Genomic Characterization of Pigeon Pox Virus in Egypt

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ABSTRACT

In this study skin scrab lesions were collected from pigeons showing symptoms of pigeon pox infection frome different localities in Egypt. The prepared tissue suspension for inoculated in chorioallentioc membrane (CAM) of specific pathogenic free (SPF) 11day embryonated chicken eggs. The characteristic pock lesions were observed on CAM at the 2nd passage and. For genetic characterization of the isolated Pigeon pox virus the Polymerase Chain Reaction (PCR) was done from both crust sample and CAM by amplification of P4B gene using the specific forward and reverse primers.The presence of bands of 570 bp is considered positive results.

INTRODUCTION

The avian pox viruses form a subgroup of viruses of varying cross-relationship degrees. They affect a wide range of sixty bird species of various families *(1)*.

Pigeon pox is caused by pigeon pox virus that classified within Order *ungrouped,* Family*: Poxviridae,* Subfamily: Chordopoxvirinae, Genus: Avipoxviruses, *Type: Pigeon pox virus* that cause disease in many species. The avipoxvirus subgroup includes a number of closely related viruses such as Fowl Pox, Pigeon Pox and Canary Pox viruses. The genome of the pigeon pox virus is more than 100 kb larger than the *Orthopoxvirus Vaccinia,* indicating that the avipox viruses have the potential to code for more proteins than other groups of poxviruses *(2)*.

PGPV pox is a transmissible disease that is spread by several kinds of vectors: biting arthropods such as mosquitoes and mites, and aerosols generated from infected birds, or the ingestion of contaminated food or water *(3)*.

There are two clinical forms of pigeon pox, probably associated with different sources of infection dry form and diphtheric form. The cutaneous form is characterized by epidermal lesions on featherless areas of the body, whereas fibrino-necrotic and proliferative lesions in the mouth, esophagus, and mucous membranes of the upper respiratory tract are characteristics of the diphtheritic form. Mortality and morbidity due to poxvirus infection may be very high in pigeons .The development of cutaneous lesions occurs in 2 weeks, with the formation of yellow scabs and desquamation of necrotic epithelium *(4)*.

Amplification of the p4b of Avi pox virus by PCR has often been used as a molecular tool for the the most sensitive techniques for the routine diagnosis detection of pigeon pox virus (PGPV) *(5)*.

The phylogenetic analysis of fpv167 (P4b) gene clustered Elsharqyia PGPV strain was clustered within subclade A2 (Turkey pox 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 (Pigeon pox virus) with the other PGPVs *(6)*.

This aime of this study is Isolation of pigeon pox virus on CAM and molecular characterization of PGPV by using of PCR in which DNA was extracted from skin lesions. The specific primers were used to amplify the P4b gene.

MATERIAL AND METHODS

Tissue samples

The study was based on local strain collected as fragments of cutaneous lesions during routine diagnostic activity.

SPF Embroynated chicken eggs (ECE)

Specific pathogen free (SPF) Embroynated chicken eggs (ECE) Fertile SPF- ECE (11 days old) for isolation and propagation of suspected pigeon pox viruses.

Primers

The specific primer pair described by Huw Lee & Hwa Lee (1997) based on P4b sequence (forward primer: 5'-CAGCAGGTGCTAAACAACAA-3': reverse primer: 5'-CGGTAGCTTAACGCCGAATA-3' *(7)*.

Thermocycler, Gene Sequencer used for amplification and sequencing of P4b gene of PGPV

DNA extraction kit: from Vivantis company (Malasia) is used for DNA extraction from both tissue crust sample, propagated PGPV in SPF EGG and Hungarian strain of PPV its consist of: Tissue Lysis Buffer (Buffer TL),Tissue Genomic DNA Binding Buffer, Buffer TB),Lysis Enhancer,-Protinase K, Washing Buffer , Elution buffer,GF-Columns, and Collection tubes, All components beside protease K are stored at room temperature while Protease K lyophilized or dissolved aliquots stored at -20°C.Absolute Ethanol (>95%), RNase (DNase-free) (20mg/ml), Phosphate Buffered Saline, The bottle labeled Wash Buffer contains concentrated buffer which must be diluted with Absolute Ethanol (>95%).add 5.6ml of Absolute Ethanol in to bottle labeled Wash Buffer.

