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First molecular characterization of *Columbid herpesvirus-1* isolated from pigeons in Egypt

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ABSTRACT

Columbid herpesvirus-1 (CoHV-1) is one of the most frequent pigeon diseases reported worldwide. It is associated with respiratory and nervous manifestations in pigeons and necrotic lesions in their livers. It may cause sudden death, especially in young and immunosuppressed pigeons, and thus, this study aimed to demonstrate the occurrence and genetic characterization of CoHV-1 in Egypt. In this study, forty-four cloacal swabs and tissue samples were collected from Qena governorate, South Egypt. These samples were screened for CoHV1 by conventional polymerase chain reaction-based assay (PCR). The PCR results revealed the presence of herpesvirus DNA in Sixteen pigeons' samples. The liver and spleen formalin-fixed tissue samples proved the presence of eosinophilic intranuclear inclusion bodies by histopathological examinations. Four samples were selected for Sanger sequencing for virus characterization. Nucleotide sequencing of the DNA-dependent DNA polymerase gene showed a high similarity with *Columbid herpesvirus-1* strains from China, Thailand, and Turkey and with Falconid herpesvirus from the USA. This characterization of CoHV-1 isolates from South Egypt helped in giving informative data about their genetic relationships with global strains and decreased the research gap in studying pigeon diseases in Egypt and worldwide.

Introduction

Columbid herpesvirus-1 (CoHV-1), or Pigeon herpesvirus (PiHV), is a pathogen affecting captive and wild pigeons worldwide (McMullin, 2020). *Columbid herpesvirus-1* belongs to the *Mardivirus* genus within the subfamily *Alphaherpesvirinae* and the family *Herpesviridae* (Vindevogel and Duchatel, 1991; Swayne *et al*., 2013). Despite pigeons are the natural host of CoHV-1, it has been reported in different birds. CoHV-1 is frequently reported in raptors like falcons, hawks, and owls proposing a predator-prey relationship with pigeons (Pinkerton *et al*., 2008). The viral genome is a single, linear dsDNA within the protein capsid that has an external diameter of approximately 180 nm. *Columbid herpesvirus-1* virus is genetically similar to Falconid herpesvirus 1 (FaHV-1), Gallid herpesvirus 2, Gallid herpesvirus 3, and Meleagrid herpesvirus 1, they all have the structure of class E of herpesvirus genomes (Guo *et al*., 2017).

Infections with *Columbid herpesvirus-1* have been reported many years ago in pigeons suffering from nervous signs, upper respiratory tract infections, conjunctivitis, crop vomiting, depression, anorexia, oropharyngeal ulceration, dyspnea, and /or diarrhea. Postmortem lesions of CoHV-1-associated disease involve liver and spleen necrosis, necrosis with diphtheritic membrane in the upper respiratory and digestive tract. High mortalities and sudden death may occur especially in young and immunosuppressed pigeons infected with *Columbid herpesvirus-1* (Zhao *et al*., 2015; Gornatti-Churria *et al*., 2023). CoHV-1 may remain latent, making pigeons susceptible to secondary infections with other bacterial, viral, and parasitic pathogens (Marlier and Vindevogel, 2006).

The presence of intranuclear inclusion bodies (INIBs) within pigeons' tissues is an important diagnostic tool in the diagnosis of Pigeon herpesvirus infection in Columbid and non-Columbid birds (Pinkerton *et al*., 2008; Abdul-Aziz and Barnes, 2018; Gornatti-Churria *et al*., 2023) .

Pigeon herpesvirus is one of the viruses associated with Young Pigeon Disease Syndrome (YPDS), a multifactorial syndrome that occurs in young pigeons and is associated with high morbidity and mortality rates. YPDS is a very common problem in young pigeons and causes commercial losses worldwide (Freick *et al*., 2008).

The first description of Pigeon herpesvirus infection was in 1943 in the USA (Smadel *et al*., 1945) when eosinophilic intranuclear inclusion bodies were discovered in the liver of US army pigeons that died during an assumed "psittacosis" disease. These intranuclear inclusions were described as caused by a herpesvirus (Cornwell *et al*., 1970). After that time, the CoHV-1 has been stated in many countries in the world and from various birds (Freick *et al*., 2008; Woźniakowski *et al*., 2013; Zhang *et al*., 2015; Phalen *et al*., 2017; Raj and Jaime, 2019; Gornatti-Churria *et al*., 2023)

In Egypt, few research papers reported the Pigeon herpesvirus (Tantawi *et al*., 1983) and the last paper studying PiHV was from about 30 years ago without any virus characterization of the Egyptian isolates (Abd-El-Motelib *et al*., 1994). Due to a lack of data regarding *Columbid herpesvirus-1* infection in Egyptian pigeon populations, and due to the research gap in studying pigeon diseases in the world. This study aimed to elucidate the genetic composition of PiHV isolates originating from field outbreaks in Qena province, South Egypt, and study their phylogenetic relationships with global strains.

