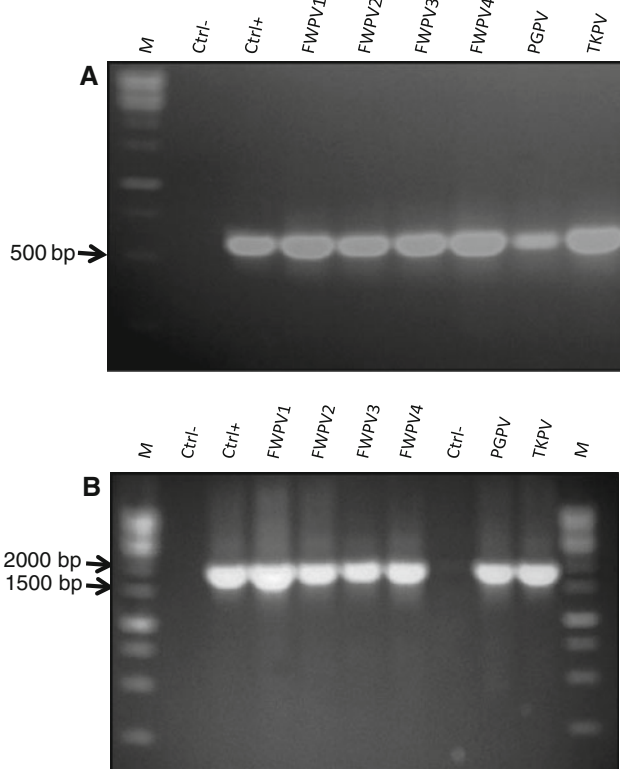

Fig. 2 a PCR amplification of the *fpv*167 (P4b) gene from APV isolates of the present study. *Lane M* 1-kbp DNA ladder (Fermentas), *CTRL−* negative control and *CTRL+* positive control (FWPVFP9). See Tables 1 and 2 for virus abbreviations. **b** PCR amplification of the *fpv*140 (H3L) gene from APV isolates of the present study. *Lane M* 1 kbp DNA ladder (Fermentas), *CTRL−* negative control and *CTRL+* positive control (FWPVFP9). See Tables 1 and 2 for virus abbreviations

named as Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, Elsharqyia_TKPV, and Elsharqyia_PGPV. The obtained nucleotide

sequences were submitted to the GenBank under accession number (Table 1).

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

The amino acid sequence alignment analysis of one hundred sixty-eight amino acids (truncated to have the same length) of the six isolates was performed using CLC main workbench. The obtained sequences were aligned with the published APVs-P4b amino acid sequence of strains FWPVFPV9, TKPV13401, and PGPVP with the accession numbers AJ581527, AY530304, and AM050385, respectively, which were used as reference strains. The alignment analysis showed that 100 % amino acid identity between the Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV strains compared to FWPVFPV9 and TKPV13401. Consistently, Elsharqyia_PGPV showed 100 % amino acid identity compared to PGPVP as a reference strain. On the other hand, the comparative alignment of Elsharqyia_PGPV revealed 98.2 % amino acid sequence identity to Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV (S1).

For the phylogenetic analysis of the studied strains, the nucleotide sequences of 37 strains isolated from 16 different bird species were aligned (Table 2). The phylogenetic analysis was performed for 428 truncated sequences by neighbor-joining (N-J) analysis with bootstrapping (1000). As previously shown, the DNA tree of APVs branched into three major clades: A (Fowlpox-like viruses), B (Canarypox-like viruses), and C (Psittacinepox-like viruses). Clade A is further subdivided into subclade A1, A2, A3, and A4, whereas clade B is subdivided into subclade B1 and B2. The five strains, Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV, were clustered in subclade A1 and showed 100 % nucleotide identity with the other FWPV strains in addition to TKPV13401 and SRPVDD1258 strains. Viruses in subclade A1 showed 90.89, 91.82, and 88.32 % nucleotide identity to the other viruses in subclade A2, A3, and A4, respectively. Furthermore, they showed an average of 76.6 and 75.85 % nucleotide identity to CNPV-like group of viruses (Clade B) and Psittacine viruses (Clade C), respectively. Elsharqyia_PGPV strain was grouped in subclade A2 together with the other TKPVs (TKPV66 and TKPV98), in addition to PGPVP, PGPVTP-2, PEPV, and OSPV strains. It showed 90.89, 100, 97.66, and 89 % nucleotide identity to the viruses in subclade A1, A2, A3, and A4, respectively. In addition, it showed an average of 75.7 % nucleotide identity to the Canarypox-like virus isolates

