Research Article

Molecular Diagnosis of Natural Pox Virus Infection in Pigeons in Babylon Province, Central Iraq

Khitam Hassan Salih Alaarajy*, Hayder Abd AL-Emier Almremdhy

Department of Pathology and Poultry Diseases, College of Veterinary Medicine, Al-Qasim Green University, Babylon, 51013, Iraq.

Abstract | Pigeons (*Columba livia domestica*) are susceptible to various diseases, many of which can cause significant economic losses for pigeon owners. One of the most important of these diseases is pigeon pox (PP). Therefore, this study aimed to investigate the tentative and molecular diagnosis of Pigeon Pox Virus (PPV) in infected pigeons in Babylon Province, located in central Iraq. A total of 90 local-breed pigeons of varying ages and sexes were collected from different areas within Babylon Province. These pigeons were suspected of being infected with the cutaneous form of pigeon pox, as they exhibited nodular lesions on the head (around the eyes and beak), legs, and around the cloacal orifice. Skin samples were collected from various infected regions for histopathological examination and molecular analysis. Polymerase Chain Reaction (PCR) was used for molecular detection of PPV and its genetic sequencing. DNA extraction targeted the core protein gene region (p4b). Histopathological analysis revealed epidermal acanthosis with papular lesions, basal vacuolation, and the presence of eosinophilic intracytoplasmic inclusion bodies in epidermal keratinocytes. PCR results confirmed the presence of PPV in all samples, producing specific cDNA bands of 582 bp. Five of these samples were submitted to GenBank and assigned accession numbers: PP537782, PP537783, PP537784, PP537785, and PP537786. In conclusion, the PPV strain detected in infected pigeons from Babylon Province appears to be a globally widespread strain. The findings underscore the importance of genetic analysis in understanding the evolutionary dynamics of avian poxviruses.

Keywords | Pigeon pox, Virus, Pathological, Babylon, Molecular detection

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***Correspondence** | Khitam Hassan Salih Alaarajy, Department of Pathology and Poultry Diseases, College of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq; **Email:** khitamhassan@vet.uoqasim.edu.iq
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INTRODUCTION

Pigeons (*Columba livia domestica*) are considered the one of the poultry species. Pigeons are ubiquitous species of birds and can be found in almost all towns and cities around the world (Marques *et al.*, 2007). The pigeon is one of the earliest species to be domesticated by humans, having been used for thousands of years for various purposes, including message delivery, competitive games (such as racing), as laboratory animals, and as cultural and religious symbols (Alemu *et al.*, 2015). In recent years, one of the most important obstacles to the expansion of pigeon breeding is due to their exposure to a variety of diseases (viral, bacterial, fungal, and infestation with internal and external parasites), which lead to high morbidity and high mortality rates. These diseases spreading due to poor management system, unhygienic, lack of vaccination and medication, hazardous seasonal variation such as heat

stress (Khan and Samad, 2008). One of important diseases infected pigeons and causes severe threated to pigeons owners is pigeon pox (PP) which diffused worldwide. Pigeon pox is a worldwide contagious viral disease infected pigeons, of all ages and sexes. Pigeon pox virus (PPV) belong the genus Avipoxvirus of family *Poxviridae* and subfamily *Chordopoxvirinae* (Weli and Tryland, 2011). PPV could be transmitted by bites of arthropods, inhalation of aerosols from infected birds or ingestion of contaminated food and water. PPV enters the body through skin wound or through the mucous membrane and cause localized infection at point of entry, then it may spread by blood to liver and bone marrow causing systemic infection (Porter and Morishita, 2022). PP occurs in two forms cutaneous form (dry form) and diphtheric form (moist form). Both forms could occur together in the same bird, that appears as greyish white nodules scattered around eye, legs and skin with yellowish nodules on the mucosa of mouth, pharynx and larynx (Jan *et al*., 2017; Audarya *et al*., 2018). Although pox disease is widespread in Iraq and is considered a major problem for pigeon producers, there is a lack of research and studies addressing this disease in pigeons, particularly in Babylon Province. Therefore, this study was designed to investigate the pathological and molecular diagnosis of the circulating pigeon pox virus in Babylon Province, located in central Iraq.