Materials and methods

Ethical approval

Birds handling and all procedures were approved by the Research Ethical Committee of the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt under approval number "VM/SVU/24(7)-02".

Sampling and birds' history

Twenty-four cloacal swabs and twenty tissue samples (liver, spleen,

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bursa, heart, lung) were collected during this study from pigeons in different areas in Qena governorate showing various signs (respiratory signs, nervous signs, vomiting, diarrhea, inappetence, sudden death). The samples were collected during 2022 to 2023 from young and old pigeons (10 days -2.5 years). The birds were subjected to postmortem examination and the samples were collected from recently dead birds or diseased birds that were euthanized. Some Tissue samples were fixed in 10% neutral formalin for pathology and other samples were stored in deep freeze at -80°C for isolation and identification.

Histopathological examinations

Tissue samples (liver and spleen) were fixed in 10% neutral formalin (pH 7.0) and processed according to routine tissue processing procedures. After embedding in paraffin wax, tissue sections were cut at 5-µm thickness from paraffin blocks and stained with hematoxylin and eosin (H&E).

DNA extraction and PCR

Total Viral DNA of forty-four samples (swabs and tissue supernatants) was extracted using a Genomic DNA Isolation Kit (Norgen Biotek Corp, Ontario, Canada, Cat. number 24700) according to the manufacturer's procedures (Qiagen, Hilden, Germany). DNA extract was stored at -20°C. The PCR for PiHV was carried out by adding a primer pair for the Polymerase gene (242 base pair), PiHV-s (GGGACGCTCTGATTAAGGAAT),

PiHV-as (CTTGGTGATCAGCAGCAGCTTG) (Raue *et al*., 2005) and PCR master mix OneTaq Hot Start 2X Master Mix with Standard Buffer (Catalog number M0484, New England Biolabs Inc.), with the following parameters: 94°C for 30 sec, followed by 40 cycles at 94°C for 30 sec and 60°C for 30 sec and 68°C for 20 sec and final extension 68°C for 5 min. Before Sanger sequencing, the PCR amplicons were visualized by electrophoresis with 1.5% agarose gel.

Inoculation in Embryonating Chicken Eggs (ECE) and hemagglutination assay

The positive PCR samples were inoculated via the chorioallantoic route in Embryonating Chicken Eggs from apparently healthy Balady flocks to check the allantoic fluids by hemagglutination assay to detect the presence of avian influenza virus or pigeon paramyxovirus serotype 1(Dufour-Zavala, 2008).

DNA sequencing and phylogenetic analysis

The amplified amplicons of four selected positive samples were sent for purification and Sanger sequencing in both forward and reverse directions by a commercial service provider (Macrogen, Inc., South Korea), using the same PCR primers.

Sequence data were compared with 60 other isolates from GenBank after aligning based on the nucleotide sequences by Clustalw (Li, 2003), and their phylogenetic relationships were investigated for building phylogenetic trees. The phylogenetic analysis based on the DNA-dependent DNA polymerase gene was carried out with MEGA7 software (Kumar *et al*., 2016) using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) with 1000 bootstrap replicates Fig. 3. Evolutionary distances between the studied sequences and representative sequences from GenBank, with pairwise comparisons of nucleotide sequences performed using SDT (Sequence Demarcation Tool) v1.2 software using Clustalw alignment (Muhire *et al*., 2014) Fig. 4. The nucleotide sequences obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) under accession numbers (PQ812281, PQ812282, PQ812283, and PQ812284).

Results

Necropsy and histopathological findings

Vomiting and diarrhea were observed on diseased pigeons. Grossly, the livers of many pigeons were swollen and fragile with pale necrotic areas. A few pigeons' lungs had a focal hemorrhage or abscessation. The kidneys were enlarged and the proventriculus was enlarged and thick. The eyelids of most pigeons were swollen, and conjunctivitis was observed.

The histopathological findings recorded in the liver and spleen of positively infected pigeons revealed that in the liver, there were areas of coagulative necrosis, marked by hypereosinophilic hepatocytes with other signs of coagulative necrosis in the hepatic nucleus including karyopyknosis and karyorrhexis. Some inflammatory cell infiltrations were noticed around the necrosed areas as well as within the central vein. Intranuclear eosinophilic inclusion bodies and chromatin margination in the hepatocytes are the pathognomic lesions in herpesvirus infection as shown in Fig.1A. By higher magnification using the oil immersion lens, the affected hepatocytes filled clearly with the intranuclear eosinophilic inclusion bodies and margination of nuclear chromatin. This was associated with the presence of macrophage cells near and around them as shown in Fig.1B.