Table 1 Details of APV strains used in this study

Strain name	Host	Nature	Date	Accession number	
				*fpv*167 locus	*fpv*140 locus
HP1-444 (FP9)	Chicken	Commercial vaccine	–	AJ581527	AJ581527
Elsharqyia_FWPV1	Chicken	Clinical isolate	June, 2011	JQ665838	JX464822
Elsharqyia_FWPV2	Chicken	Clinical isolate	June, 2011	JX464819	JX464823
Elsharqyia_FWPV3	Chicken	Clinical isolate	August, 2011	JX464820	JX464824
Elsharqyia_FWPV4	Chicken	Clinical isolate	September, 2011	JX464821	JX464825
Elsharqyia_TKPV	Turkey	Clinical isolate	September, 2011	JQ665839	JX464826
Elsharqyia_PGPV	Pigeon	Clinical isolate	November, 2011	JQ665840	JX464827

(Clade B) and 75.15 % nucleotide identity to Psittacinepox-like virus isolates (Clade C) (Fig. 3).

Alignment and phylogenetic analysis of *fpv*140 (H3L) gene

The alignment analysis of the full length amino acid sequences of *fpv*140 (H3L) gene from the six isolates was performed using CLC main workbench. The obtained sequences were aligned together and with the published APV-H3L amino acid sequence of strains FWPVFPV9, TKPV66, and PGPVP with the accession numbers AJ581527, AM071390, and AM071389, respectively. The alignment analysis revealed 100 % amino acid identity between the Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV isolates. The five isolates showed 99.7 and 87.13 % amino acid identity when they were aligned with the reference strains FWPVFPV9 and TKPV66, respectively. Elsharqyia_PGPV showed 84.90 % amino acid identity compared to PGPVP as a reference strain. On the other hand, the comparative alignment of Elsharqyia_PGPV revealed 74.26 % amino acid identity to Elsharqyia_FWPVs and Elsharqyia_TKPV. Remarkably, insertion of 13 amino acid residues (FILSSYVL amino acids 279–286) and (WEIRY amino acids 293–297) were observed in Elsharqyia_PGPV-*fpv*140 and were not present in PGPVP strain (S2). In addition, Elsharqyia_PGPV-*fpv*140 has a deletion in 9 amino acid residues (GLAIFDVNN amino acids 310–318) which are present in PGPVP strain.

For the phylogenetic analysis of the studied strains, the nucleotide sequences of 16 strains isolated from eight different bird species were aligned (Table 2). The phylogenetic analysis based on *fpv*140 gene sequences was performed for 896 truncated sequences by neighbor-joining (N-J) analysis with bootstrapping (1000). As previously shown [11], the DNA tree of APVs based on *fpv*140 gene sequences branched into two major clades: A and B. Clade A is further subdivided into subclade A1 (Fowlpox virus), A2 (TKPV), A3 (Falcon and Albastrosspox Virus), and A4 (PGPV), whereas clade B is a Canarypox virus. Elsharqyia_FWPVs

and Elsharqyia_TKPV strains were clustered in subclade A1 with the other FWPV strains. They showed an average of 99.87, 87, 89.39, and 88.24 % nucleotide identity to the other strains in subclade A1, A2, A3, and A4, respectively, and 66.67 % nucleotide identity to CNPV-like group of viruses (Clade B). Elsharqyia_PGPV strain was clustered in subclade A4 together with the other PGPVs (PGPVP and PGPVIndia_08_2009). Hence, it showed an average of 87.58, 94.8, 97, and 98.7 % nucleotide identity to subclade A1, A2, A3, and A4, respectively, and 66.50 % nucleotide identity to the CNPV-like group of viruses (Clade B) (Fig. 4).

