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RESEARCH ARTICLE
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Molecular identification of local field isolated fowl pox virus strain from Giza governorate of Egypt

Susan S. El-Mahdy[1], M. H. H. Awaad[2] and Y. A. Soliman[1]

1. Central Laboratory for Evaluation of Veterinary Biologics (CLEVB),-El-Seka El-Baida St-Abbasia 131,Cairo,Egypt;
2. Department of Poultry Diseases, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.
Corresponding author Susan S. El-Mahdy, email: prof.s.elmahdy@gmail.com
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Abstract

Aim: Molecular identification of field isolated pox virus from infected chickens in Egyptian farms in 2012 by polymerase chain reaction (PCR).

Materials and Methods: Isolation and identification of fowl pox virus (FPV isolate ch-08TK) from 30 day-old chickens manifesting pox lesions. The isolate was propagated successfully on chorioallantioc membrane of specific pathogen free (SPF) embryonated chicken eggs and clear pock lesions were observed. These lesions were homogenated and used to infected SPF chickens and pigeons (via wing web route for chickens and via feather follicle route into the thigh of pigeons) using $10^{4.5}$ EID_{50}/mL; uninoculated birds of each species were used as negative controls for determining the effect of isolated FPV strain.

Result: The isolated strain gave pathogenic takes 100% in chickens and 75% in pigeons. Virus identification by PCR was done using dream Tag master mix kit using the primers that targeted thymidinekinase (TK) gene. These primers were designed using Lasergene DNASTAR software Version 10. We used these primers to amplify a 305 bp fragment of the TK gene of FPV. Phylogenetic analysis of sequenced TK amplicone which reflects a new emerging isolate of the field isolated FPV gave very limited similarity (not exceeding 60%) with the published sequences. Thus FPV isolate ch-08 TK gene (with Accession No. KF314718 in Gen Bank) is different than canary, Egypt, 2012, P4b and Elsharqyia-FWPV2 FPV140 (FPV140); TKPV FPV140 (FPV140) and PGPV FPV (FPV140) which have been isolated from cases of avipox virus in 2011 from skin crust of different domesticated birds reared under the Egyptian backyard management system. Our sequencing and phylogenetic analysis of newly isolated virus using DNASTAR software Version 10 revealed that this virus differ from canary, Egypt, 2012, P4b and Elsharqyia-(FWPV2 FPV140 (FPV140); TKPV FPV140 (FPV140) and PGPV FPV (FPV140)) according to published data in Gen Bank.

Conclusion: FPV (isolated ch-08 TK gene with Accession number KF314718 in Gen Bank**)** was isolated from 30 day old laying chickens suffering from pox lesions. We believe that this study is the first molecular identification of FPV strain from laying chickens in Egypt.

Key words: Egypt, fowl pox virus strain, local field isolate, molecular identification.

Introduction

Avipox virus (APV) infection is a highly contagious disease of birds and has been reported in more than 200 species of birds and affects domesticated and free-ranging birds around the world [1]. APV infection is caused by a large DNA virus that belongs to the genus Avipox virus within the subfamily Chordopoxvirinae in the family Poxviridae [2]. Pox viruses are different from other DNA viruses in that the viruses replicate and mature in the cytoplasm of the infected cells [3]. Wild birds [4,5] and insects [6] play an important role in the spread of pox infection. The DNA of fowl pox virus (FPV) contains approximately 288 to 300 kilo base pairs (Kbp) [2,7]. The shape of pox viruses resemble around brick [8]. It is slow spreading and is characterized by formation of proliferative lesions and scabs (dry form) on skin, and diphtheritic lesions (wet form) in the upper part of digestive and respiratory tracts [2].