Pigeon pox virus vaccine: Pigeon pox virus strain was kindly supplied from Pox Vaccine production and Research department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

Antibiotic stock solution: Penicillin-G sodium 1.000.000 IU, Streptomycin sulfate 1000 mg, Nystatin.

Method

Chicken embryo inoculation

Samples from nodular skin lesions of naturally infected Pigeons were collected. 10% W/V tissue homogenate were prepared in PBS containing penicillin (100IU) and streptomycin (1mg/ml)The solution was centrifuged and supernatant fluid was inoculated in 11 days SPF ECE (obtained from Quem Ochem Company, Fayoum, Egypt). Inoculated eggs were incubated at 37°C, observed and candled for 5 days. Five non inoculated control eggs were left as a negative control. 5 days post inoculation (PI). Embryos were chilled (over night) and CAMs were examined for presence of pock lesions areas were collected, pooled were used for further passages.

Pigeon pox virus DNA ampification

DNA extraction: DNA was extracted from clarified suspension of collected skin scrab or pock lesions produced by PGPV isolate and vaccine strains using the GF-1 tissue DNA extraction kit (vivantis Company, Malaysia) following manufacturer instructions.The tissue samples were cut in to small pieces with aclean scalpel then250 µl buffer tissue lysis and 10 µl of protein Kinas K were added to the prepared sample. Mix thourghly by pulsed vortexing to obtain homogenous solution.Add 12 µl of lysis enhancer and mix immediately. Incubate at 65 °C for 1-3 hours in shaking water bath or mix during incubation to ensure thorough digestion of the sample. After that make Homogenization in which two volumes 560 µl of buffer tissue binding was added then mix thoroughly by pulsated vortexing until homogenous solution is obtained. Incubate 10 min at 65°C.200 µl of absolute ethanol was added; mix immediately and thoroughly by pulsated vortexing to obtain homogenous solution. Loading to column by Transfer approximately 650 µl of sample in to a column assembled in clean collection tube .centrifuge at 5000xg for 1 min.discard flow through.Repeat for the remaing sample from step 4.The column was wsashed with 650 µl wash buffer and centrifuge at 5000xg for 1min.discard flow through. Repeat column washing once again.Column drying by

Centrifuge the column at 10000xg for 1min to remove all traces of ethanol. DNA Elution by placing the column in to aclean micro centrifuge tube. Add 200 µl of preheated elution buffer, or sterile water directly on to column membrane and stand at room temperature for 2min.centrifugion at 5000xg for 1min to Elute DNA.store DNA at 4°C or -20°C until PCR amplification.

PCR amplification: A primer pair was used to amplify a 578 bp fragment of the P4b gene (fpv167). Primers were used to amplify the P4b gene (fpv167 locus) (Forward primer: 5'-CAGCAGGTGC TAAACAACAA-3 '& reverse primer: 5'-CGGTAGCTTA ACGCCGAATA-3'). PCR amplification was performed in 50 µl reaction volume and contained 25 µl Tag. Master mix, 2µl of DNA template, 1 µl forward Primer, 1 µl reverse Primer, 0.5µl MgCL 2, and 20.5 µl water nuclease free (up to 50 µl).