Discussion

Most of our knowledge about the situation of APVs in Egypt was obtained from a very few reports [2, 7, 17] concerning the epidemiology and the infection biology of the virus. The incidence of APV infection has been recorded before among different avian species in the Elsharqyia Province in a period of 2 years (Late summer 1996–Spring 1998) [6]. However, and up till now, there was no available record of the molecular composition of APVs circulating in Egypt. In this study, a small scale survey was conducted to understand the nature of APVs isolated from different backyard domesticated birds in the Elsharqyia province (Egypt). The samples were collected during the summertime in 2011 as it is the prime mosquito season in Egypt [19, 22]. Six APVs were isolated from chickens, turkeys, and pigeons on CAM of ECE. For most of the samples, the pocks were clear after the first passage suggesting their ability for embryonic adaption as previously reported [13]. The evidence of virus growth was confirmed by the histopathologic examination of Pox virus lesion on CAMs. Moreover, the isolated viruses were molecular biologically analyzed and characterized. As previously shown, PCR amplification of *fpv*140 and *fpv*167 gene loci were used in many reports for the molecular identification and characterization of the APV genome in a different era all over the world [11–13, 20]. Consistently, in

Table 2 Details of APVs obtained from GenBank

Strain name	Abbreviation	Host	Accession number	
			*fpv*167 locus	*fpv*140 locus
HP1-444 (FP9)	FWPVFPV9	Chicken	AJ581527	AJ581527
Nobilis Variole W	FWPVN	Chicken	AM050379	AM071394
Diftosec CT	FWPVD	Chicken	AM050380	AM071395
AT_Reidling/68/7/2008	FWPVAT_Riedling_68_7_2008	Chicken	GU108506	–
HP-B	FWPVHPB	Chicken	AY530302	–
PA10-14263	FWPVPA10_14263	Turkey	FR852585	FR852587
GB 134/01	TKPV13401	Turkey	AY530304	–
10/12/98	TKPV98	Turkey	AM050388	AM071391
2/11/66	TKPV66	Turkey	AM050387	AM071390
2008/India/02	FWPV2008_India_02	Golden pheasant	HM481403	HM481411
2008/India/05	FWPV2008_India_05	Silver pheasant	HM481406	HM481414
Peekham 19/11/75	PGPVP	Pigeon	AM050385	AM071389
TP-2	PGPVTP-2	Pigeon	AY530303	–
950 24/3/77	PGPV950	Pigeon	AM050386	–
B7	PGPVB7	Pigeon	AY453177	–
India_2006	PGPVIndia_2006	Pigeon	DQ873811	–
India_08_2009	PGPVIndia_08_2009	Pigeon	HM481409	HM481417
San92	PEPV	Penguin	FJ948105	FJ948106
GB 724/01-20	OSPV	Ostrich	AY530305	–
Fort Dodge	CNPV	Canary	AM050384	–
1445/97/33	CNPV1445	Canary	AM050375	AM071512
1381/96	FLPV1381	Falcon	AM050376	AM071515
GB362-02	FLPV36202	Falcon	AY530306	–
APIII	AGPV	Agapornis	AY530311	–
353/87	ABPV	Black-browed albatross	AM050392	AM071388
GTPV-256	GTPV-256	Great tit	AY453175	–
GTPV-A310	GTPVA310	Great tit	AY453173	–
GTPV-A311	GTPVA311	Great tit	AY453174	–
GB 182/98	SCPV18298	Stone curlew	AY530310	–
1305/86	MCPV	Macaw	AM050382	–
27	SLPV	Starling	AM050391	–
364/89	PRPVCVL364_89	Parrot	AM050383	–
2009/India/06	SRPV06	Sparrow	HM481407	–
DD1258	SRPVDD1258	Sparrow	AY530307	–
GB 320/02	SRPV32002	Sparrow	AY530308	–
9037 31/5/66	SRPV9037	Sparrow	AM050389	AM071511
9037 31/5/66/23	SRPV23	Sparrow	AM050390	AM071510