Fowl pox (FP) is of considerable economic importance as the disease can result in a drop in egg production, or retarded growth in younger birds. The chances of mortality increase when the dry form occurs together with the wet form [9]. The general signs of the diseases include weight loss, loss of feathers and scaly skin of the head, neck and back. Secondary bacterial infections are common with both forms of the disease, having the potential to cause pneumonia or other bacterial infections at the site of blistering [5]. Integration of reticuloendotholiosis virus (REV) sequences has been observed in the genome of FPV [10,11]. Virulence is enhanced by the presence of REV provirus in the genome of field strains of FPV. Complete sequence of the genome of a vaccine-like strain of FPV has been determined [7,12]. The virus tends to persist in the poultry environment for extended periods of time where other viruses may not survive. In this regard the presence of photolysis gene and A-type inclusion body gene in the virus genome appear to protect the FPV from environmental insults [13,14]. Antigenic cross reactivity is observed among APVs and it appears that

many genes are conserved. Restriction fragment length polymorphism (RFLP) analysis can be used for comparison of field isolates and vaccine strains of FPV [15,16]. Cloned genomic fragments of FPV can be used effectively as nucleic acid probes for diagnosis of fowl pox. Viral DNA isolated from lesions can be detected by hybridization either with radioactively or non-radioactively labeled genomic probes [17]. Genomic DNA sequence of various sizes can be amplified by the polymerase chain reaction (PCR) [17,18]. PCR technique is very sensitive, especially when small amount of virus in present in the sample [7].

Our study was planned for molecular identification of field isolated pox virus from infected chickens in Egyptian farms in 2012 by PCR using specific primers.

Materials and Methods

Sample collection: Nodular lesions were collected aseptically from a commercial layer chicken flock in Giza governorate at end of 2012. The flocks have nodular lesions on the combs; corners of the mouth and around the eyelids. The lesions started from 19th day of age and with 10% mortality rates; these lesions persisted in the flock till 30 day of age. There was no history of previous pox vaccination in this flock.

Virus isolation using embryonated SPF eggs [19,20]: Nodular lesions from infected birds were removed with sterile scissors and forceps by cutting deep into the epithelial tissue. The materials are ground and the 10% W/V tissue homogenates were prepared in Hank's balanced salt. Suspension is centrifuged for 10 min at about 700 Xg. to remove the large tissue particles. Antibiotics (penicillin & streptomycin) were added to the supernatant to give respective final concentrations of 1000 IU/ml and 1mg/ml. The suspension was held at room temperature (25 ^{O}C) for 30 min. About 0.1 ml of suspension was inoculated onto the chorioallantoic membranes (CAMs) of 10-12 day- old SPF embryonating chicken eggs obtained from SPF production farm; Koum Osheim; El-Fayoum, Egypt. This farm is a part of the Ministry of Agriculture. Inoculated embryos were incubated at 37^{o} C, observed and candled for 5 days. Five non inoculated eggs were kept as negative controls. Five days post inoculation; CAMs were cut, opened and the white pock lesions and generalized thickening areas were collected, pooled and used for further passages on CAMs.

Virus titration: Titration of Fowl Pox isolate was performed in SPF emberyonated chicken egg (ECE) [21]. Ten-fold serial dilutions of isolate were obtained and 5 embryos (10-12 days old) used for dilution were inoculated with 0.2 ml in PBS with pH 7.2 via the CAM route. All deaths within 24 hours post inoculation were not considered. The survived embryos were examined for evidence of infection. Demonstrated pock lesions or generalized thickening of the CAM, on the 5th day post inoculation ID_{50} was calculated as described earlier [22].

Pathogenicity test: Pathogenicity test was carried out as per method described by AAAP [23] for determining the effect of isolated FPV in different species. 25SPF chickens and 20 pigeons (28 days old) were inoculated with $10^{4.5}$ EID_{50}/ml via wing web route for chicken and via feather follicle route in the thigh to pigeons. Five birds per each species were left without inoculation as negative control. All birds were examined for takes formation in case of chickens and for thickening of skin of the thighs in case of pigeons at inoculation site at 3rd to 10th day post inoculation. Additionally, birds were examined for any deaths or symptoms related to avian pox virus.