Table 1. PCR cycle conditions of PGPV

Cycle Name	Temperature degree and time
1-Initial Denaturation(one cycle)	95 °C for 5 minutes
2-Denaturation(35 cycles)	95°C for 30 seconds
3-primer annealing(35 cycles)	55°C for 30 seconds
4-Extension (35 cycles)	72°C for 30 seconds
5-Final Extension(one cycle)	72°C for 7 minutes

Agarose gel electrophoresis

5 µl of the amplified PCR products were separated by 1-2% agarose gel stained with Ethidium Bromide (50 ml of 2% agarose in 1X TEA buffer were prepared and then were placed in baker of boiling water until melted. Allow to cool to about 45 °C and add 1ul Ethidium bromide .Pour gel and insert well former (comb). Pour buffer1x TEA into tank and remove comb from gel. 5 µl PCR products were loade into the wells formed in the agarose gel.Negative control includes all the reagents without template and using reference PGPV as Positive control.Molecular weight marker was also prepared and loaded in to the well formed in the gel. The tank covered with its led and attached to power supply which adjusted on 100 volts.Electrophoreses at 100 volts for 20nm (minimum) or 10 volts overnight, after complete migration of the DNA the electric current turned off, and the lid was removed. 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 and amplification pattern of each virus was determined according to molecular size of the amplified products.

RESULTS

Pigeon pox virus isolation on ECE

Results obtained showed that the virus isolation from suspected tissue samples revealed at the second passage there is only thickening of CAM of the inoculated eggs with few fine grayish yellow lesions on CAM. Pock lesions increased in numbers and size by successive serial passages of the inoculated chicken embryos. Different stages of the pock lesions developed illustrated in figure(1), (2), (3).While the CAM of negative control SPF eggs showing no pathological change as shown in figure. (4).

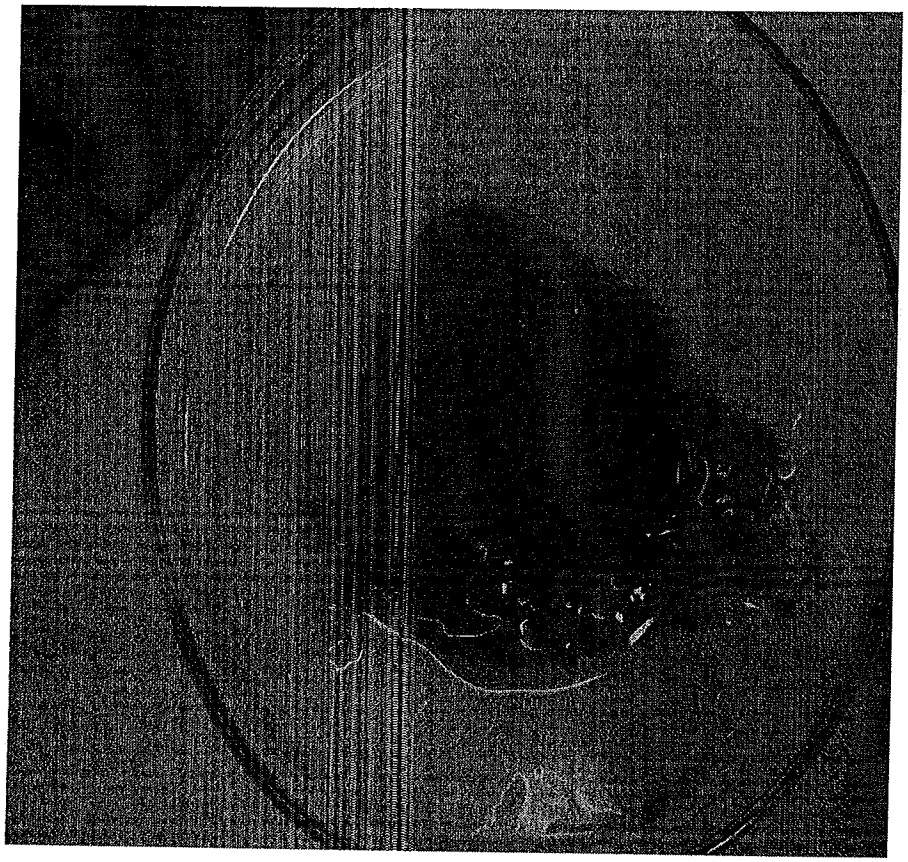

Fig. 1. CAM of SPF-ECE: passage no.3 generalized thickening of the inoculated CAM with supernatant fluid of samples collected from infected pigeon.