our studies, PCR amplification of the P4b (*fpv*167) gene revealed the highly conserved 578-bp APV-specific DNA fragment. Based on the length of the amplicons, the amplification of *fpv*140 locus allows the discrimination between FWPV and CNPV [11]. Since the PCR of *fpv*140 gene locus revealed 1,800-bp PCR products, all the studied isolates in the present study were classified as FWPV or FWPV-like viruses [11]. This result in contrast to the

previous finding [13], as it was suggested the inapplicability of *fpv*140 gene as a diagnostic marker for APVs. Sequencing of the P4b-578-bp DNA fragment was performed on six isolates from different host species. The sequencing results revealed that all the isolates belonged to the major clade A according to [11]. Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV showed 100 % identity
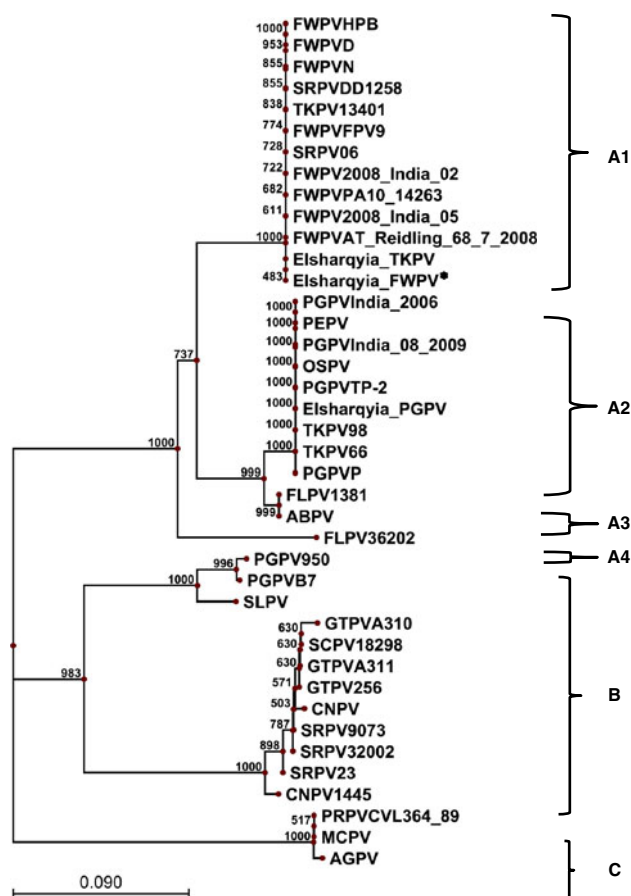
Fig. 3 Phylogenetic analysis of APVs based on *fpv*167 (P4b) gene nucleotide sequences. Phylogenetic tree was constructed via multiple alignments of 428-bp nucleotide sequence of P4b gene from 43 APV strains. The tree was analyzed by neighbor-joining (N-J) analysis with bootstrapping (1000). APV clades and subclades are labeled. (*Asterisk*) Elsharqyia_FWPV label represents Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, and Elsharqyia_FWPV4. See Tables 1 and 2 for virus abbreviations

and clustered in the minor subclade A1 (FWPVs) with the two commercially available vaccines FWPVN and FWPVFPV9, as well as the other poxviruses detected from chickens, turkeys, and sparrows. It is not obvious if the clustering of the Elsharqyia_TKPV with the FWPVs in the same subclade is due to the emergence of FWPVs to the turkey host or both viruses are closely related [1, 12]. The five strains, Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV, revealed mean distance percentage of 28 and 29 % with clade B and C, respectively.