MATERIALS AND METHODS

Study Ethics

All procedures were carried out in accordance with the laws governing animal care and the animal welfare guidelines of the Pathology Department, College of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq. The ethical standards for animal use in research were approved and followed as established by the local Animal Welfare Committee (Approval No: 2023, dated 5/09/2023).

Sample Collection and Clinical Examination

This study was conducted in the Department of Pathology and Poultry Diseases, College of Veterinary Medicine, Al-Qasim Green University. A total of 90 local-breed pigeons of varying ages and sexes were obtained from live bird markets, private veterinary clinics, and the veterinary teaching hospital in Babil (Babylon) Province, Iraq, during the period from June to November 2023. The pigeons exhibited nodular lesions on the head (around the eyes and beak), legs, and around the cloacal opening. Clinical signs observed in the affected pigeons were documented.

Following clinical evaluation, the pigeons were humanely euthanized to collect sterile skin tissue samples from papules, pustules, and scab lesions located in featherless areas of the head, legs, feet, and toes. These samples were divided into two parts: one portion was placed in plastic containers containing 10% neutral buffered formalin for histopathological analysis, conducted according to the method described by Suvarna *et al*. (2018). The second portion was pooled in a sterile container with 5 mL of 50% glycerol phosphate buffer solution and stored at –20°C for subsequent polymerase chain reaction (PCR) analysis.

DNA Extraction and PCR Amplification

The viral DNA of avian pox virus was extracted from cutaneous lesions of infected pigeons, also, from fowl pox vaccine using Genomic DNA Mini Kit (Tissue) Gene aid Biotech Ltd. Taiwan according to the manufacturer's instructions. The presence of pigeons pox virus in the DNA extracted from clinical samples was confirmed by performing poxvirus-specific PCR with the primers designed by (Macrogen Inc. Geumchen, Seoul, South Korea) for amplifying the 582 bp pox virus p4b gene (virus core protein) as described in Table 1. The thermo cycler condition of real time PCR reaction were presented in Table 2.

Table 1: *The P4b gene primers used in this study.*

Product size	Sequence	Primer
N C B I 582 bp (primer blast)	CAGCAGGTGCTAAA- F CAACAA	P4b
	CGGTAGCCTTAACGC- R CGAATA	

Table 2: Real time PCR thermo cycler conditions.

No.	Step	Temperature	Duration	Cycle
1	Pre-denaturation	95 c°	5 min	1
2	Denaturation	95 c°	15 sec	40
3	Annealing	60 c°	45 sec	
4	Melt cure	In accordance with the instruments instructions		

Table 3: Show the sequence Thermo cycler applied in conventional PCR procedure.

No.	Step	Temperature	Time	Cycles
1	Pre-denaturation	94 c°	5 min	One cycle
2	Denaturation	94 c°	30 sec	35 cycles
3	Primer annealing	60 c°	30 sec	
4	Extension	72 c°	40 sec	
5	Final extension	72 c°	5 min	One cycle

For the conventional PCR procedure, the reaction mixture consisted of 10 µL of master mix, 2 µL of primer mix, 2 µL of extracted DNA, 0.5 µL of $MgCl_2$, and 10.5 µL of double-distilled nuclease-free water, making up a final volume of 25 µL. The PCR was performed using a thermal cycler, with cycling conditions described in Table 3. After amplification, 5 µL of the PCR products targeting the p4b core gene were separated by electrophoresis on a 2% agarose gel. The gel was stained with ethidium bromide, and

DNA bands were visualized using a UV transilluminator. Fragment sizes were determined by comparison with a 250 bp DNA ladder.

Figure 1: Signs of pox disease in pigeon. A: skin nodules in the neck region **B-D:** skin nodules in the beak/head regions **E:** Pox lesion around the cloaca orifice and toes. **F:** Fibro-necrotic lesions in the oral cavity mucosa membrane.