Fig.2. CAM of SPF-ECE: passage no.4 focal white opaque pocks with generalized thickening of the inoculated CAM .

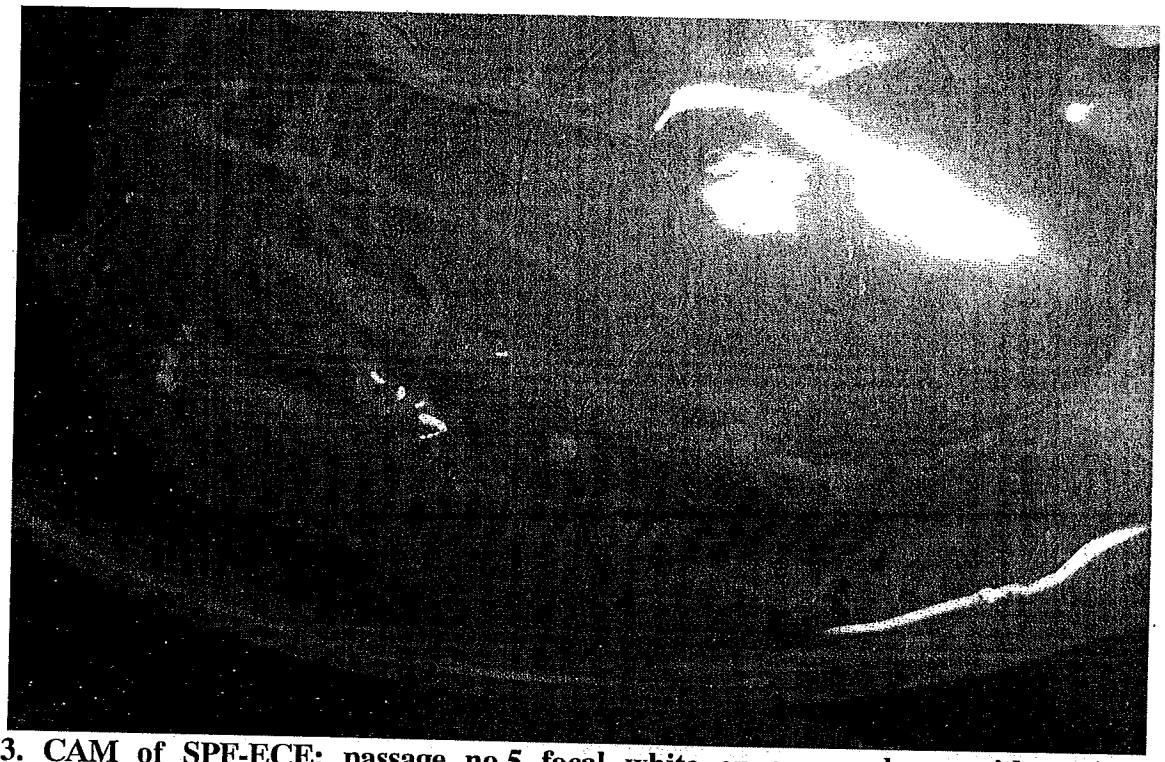

Fig.3. CAM of SPF-ECE: passage no.5 focal white opaque pocks with generalized thickening of the inoculated CAM.