In the spleen, the lesions were clearly seen in the red pulp and characterized by intranuclear eosinophilic inclusion bodies within the infected lymphocytes with macrophage infiltration as shown in Fig.1C. Another section of the spleen was photographed by an oil immersion lens revealing the intranuclear inclusion bodies within the infected lymphocytes with the macrophage cells distribution. Splenic depletion was the hallmark of necrosis which is characterized by wide spaces in between the splenic lymphocyte population as shown in Fig.1D.

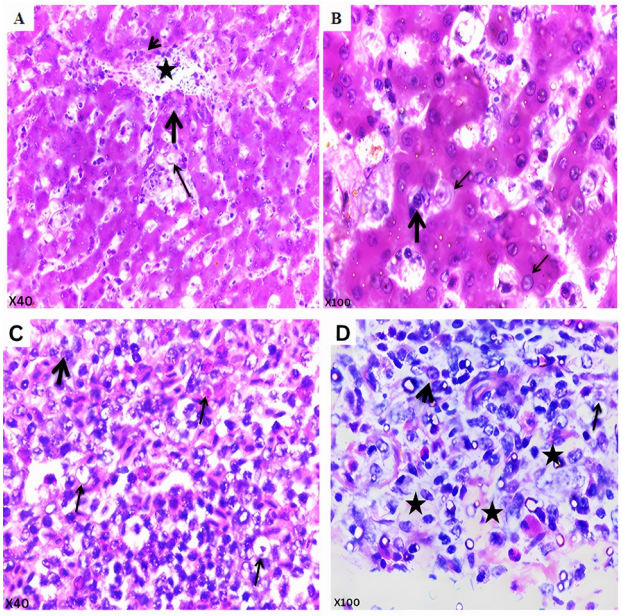

Fig.1. Histomicrograph of liver showing A) area of coagulative necrosis, characterized by hypereosinophilic hepatocytes (arrowhead) with karyopyknosis and karyorrhexis (thick arrow). Some inflammatory cell infiltrations were noticed around the necrosis and within the central vein (star). Intranuclear eosinophilic inclusion bodies and chromatin margination occurred in hepatocytes (thin arrow). B) Higher magnification of hepatocytes showing intranuclear eosinophilic inclusion bodies and chromatin margination within hepatocytes (thin arrow) with the presence of macrophage around (thick arrow). Histomicrograph of spleen showing C) red pulp of spleen with intranuclear inclusion bodies within the infected lymphocytes (thin arrow) with macrophage infiltration (thick arrow). D) Higher magnification of the spleen revealed the intranuclear inclusion bodies appeared (thin arrow) with the macrophage cell distribution (arrowhead). Splenic depletion is the hallmark of necrosis and is characterized by wide spaces in between (star). (H&E… X 40(A, C) and X 100(B, D)).

PCR screening results and hemagglutination assay

In this study, a total of 44 pigeon samples were analyzed. Out of the forty-four samples, 16 isolates were positive (7 positive swab samples and 9 tissue samples) (Fig.2). These positive samples were isolated from

different ages (4 isolates from old pigeons; more than 1 year old, and 12 isolates from young pigeons; less than 1 year old). The hemagglutination assay of allantoic fluids was negative for the hemagglutinating viruses (Avian influenza virus and pigeon paramyxovirus serotype 1).