Virus purification and DNA extraction: The procedure for virus purification was as detailed earlier [24]. After observation of white pock lesions on CAMs in infected SPF eggs with nodular lesions; these CAMs were scrapped into the fluid Tris-EDTA (TE pH 8.0) and pelleted by centrifugation at 5000 Xg for 30 min. The pellet was suspended in 1 m M Tris-HCL pH 8.0 and sonicated at 80% amplitude in an ultrasonic disintegrator five times for 10 s with intervals of 30 s. The supernatant was collected by low speed centrifugation and suspended in proteinase K buffer and digested with proteinase K (1 mg /mL) at 37^{o}C for 2h. The viral DNA was extracted according to the standard procedures by using Mega DNA extraction kit (Biobasic Cat # 00086242) according to the manufacture procedures.

Virus identification by PCR: The CAMs collected at 5 days post inoculation in SPF eggs were serially diluted and used in reverse transcriptase PCR (RT-PCR). Specific primers were chosen accordingly to the TK gene sequence of an FPV strain [25]. Direct PCR was done using dream Taq master mix Kit (Fermantase cat#00041067) using the primers that target thymidine kinase (TK) gene These primers were designed using Lasergene DNASTAR software Version 10. The amplicons were electrophoresed on a 1% agarose gel and visualized under a U.V. transilluminator. A 305 bp fragment of TK gene FPV was amplified. The primer pair designated and had the following sequences:

FP-F 5'-TAG-AAG-CAT-CCA-TGT-TAT-TACA-3'

FP-R 5'-GTT-AAG-CGC-GGC-CAC-AA-AC-3'

A commercial FPV was used as positive controls in PCR.

DNA sequencing: The complete nucleotide sequence of the amplicon was performed in (Macrogen USA). For preparation of the gene for sequencing, the PCR product was separated on 1% low melting agarose. The bands were sliced off and purified with the biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacturer. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), Sequencing Technology (Sanger dideoxy sequencing).

Figure-1. Head of the infected chicken: Notice that infected head showing crusts formation on the comb and on the eyes.

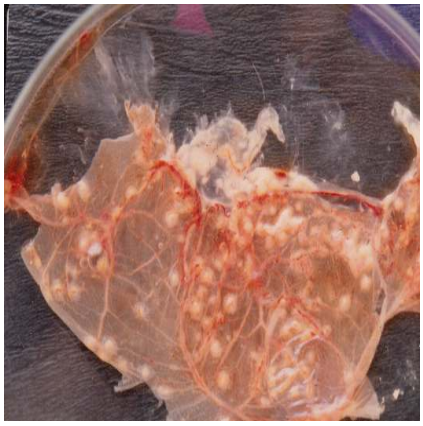

Figure-2. CAM of SPF embryonated chicken eggs inoculated with supernatant fluid of samples collected from infected chicken: Notice that focal white opaque pocks with a generalized thickening of the inoculated CAM with supernatant fluid of samples collected from infected chicken.

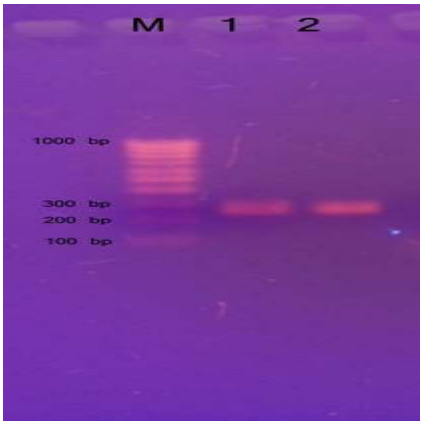

Figure-3. PCR of genomic DNA extracted from Pock lesion: PCR amplification: A 305 bp length amplicon was amplified either from the crusts or the propagated virus on CAM. The amplified TK conservative region from either the crusts (Lane 1) or propagated virus on CAM (Lane 2). Note that the amplicons migrate at about 305bp . M a 100 bp DNA ladder.

Phylogenetic analysis: The nucleotide sequence analyses and construction of the phylogenetic trees were performed using Lasergene DNASTAR software Version 10.