The PCR products of the poxvirus p4b gene were purified and sequenced by Macrogen Inc., Seoul, South Korea. The amplified PCR fragments were subjected to Sanger sequencing to assess genetic polymorphism among the viral samples. The sequencing data were then edited, aligned, and analyzed using BioEdit Sequence Alignment Editor version 7.1 (DNASTAR, Madison, WI, USA). Sequence alignment was performed according to the protocol described by Al-Shuhaib and Hashim (2023), and the results were compared with reference sequences in public databases. To investigate the evolutionary relationships, a phylogenetic tree was constructed based on the p4b gene sequences using a comprehensive rectangular cladogram. This analysis provided insights into the genetic relatedness and evolutionary history of the circulating pigeon pox virus strains.

RESULTS

Clinical Examination

Clinical examination of the affected pigeons showed that most of them had the cutaneous form of pigeon pox, with the unfeathered areas around the beak, eyes, neck, cloacal opening, and toes covered with papules and pustules. Some of these pustules were covered with a thick yellow or brown crust, as shown in Figure 1A, 1B, 1C, 1D and 1E).

The study also showed that a small number of pigeons were infected with the wet and cutaneous forms of pigeon pox, where fibrous necrotic lesions were observed in the oral mucosa, as shown in Figure 1F. On the other hand, infected pigeons suffered from loss of appetite, emaciation, fever, and decreased body weight.

Histopathological Changes

The histopathological results in this study revealed hyperplastic epithelial cells containing vacuolar inclusions and eosinophilic intracytoplasmic inclusion bodies (Bollinger bodies). These inclusions caused cytoplasmic swelling, leading to cellular necrosis. The findings are illustrated in Figures 2 and 3.

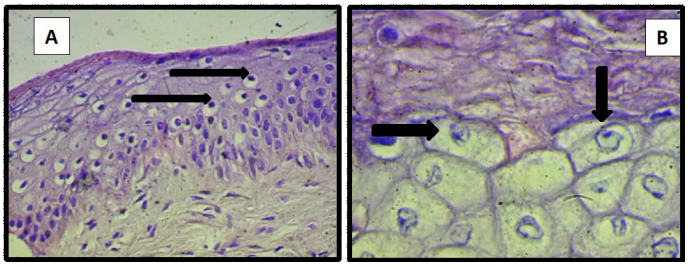

Figure 2: A: Section of pigeon skin infected with pox virus showing ballooning degeneration in epithelial cells (black arrow) (H&E, stains,400x). **B:** Section of pigeon skin infected with pox virus showing presence of large cytoplasmic vacuoles with nuclear degeneration (black arrow) (H&E, stains,1000x).

Molecular Results

In this study, all samples collected from infected pigeons were tested for the presence of pigeon pox virus using real-time PCR, and all samples tested positive. Figure 4A and 4B illustrates the amplification and melting curves, respectively. Figure 5 shows the gel electrophoresis results of the *P4b* gene of pigeon pox virus. Five positive samples were selected for sequencing. These samples, which showed an amplification product of 582 bp by conventional PCR,

were subjected to Sanger sequencing. The resulting sequences were submitted to the NCBI GenBank database under the accession numbers: PP537782, PP537783, PP537784, PP537785, and PP537786. Alignment of the *P4b* gene sequences from the pigeon pox virus samples revealed no nucleotide variations when compared with the closest reference sequence (GenBank Accession No. NC_024447.1), as shown in Figure 6.