As previously mentioned by Jarmin et al. [11], PGPV would not be used as an independent taxonomic unit based on the phylogeny of *fpv*167 (P4b) gene as viruses from clades A and B can infect and cause disease in pigeons. Elsharqyia_PGPV was clustered in the minor subclade A2 together with TKPVs, PEPVs, OSPVs, and other PGPVs. Hence, it was the nearest phylogenetic neighbor and
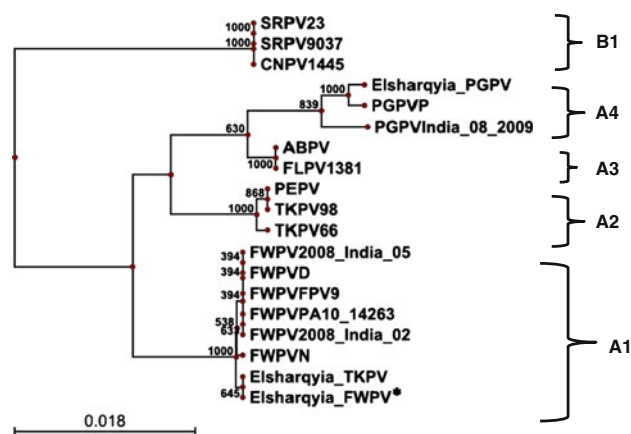

Fig. 4 Phylogenetic analysis of APVs based on *fpv*140 (H3L) gene nucleotide sequences. Phylogenetic tree was constructed via multiple alignments of 892-bp nucleotide sequence of H3L gene from 22 APV strains. The tree was analyzed by neighbor-joining (N-J) analysis with bootstrapping (1000). APV subclades A1, A2, A3, A4, and B1 are labeled. (*Asterisk*) Elsharqyia_FWPV represents Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, and Elsharqyia_FWPV4. See Tables 1 and 2 for virus abbreviations

showed 100 % nucleic acid identity with the wood pigeon Indian isolate (PGPVIndia_08_2009) and the unknown origin pigeon isolates (PGPVP & PGPVTP-2) [11, 12]. In addition, Elsharqyia_PGPV revealed the mean distance percentage of 28 and 30 % with clade B and C, respectively.

The phylogenetic analysis of the *fpv*140 gene allows easily differentiation between the different viruses: subclades A1 (FWPV), A2 (TKPV), A3 (FLPV), A4 (PGPV), and B1 (CNPV) [5, 11, 15]. Consistently, in our study, the phylogenetic analysis of the *fpv*140 from the Egyptian APVs clustered the five viruses isolated from chicken and the one from turkey in subclade A1 (FWPV). The five strains revealed a distance percentage of 56.9 % from clade B. On the other hand, it places Elsharqyia_PGPV with the other PGPVs in the subclade A4 with a distance percentage of 53.9 % from clade B. Our study also supports the previous finding [11, 15] that the phylogenetic analysis of APVs based on the *fpv*140-H3l gene allows clustering of the PGPV strains in an independent taxonomic unit which correlates with the pigeon host species.

In conclusion, sequence analysis of the APV-*fpv* 167 and APV-*fpv*140 revealed that Elsharqyia_FWPV1, Elsharqyia_FWPV2, Elsharqyia_FWPV3, Elsharqyia_FWPV4, and Elsharqyia_TKPV belong to Fowlpox-like viruses (subclade A1). None of the analyzed strains belong to either the Canarypox-like virus isolates (Clade B) or Psittacinepox-like virus isolates (Clade C). The phylogenetic analysis of the *fpv*140 allows better discrimination for Elsharqyia-PGPV (subclade A4) from the FWPV strains. Taken together, this small scale study provides the first molecular characterization of Egyptian APVs. To have a better understanding about the genetic

composition of APVs and their relationship to each other and to their host species a larger scale survey needs to be performed. Hence, it should include a wider range of hosts and kinds of management system to study the evolution of APVs in Egypt.