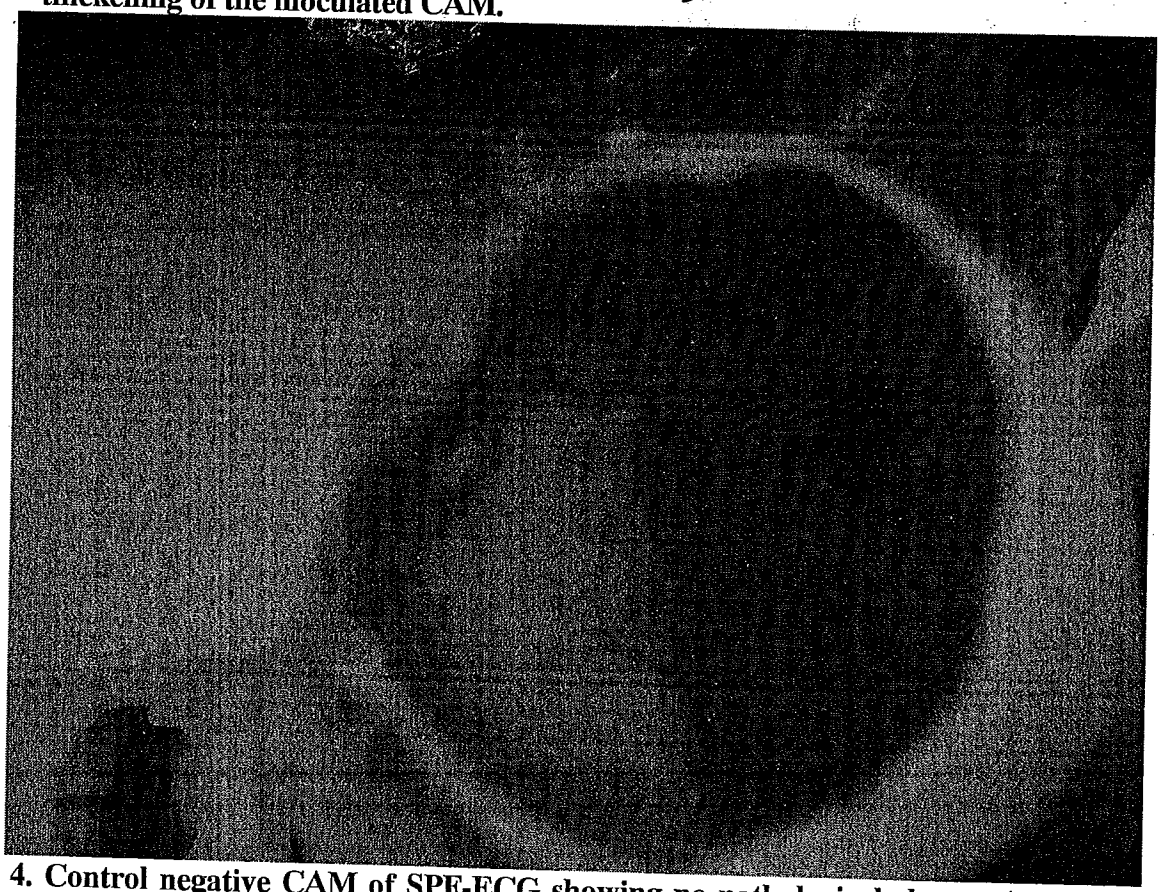

Fig. 4. Control negative CAM of SPF-ECG showing no pathological change (no thickening nor pock lesion).

Genomic characterization for virus PGPVextracted from crust samples and the isolated propagated PGPV virus.

Application of polymerase chain reaction (PCR) on crust sample for P4B gene and the isolated propagated PGPV

PCR was applied on the DNA extract (using VIVANTIS DNA extraction mini kit) from both isolated propagated Pigeon pox virus CAM and crust sample using the P4B gene forward and reverse primers, Results showed that the selected primers allowed amplification of DNA from all isolates (that had been obtained from different pigeons from several countries), propagated and vaccinal strain of PGPV.

The PCR amplification of the tested samples result in the characteristic PCR positive bands of 570 bp size fragment of pigeon-pox virus for both isolated propagated PPV in CAM and crust sample as shown in photo (5).