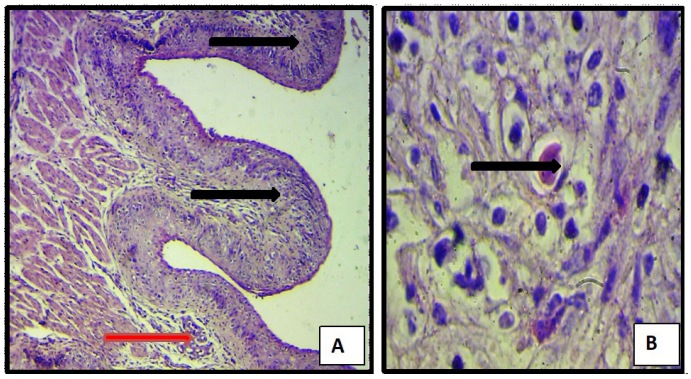

Figure 3: A: Histopathological section of pigeon skin infected with pox virus showing severe hyperplasia of the epidermis (black arrow) with inflammatory cells infiltration in the dermis layer (red arrow) (H&E stains, 100x). **B:** Histopathological section of pigeon skin infected with pox virus showing eosinophilic cytoplasmic inclusion bodies(black arrow) (H&E, stains,1000x).

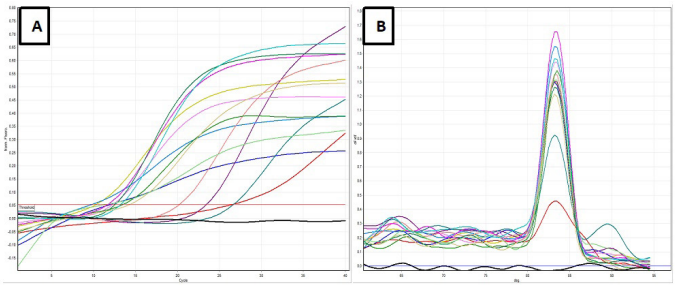

Figure 4: Detection of pigeon pox virus by real time PCR; **A:** amplification curves; **B:** melting curve analysis, the black curve represent negative control, other curve represent 16 different samples.

To further understand the phylogenetic relationships between the examined samples and other relevant reference strains, a phylogenetic tree was constructed based on the nucleotide sequences of the *P4b* amplicons. The tree incorporated both the amplified samples and related reference sequences. A rectangular cladogram was generated to represent the viral sequences, organizing them into three distinct phylogenetic clades, as illustrated in Figure 7.

Within the major pigeon pox virus clade, it was found that the investigated viral samples were positioned in the immediate vicinity of several strains that have been isolated from Egypt (Gen Bank JQ665840.1, MN892361.1, OR027037.1, and MT219996.1), India (Gen Bank OK483027.1 and DQ873811.1), Tanzania (Gen Bank KJ913659.1), Canada (Gen Bank MH175237.1), Iran (Gen Bank MF102271.1), Germany (Gen Bank AY530303.1), and South Africa (Gen Bank FJ948105.1). Accordingly, the analysis suggests that the investigated viral samples may have originated from multiple international sources.

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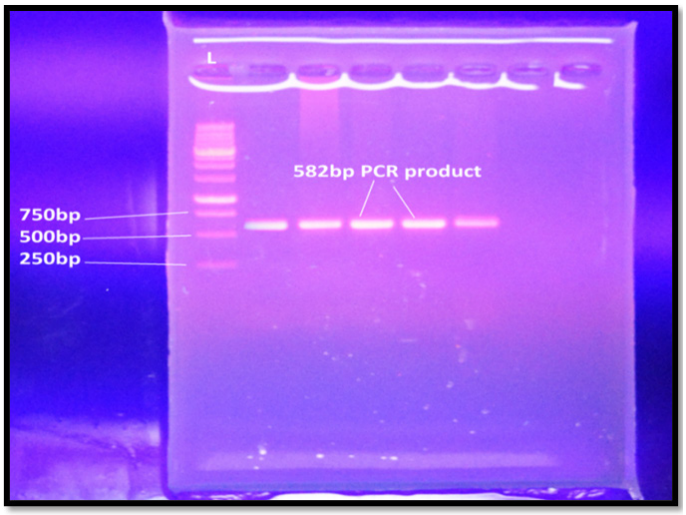

Figure 5: Gel electrophoresis of P4b gene of pigeon pox virus; L-Ladder.

In the phylogenetic tree, two related viral clades canary pox virus and fowl pox virus were included as outgroups to support the classification and evolutionary placement of the pigeon pox virus clade.