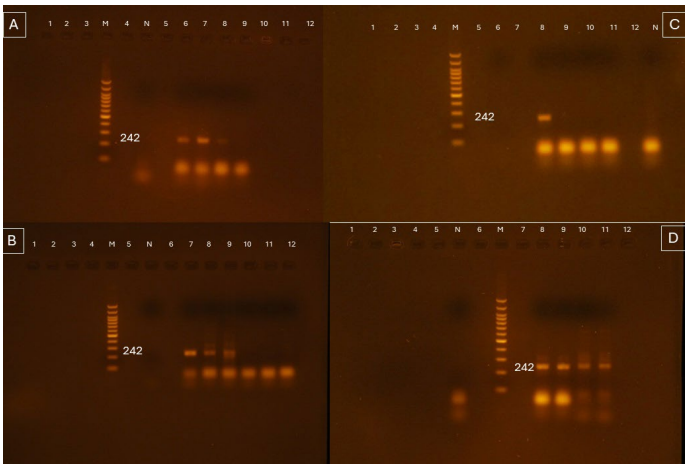

Fig. 2. Representative pictures of agarose gel electrophoresis of PCR amplification products (242bp) of CoHV-1 DNA-dependent DNA polymerase gene in 1.5% agarose gel stained with ethidium bromide after gel electrophoresis; A) Lane M: 100bp DNA ladder. Lanes 6,7 and 8: CoHV-1 positive isolates, lane 9: negative sample, lane N: negative control. B)Lane M: 100bp DNA ladder. Lanes 7,8 and 9: CoHV-1 positive isolates, lanes 10,11,12: negative samples, lane N: negative control. C) Lane M: 100bp DNA ladder. Lane 8: CoHV-1 positive isolate, lanes 9,10,11: negative samples, lane N: negative control. D)Lane M: 100bp DNA ladder. Lanes 8,9,10,11: CoHV-1 positive isolates, lane N: negative control.

Distance and phylogenetic analysis of polymerase gene

Four representative amplified amplicons of the PiHV polymerase gene were Sanger sequenced and Blasted in the NCBI (https://blast.ncbi.nlm.nih.gov) to compare these sequences with the genomes of Pigeon herpesvirus. The isolates labeled, EGY/COHV-1/4S, EGY/COHV-1/17S, EGY/COHV-1/5T, EGY/COHV-1/13T matched most closely with Columbid herpesvirus1 isolates: KJ995972.1 (China), KX589235.1 (China), KM010015.1(Thailand), MZ694942.1 (Turkey) and NC 024450.1 Falconid herpesvirus 1 strain (USA) (Fig. 3. and Fig. 4.).

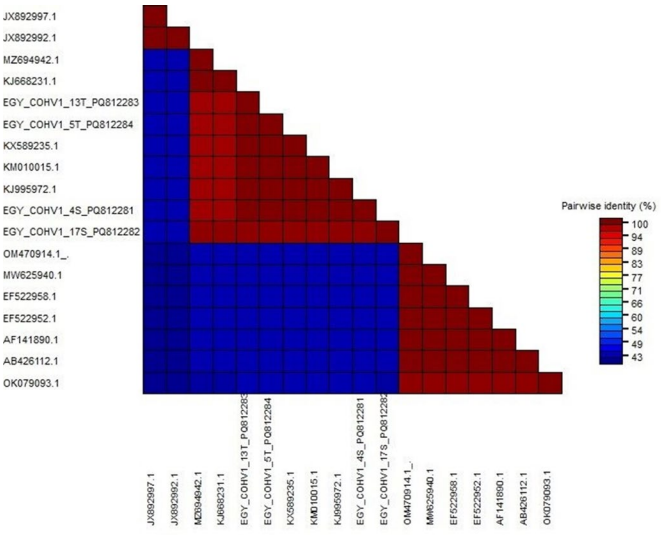

Fig. 3. A molecular phylogenetic tree which was constructed from DNA-dependent DNA polymerase gene partial sequences of Pigeon herpesvirus using the maximum likelihood method with the Tamura 3-parameter model for nucleotides with 1000 bootstrap replicates.

After blast in NCBI, the EGY/COHV-1/4S, EGY/COHV-1/5T, EGY/COHV-1/13T sequences were found to be closely related to KJ995972.1 (China), KX589235.1(China), and KM010015.1(Thailand) with 100% similarity and to MZ694942.1 (Turkey) and NC 024450.1 Falconid herpesvirus 1 strain (USA) with 98.77% similarity. The color-coded matrix of pairwise comparisons of nucleotide sequences in Fig. 4.) clarify this relationship with color scale.

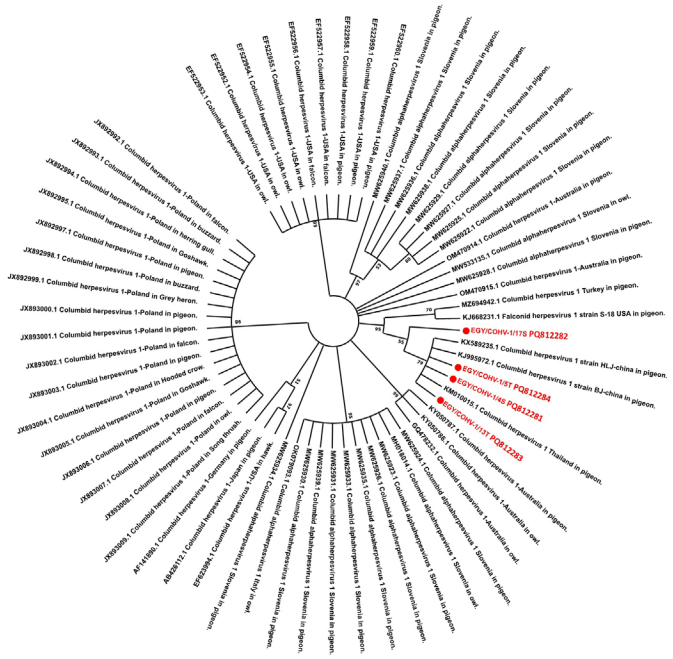

Fig 4. Color-coded matrix of pairwise comparisons of nucleotide sequences were performed using SDT (Sequence Demarcation Tool) v1.2 software.