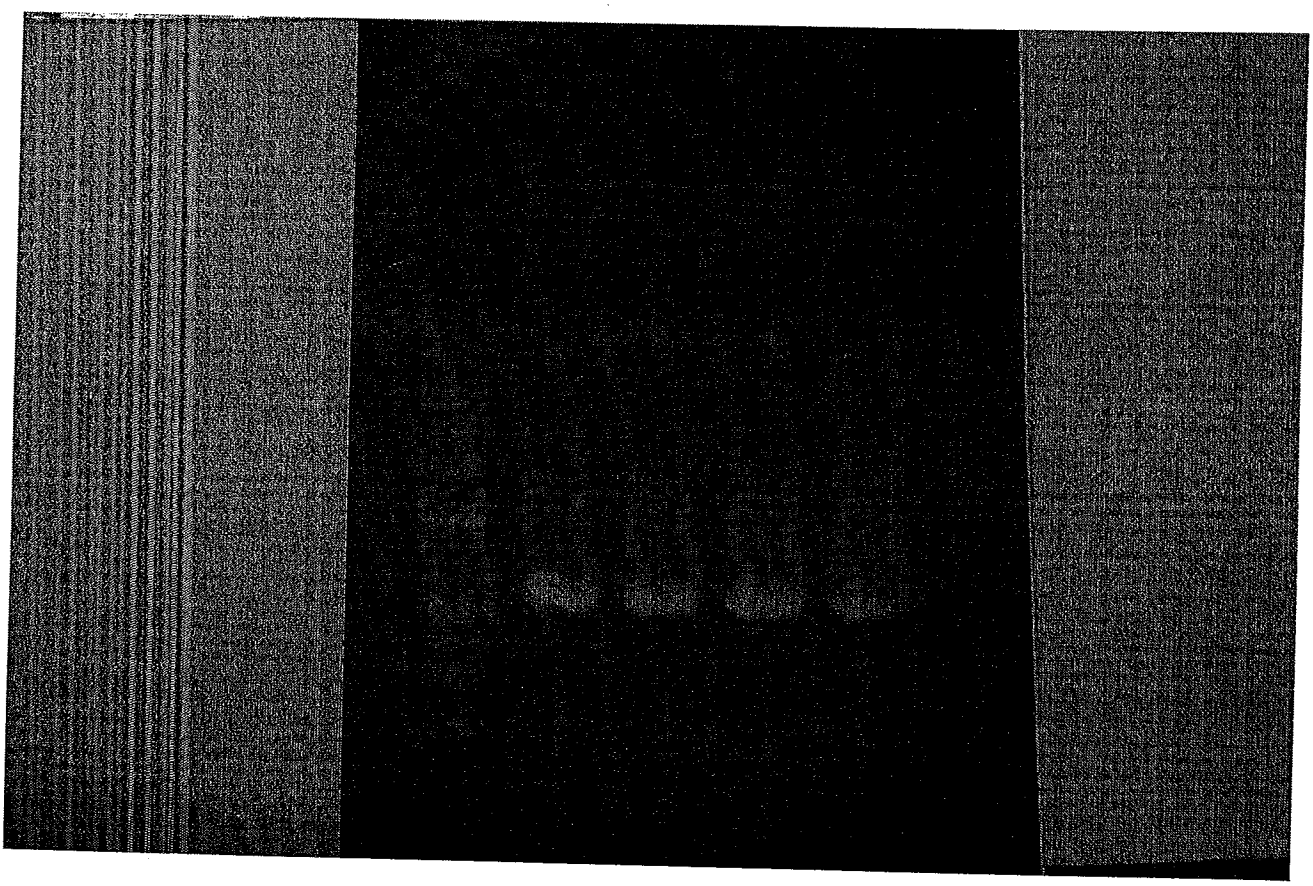

Fig. 5. PCR amplification of the p4b (fpv167) gene from some of the samples of this study.
Lane marker: DNA size marker (100 bp DNA ladder.
Lane 1: skin scrab sample show band of 570 bp size,
Lane 2: isolated PGPV on CAM show band of 570 bp size,
Lanes 3: Vaccinal strain show band.
Lanes 4: control positive results in PCR.
Lane 5: control negative show no band.