DISCUSSION

The clinical signs and gross lesions recorded in the present study were consistent with those reported in previous studies. These findings confirm that avian pox is characterized by the presence of multiple yellowish nodules on both feathered and unfeathered areas of the skin particularly around the eyes, cloaca, wings, beak, legs, and feet as well as fibronecrotic lesions on the oral mucous membranes of pigeons (Tripathy and Reed, 2013; Alehegn *et al.*, 2014; Mahmoud, 2015; Al-Bayati, 2017; Jan *et al.*, 2017; Audarya *et al.*, 2018; Lebdah *et al.*, 2019; Hartati *et al.*, 2021; Shalaby *et al.*, 2021; Khaleefah *et al.*, 2024). The current study also observed pigeon pox lesions around the cloacal opening (Figure 1), which may be attributed to mite infestations, as mites often parasitize the cloacal region—a common site for their activity. Furthermore, the role of mites in the transmission of fowlpox virus has been documented. Shirinovf *et al.* (1972) demonstrated the ability of *Dermanyssus gallinae* (red poultry mite) to transmit the fowlpox virus.

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10 20 30 40 50 60 70 80 90 100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
CAGCAGGTGCCAAACAACAACGGCCCGTGCGTCTTCGTAGTAGTAAACCAAAACCCGA
TATATGTAAAGGAGTTTTAGATTCTGGAAAACAAAAAAATAC
W3 ..........
W10 ..........
W16 ..........
W17 ..........
W19 ..........
110 120 130 140 150 160 170 180 190 200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
AATTATCAATATAGATGAAATTACATCAACTCATGACTGGCAATATAATCTCAGAAAAG
ATGCAGATGCTATAGTAAGATATCTTATGGATAGAAAATGT
W3 ..........
W10 ..........
W16 ..........
W17 ..........
W19 ..........
210 220 230 240 250 260 270 280 290 300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
GACATAAATAACTTTACGATACAGGATCTTATTAGAGTTATGAGAGAATTAAATATTAT
TAGGAACGAAAGACAAGAGTTATTCGAGTTACTATCTCACG
W3 ..........
W10 ..........
W16 ..........
W17 ..........
W19 ..........

310 320 330 340 350 360 370 380 390 400 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
TTAAAGGATCGCTTTCTAGTAATAGTGTTTCTGTCAAAACTAGTCATCCACTAATGGTT
ATTTATTCA.CATTCAGATAACAAGATAGGGGAACAGTTAAA
W3 ..........
W10 ..........
W16 ..........
W17 ..........
W19 ..........

410 420 430 440 450 460 470 480 490 500
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ref.
ACTACTAGAAAATACTTACGATCCATCTAGATATCAGGCTCTGATAGATACTACGAGGT
TTCAATCTACAAACTTTGTGGATATGTCAACGTCTAGTGAT
W3 ..........
W10 ..........
W16 ..........
W17 ..........
W19 ..........

510 520 530 540 550 560 570 580
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.
ATGTTGTTTAGATTTAAAGACCAGGATTCCATAGGTTACGTTCATCCTATATTAGTTGC
TTTATTCGGTGTTAAGCTTCCG
W10 ..........
W16 ..........
W17 ..........

W3 ..........
W19 ..........
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Figure 6: Nucleic acid sequences alignment of five viral samples with their corresponding reference sequences of the P4b genomic sequences. The symbol "ref" refers to the NCBI referring sequence of pigeonpox virus (GenBank acc. no.NC_024447.1).The letter "W#" refers to the sample code