Materials and methods

Viruses

Six isolates of APV from different avian species were used in this study. These viruses were isolated from chickens, turkeys, and pigeons which were reared in the backyard management system of some villages in Elsharqyia province, Egypt. These birds were suffering from the skin lesions of APV infection on the comb, eyelids, beak, and wattles as well as the diphtheritic membrane on the mucous membrane of the upper respiratory tract. The affected flocks suffered from low or even no mortality except in case of pigeon; hence, there was a high level of mortality among the squabs reach to 30 %. Commercially available live-vaccinal strain against FWPV (FWPVFPV9) was also included in the study as shown in Table 1.

Virus isolation in embryonated chicken eggs (ECE)

Scab samples from each clinical case were minced separately using sterile iced mortar and pestle, suspended in phosphate buffer saline containing 1,000 IU/ml penicillin, and 100 μg/ml streptomycin. Sample suspensions were centrifuged at 2,000 rpm/10 min, thereafter 100 μl from each sample supernatant was separately inoculated onto chorioallantoic membrane (CAM) of 13-day-old ECE from non-vaccinated flock. The inoculated eggs were incubated at 37 °C for 5 days and then examined for focal white pock lesions or generalized thickening of the CAMs. Portions of CAM showing lesions were frozen at-20 °C and were used for DNA extraction.

Histopathology

Both skin and pock lesions were fixed in 10 % buffered formalin and embedded in paraffin. Thin sections of the fixed lesions were stained with hematoxylin and eosin stain and examined microscopically for the presence of intra-cytoplasmic inclusion bodies.

DNA extraction

DNA was extracted from 20 mg of frozen cutaneous lesion and CAMs as well as 20 mg of lyophilized vaccine using GeneJET™ genomic DNA Purification Kit (Fermentas) following the manufacturer's instructions.

PCR amplification

PCR for amplification APVs specific fragments (fpv167 and fpv140 genes) were performed. Primers described previously [10, 13] were used to amplify the FWPV P4b gene (fpv167 locus) (Forward primer: 5′-CAGCAGGTGC TAAACAACAA-3′ & reverse primer: 5′-CGGTAGCTTA ACGCCGAATA-3′) as well as fpv140 gene (Forward primer: 5′-GAAGTAGAGTTATCGGTTC-3′ & reverse primer: 5′-GGTGATCCATTTCCATTTC-3′). Amplification was performed in an Eppendorf thermal cycler (MWG) under the following conditions: cycle at 95 °C for 2 min (initial denaturation), 40 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min), and cycle of final extension at 72 °C for 10 min. Negative controls were included in each assay for detection of any contamination using Dream Taq™ Green PCR Master Mix (2×) (Fermentas). Five microliters of amplified PCR products were separated by 1 % ethidium bromide strained agarose gel electrophoresis at 120 V for 20 min. 1-kbp DNA Marker (Fermentas) was used as standard and the amplified products were visualized using ultraviolet light transilluminator (Spectroline).

Purification and sequencing of PCR product

PCR products were purified using Gene JET PCR purification kit (Fermentas) and resuspended in 50 μl H₂O. Each purified amplicon was sequenced in both forward and reverse directions using the amplification primers. The sequencing reaction was performed in an automated sequencer (Macrogen Inc., Korea ABI 3730XL DNA analyzer). The sequences were submitted to the Genbank database under accession numbers which were listed in Table 1.

Phylogenetic analysis

Comparative alignment and phylogenetic analyses were performed computationally by means of CLC main workbench program. The phylogenetic tree was generated by N-J tree method and the liability of internal branches was assessed by 1000 bootstrap replications.

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