Results

Egg passage: After Passage in SPF embryonated eggs, lesions on CAMs were observed in the form of focal white opaque pocks with a generalized thickening of the CAM (Fig.2).

Virus titration: Isolated pox virus has $10^{4.5}$ EID_{50}/ dose.

Fowl pox DNA extraction and detection of DNA of FPV strains: Following proteinase K digestion of the purified virions the extracted FPV DNA from a representative strain VI was treated with Eco R1 and was separated on a 1-2 % agarose gel. The extracted DNA was identified as the FPV genome and used for detection of FPV strains using PCR. A DNA fragment with a length of 305 bp was amplified from FPV strain in accordance with the reaction cycle (Fig. 3).

Virus specificity and sensitivity: PCR of FPV strain VI was carried out by applying the primer set for testing the specificity of detection of viral DNA.

Uninfected CAMs were used as negative control and total nucleic acids extracted from CAMs containing FPV were used as positive control. The results showed that no fragment could be amplified with the nucleic acids from any of control cells, however, it could be detected with the nucleic acids extracted from the FPV strain VI.

To assess the sensitivity of detection viral DNA by PCR of a fragment of size 305bp was simplified in a serial 10-fold dilution of infected CAMs with strain VI. After electrophoresis, specific bands of expected size were visible in the sample.

Phylogenic analysis of Avipox viruses TK gene: The isolated strain from layer chicks is shown in (Fig.4-6).

Pathogenicity test: Inoculation of the isolated virus in SPF chickens revealed that about 100% showed takes at fourth day post inoculation at the sites of inoculation. While 15 of the inoculated pigeons showed thickening of the thighs at sites of inoculation in pigeons neither negative control chickens nor pigeons showed lesions, symptoms related to avian pox infection.

Discussion

Genus Avipox includes FPV or FWPV; Pigeons Pox Virus (PPV); Turkey Pox virus (TKPV); Canary Pox virus (CNPV); penguin pox virus (PEPV) and other species [26]. Fowl pox has a world-wide distribution and is caused by a DNA virus of the genus Avipox viruses of the family poxviridae [2]. It is a slow spreading virul disease of chickens. All avian species are susceptible to avian pox-as indicated by the fact that natural pox infections have been reported in several species; wild birds of different families as well as in domestic birds [5,27,28]. Most of field strains contain REV provirus. Virulence of virus is enhanced by presence of REV provirus in the genome of field strains of FPV. Restriction fragment length polymorphism (RFLP) analysis can be used for comparison of field isolates and vaccine strains of FPV [29]. DNA of FPV can be detected successfully by using PCR [25,30].

Virus isolation: In this study, poxvirus was isolated on CAMs of SPF chicken egg and further identification was carried out by PCR and sequencing. This technique is useful when there is only extremely small amount of viral DNA in the sample [21]. Sample collection was carried out according to [2]. Lesions detected in Egyptian farms in 2012 in our study agree with previously reported study [31] which isolated FPV from one month old local breed chickens in Grenada with gross lesions in the skin of the head region. Virus isolation on CAM was done [21] and we observed focal white opaque pocks with a generalized