Discussion

Pigeon production plays an important role in the Egyptian economy and the social lives of most Egyptian peoples (Omar *et al*., 2016; Adawy and Abdel-Wareth, 2023) , so studying pigeon diseases is very important to overcome outbreaks, especially those caused by YPDS. This syndrome frequently occurs in young pigeons and negatively affects the Egyptian pigeon industry. In Egypt, for years there was a research gap in studying the Pigeon herpesvirus despite its importance as one of the pathogens that causes YPDS. To our knowledge, no documented reports on genetic characterization of Pigeon herpesvirus isolates in Egypt. The essential purpose of the current paper is the isolation, identification, and genotyping of CoHV-1 isolates from infected pigeons in Qena governorate, South Egypt.

In this study, the use of a histopathological assay together with conventional PCR facilitated the detection of herpesvirus in pigeon samples. Histopathologically, the presence of Cowdry-type inclusions in affected tissues confirmed the presence of Pigeon herpesvirus (Abdul-Aziz and Barnes, 2018; Gornatti-Churria *et al*., 2023). Some authors noted microscopic necrosis of the splenic and liver tissues with eosinophilic intranuclear inclusion bodies in affected birds (Zhao *et al*., 2015; Gornatti-Churria *et al*., 2023), these histopathologic findings were proved in our study. By PCR, sixteen tissue and swab samples were positive, this proved the previous papers' theories about the possibility of isolation of CoHV-1 from both tissue and swab samples (Kaleta and Lierz, 1998; Stenzel *et al*., 2012; Žlabravec *et al*., 2021a; Žlabravec *et al*., 2021b; Gornatti-Churria *et al*., 2023). Furthermore, these positive samples were isolated from both young and old pigeons. This result agrees with those who reported CoHV-1 in birds of different ages (Freick *et al*., 2008; Stenzel *et al*., 2012; Gornatti-Churria *et al*., 2023).

The negative HA test reveals that the nervous signs of pigeons were caused by CoHV-1, not by pigeon paramyxovirus serotype 1 or avian influenza, which clarifies a possible neurotropic form of herpesvirus infection, such as those documented before (Tantawi *et al*., 1979; Kamionowski, 1981; Tantawi, 1981).

Our Egyptian isolates with numbers (PQ812281, PQ812282, PQ812283,

and PQ812284) in NCBI were found to be closely related to KJ995972.1 (China), KX589235.1(China), KM010015.1(Thailand), MZ694942.1 (Turkey), and NC 024450.1 Falconid herpesvirus 1 strain (USA), the possible cause of this relation may be due to the international trade through importation and exportation between Egypt and these countries. The possibility can't be excluded that wild birds, especially migratory ones, play a role in the spread of the virus. This relation documented the fact of genetic similarity between FaHV-1 and CoHV-1 (Spatz *et al*., 2014).

It is noteworthy that, to our knowledge, this is the first Egyptian isolates in Gene Bank, so this will help in increasing the knowledge about CoHV-1 in Egypt. Despite, the presence of commercial vaccines for Pigeon herpesvirus in many countries like the Slovak Republic, Czech Republic, Hungary, Poland, Belgium, Germany, and Netherlands. there is no commercial vaccine for PiHV in Egypt. So, we hope this paper helps in giving note to commercial companies in Egypt to save a commercial vaccine against Pigeon herpesvirus to facilitate decreasing herpesvirus outbreaks in pigeons and lowering CoHV-1 spread inside the loft or between lofts.

Despite this study's importance in increasing the knowledge about CoHV-1 in Egypt, additional studies with larger numbers of different bird species and from different areas all over Egypt are needed.

Conclusion

CoHV-1 is closely related to other isolates from different countries. This is the first genetic characterization of the DNA polymerase gene of CoHV-1 in Egypt. The continuous disease monitoring and surveillance are required not only to elucidate sequence characteristics of prevailing strains but also to revise appropriate vaccines against the disease.

Conflict of interest

The authors declare that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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