DISCUSSION

The pock lesions increased in numbers and size by successive serial passages with survival of the inoculated chicken embryo post virus inoculation. These results agree with *(8,19)* who reported an inflammatory edematous areas on the CAM with primary lesions usually appeared to be of 2-5 mm in diameter, raised above the surface of the membrane and surrounded by an inflammatory edematous area with scattered secondary lesions, the latter was of two types, some were minute pin headed grayish white in colure while others were large 1-2 mm in diameter and not raised above the surface of the membrane. Also similar result obtained by *(10)* who described the characteristic Pock lesions of PGPV on CAM as circular, opaque, white enlarged areas on the 5th day post inoculation at the second passage.

For genomic characterization Polymerase Chain Reaction (PCR) were applied on the collected skin crusts and the isolated propagated PGPV on CAM for P4B gene after the DNA extraction from crust samples and the isolated propagated virus on CAM.PCR assay was done for amplification of the P4B gene of the extracted DNA from isolated propagated PGPV on CAM primer pair (forward primer: 5'-CAGCAGGTGCTAAACAACAA-3': reverse primer: 5'-CGGTAGCTTAACGCCGAATA-3').The gene sequence encoding P4B will be amplified and the size of the PCR product, specific for the pigeon pox virus (PGPV) was 570 bp. These results agree with those obtained by *(7)* who amplified the P4B gene of PGPV isolated from skin crust material of affected pigeon and the size of amplified product were 570bp., also similar results recorded by *(11)*, in which PCR was applied on the DNA extract from isolated propagated PGPV on CAM in order to amplify P4b gene using the primer pair (forward primer: 5'-CAGCAGGTGCTAAACAACAA-3': reverse primer: 5'-CGGTAGCTTAACGCCGAATA-3') of the P4b gene. The PCR amplification of P4b gene resulted in the characteristic 570 bp product similar result obtained by *(12)*.Who found that P4b (fpv167) PCR amplification in combination with restriction end nuclease analysis and sequencing are rapid and effective diagnostic tools for PGPV.

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الملخص العربي

التوصيف الجينى لفيروس جدرى الحمام فى مصر

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تم فى هذه الدراسه عزل فيروس جدرى الحمام من مناطق مختلفه من جمهوريه مصر العربيه وعمل التوصيف الجزيئى للفيروس المعزول. حيث تم جمع عينات اصابات جلديه (بثرات) من حمام عليه اعراض جدرى الحمام وقد تم عزل الفيروس على (CAM) لاجنه الدجاج الخالى من المسببات المرضيه (SPF) وتمريره عدة تمريرات متتابعه حتى ظهرت (pock lesions) على شكل عقد رماديه اللون وحبيبات بيضاء،مما يدل على اقلمه الفيروس المعزول على البيض. ولعمل التوصيف الجينى لفيروس جدرى الحمام المعزول تم اجراء تفاعل البلمره المتسلسل (PCR) على الحمض النووى المستخلص من البثرات الجلديه والفيروس المعزول والممرر على ال (chorioallentioc membrane)للبيض الخالى من المسببات المرضيه (SPF) بالاضافه الى فيروس جدرى الحمام عتره اللقاح (عترة الهانجرين) وذلك بالاكثار من كميه الجين (P4B-Gene) باستخدام البريمرات الخاصه بالجين حيث تم الحصول على نتائج ايجابيه عند 570 bp لكل من الحمض النووى المستخلص من البثرات الجلديه والفيروس المعزول وكذلك لعترة اللقاح (عترة الهانجرين)وهى نتيجه داله على ان الفيروس المستعمل فى نتاج لقاح جدرى الحمام ينتمى لفيروس جدرى الحمام.وبذلك اظهر العزل الفيروسى على ال (chorioallentioc membrane) للبيض الخالى من المسببات المرضيه (SPF) والتعرف على الحمض النووى للفيروس الممر والمستخلص من البثرات الجلديه ان ما ظهر من اصابات جلديه بالحمام هو فيروس جدرى الحمام.