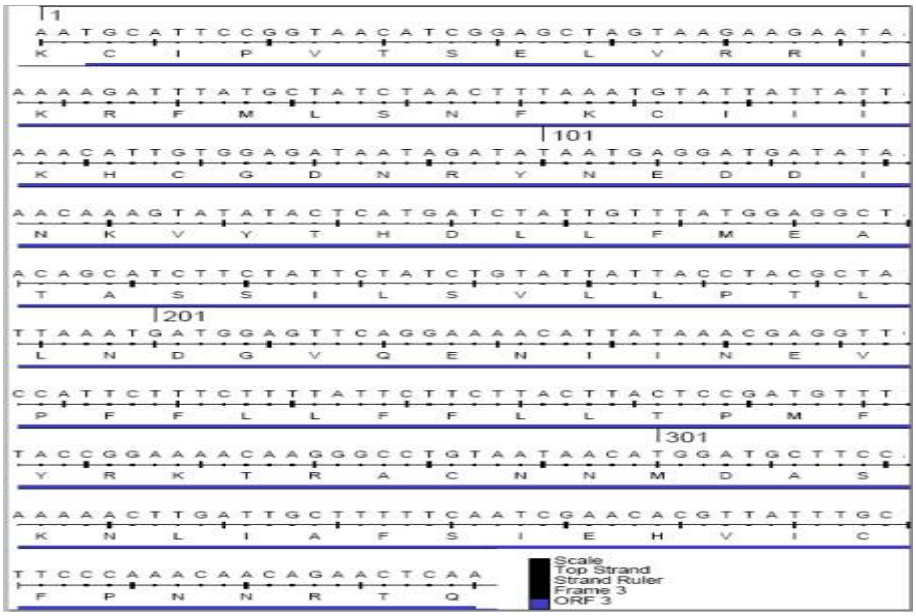

Figure-4. Phylogenetic analysis of Avipoxviruses TK gene. Notice the sequence analysis of the TK gene of the field isolate of FPV

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Fowlpox virus (isolate HP-438[Munich]), passage 438 clone FP9, complete genome	370	370	60%	5e-99	95%	AJ581527.1
Fowlpox virus thymidine kinase gene, complete cds	370	370	60%	5e-99	95%	AF396867.1
Fowlpox virus, complete genome	370	370	60%	5e-99	95%	AF198100.1
Fowlpox virus strain HP-440 DNA, isolate FP9, 14.6 kb fragment	370	370	60%	5e-99	95%	AJ223385.1
Fowlpox virus thymidine kinase gene, complete cds	370	370	60%	5e-99	95%	M16617.1
Fowlpoxvirus genes for unknown ORF and thymidine kinase, complete cds	370	370	60%	5e-99	95%	D00321.1
Fowlpox virus vaccine strain thymidine kinase gene	370	370	60%	5e-99	95%	X52860.1
Fowlpox virus nonfunctional thymidine kinase gene, partial sequence	318	318	54%	2e-83	94%	FJ986112.1
Fowlpox virus TK gene for thymidine kinase	73.1	272	38%	2e-09	100%	X12700.1

Figure-5. The blast search analysis of the sequenced TK amplicon of the field isolated FPV. Notice that there is a very limited similarity (not exceeding 60%) with the published sequences.

thickening of the CAM (Fig. 2). Similar results have been previously reported with variable levels of thickening, ranging from mild to severe, in CAMs infected with avian pox virus isolated from Italy [32]. There have been many reports that describe differences in growth characteristics of the orthopoxviruses and factors that influence poxvirus growth on CAMs which include incubation temperature, age of the embryos and the source of eggs; so the variability in pock color has also been ascribed to mutation of specific viral genes. [33].

Extraction and detection of DNA: Results of-extraction and detection of DNA of FPV strains which gave a fragment of 305bp (Fig. 3) agree with that reported by other authors where application of the PCR for the diagnosis of FPV infection gave a positive 578bp fragment [34] and with others who also used PCR for identification and characterization of FPV strain [11,35]. Data obtained by others [30] also adds more support to our results.

Specificity and sensitivity of FPV: Our results agree with previous studies [36-38]. Based on the definition, at least one infectious unit is required for the isolation of virus, indicating that PCR may also detect noninfectious virus particles present in the sample preparations. In addition, this primer set appears to be specific for FPV DNA, because it did not amplify the DNA sequences of nucleic acid preparation from uninfected tissue. Furthermore, nucleic acid extracted from unrelated pox virus was not amplified with this primer set. Thus, PCR seems to be rapid, specific and highly sensitive and could become a powerful tool for the detection sensitivity of FPV infections [34].

Pathogenicity test: Inoculation of the isolated virus into SPF chickens revealed that about 100% showed takes at fourth day post inoculation in wing web sites of inoculation, while 75% of the inoculated pigeons showed thickening of the thighs (sites of inoculation in pigeons). In addition, neither negative control chickens nor pigeons showed lesions or symptoms related to avian pox infection. These results indicate that the isolated strain is more related to chicken than pigeon.

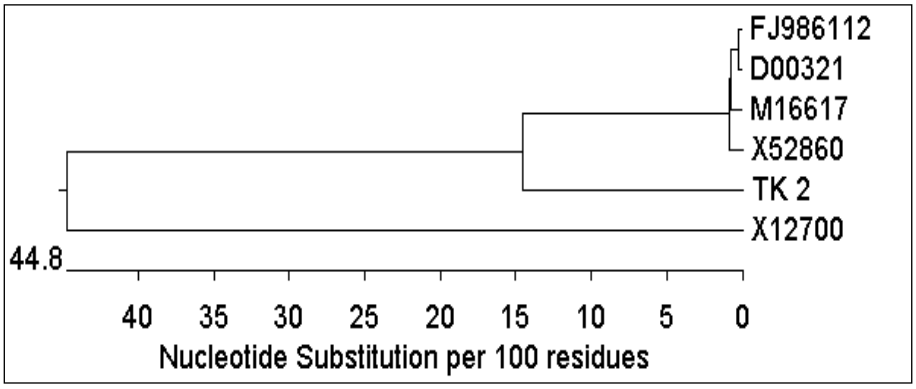

Figure-6. The phylogenetic tree analysis of the sequenced TK amplicon of the field isolated FPV.

Phylogenic analysis of Avipox viruses TK gene: Our results are different from that previously reported on inoculation of canary, Egypt, 2012, P4b virus in SPF chickens where 7out of 15 chickens (about 46%) showed takes at 3rd day post inoculation into the wing web; while none of the inoculated pigeons showed thickening of the thighs at the sites of inoculation in pigeons [39]. Therefore, in this study as well as in others [25,32,33,40], APVs from the same species of bird are classified in different sub clades. Conversely, it has also been shown that the same viruses can infect different birds. By using phylogenic analysis; the strains isolated in the present work showed homology to the PCR products of chicken sample on purification and sequencing (Fig 4-6). A common sequence of nucleotides was obtained, allowing comparison with equivalent previously published sequences in the Gen Bank. The derived and aligned nucleotides sequences were used to generate a phylogenetic tree for Pub encoding sequence with other 9 Avi poxviruses with TK gene. So that there is a very limited similarity (not exceeding 60% with (AJ581527 [41]; Af396867; Af198100 [42]; AJ223385[43]; M16617[44]; D00321 [45]; X52860 which isolated at 2003; 2001; 1999; 1987;1987;1988;1990 from Germany; India; USA; UK and USA ; respectively) and 55% with Fj986112 [46] which isolated at 2008 from Grenada and with X12700[47] which isolated from Australian at 1987 with very minute similarity (38%) (Fig.5).

Conclusion

FPV (FPV isolated ch-08 TK gene with Accession KF314718 in Gen Bank) which is isolated from laying chicken aged 30 day old and suffering from pox lesions were different from avian pox like lesions which-were observed from canary in Egypt in 2012 (Canary/ Egypt/2012/p4b) [39]; and from that isolated six clinical cases of avipoxvirus at 2011 from skin crust of different domesticated birds reared in the Egyptian backyard mangment system [40] and were propagated on CAMs of embryonated chicken eggs and virus isolation was confirmed via PCR as Elsharqyia-FWPV2 FPV140(JX464823);TKPV FPV140 (JX 464 826) and PGPV FPV(JX464827). Thus, according to our knowledge this study is considered as the first molecular identification of FPV strain from laying chickens in Egypt.

Authors' contributions

MHHA was responsible for collection of samples from commercial layer chicken flocks in Giza governorate at end of 2012. YAS did the PCR identification and phylogenic analysis. SSEM did the virus isolation, titration, pathogenicity, drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests

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