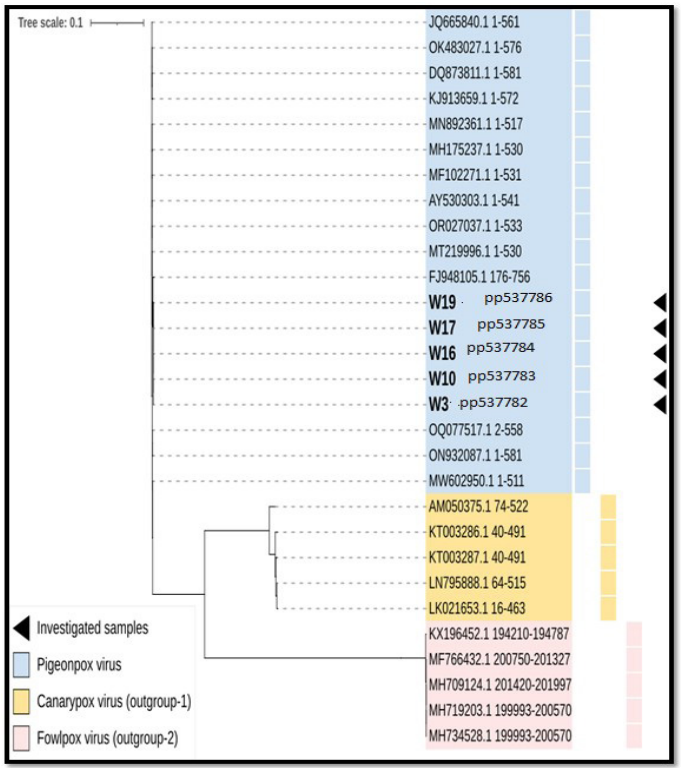

Figure 7: The comprehensive rectangular cladogram phylogenetic tree of P4b sequences of pigeonpox virus. All the mentioned numbers referred to GenBank accession number of each referring species. The number at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "W#" refers to the code of the investigated samples.

Histopathological examination of skin lesions in infected pigeons revealed vacuolated cytoplasm and single, round, dense eosinophilic intracytoplasmic inclusions (Bollinger bodies), which are pathognomonic for poxvirus infections. These findings are in agreement with the studies of Mahmoud (2015), Lebdah *et al.* (2019), Shalaby *et al.* (2021), Pandiyan *et al.* (2022), Faisal and Al-Azzawi (2023), and Khaleefah *et al.* (2024), who reported that fowlpox virus induces marked histopathological changes in the skin tissue of infected pigeons. These changes include enlargement of stratified squamous epithelial cells, acanthosis, and the presence of severe inflammatory infiltrates composed of heterophils, macrophages, and lymphocytes. The stratified squamous epithelium exhibited hypertrophic degeneration, and the epithelial cells appeared swollen, rounded, and widely distributed. Large eosinophilic intracytoplasmic inclusions (Bollinger bodies) were frequently observed in hyperplastic epithelial cells along with constant cytoplasmic vacuolization, appearing as ring-shaped eosinophilic inclusions.

Although histopathological lesions of pigeonpox may resemble those caused by fungal infections, they can be distinguished by the presence of acidophilic intracytoplasmic

inclusion bodies, which are a hallmark of poxvirus infection. In contrast, fungal infections may present with hyphae within the affected tissues, providing a key differential diagnostic feature.

The P4b core protein gene is a conserved specific gene used in molecular diagnosis and phylogenetic analysis of fowl pox virus. We relied solely on the p4b gene of the avian pox virus core protein, which has been used in previous studies. Our results were not compared with other conserved genes of the avian pox virus. P4b was chosen because it is relatively conservative among Avipoxvirus strains. This indicates the high effectiveness of the molecular approach in confirmatory identification of fowlpox virus (Lüschow *et al*., 2004; Masola *et al*., 2014). Regarding molecular biological analysis, all isolates in this investigation generated gene P4b amplification products of the anticipated size. Hence, PCR is an excellent method for diagnosing pox virus infections. According to previous studies Ababneh *et al*. (2020) reported that real time PCR was reported to be effective tool and as sensitive as to characterize different virus strains and genotypes. PCR was used in this study for genomic characterization of pigeon pox virus by using P4B gene, the results showed the presence of P4B gene in all tested sample, this results was agreement with results that obtained by Abd El-Hafez *et al* . (2021), Khaleefah *et al*. (2024), who reported that the P4B gene was detected in all 30 tested samples. In addition, the genetic similarity between local samples and globally distributed strains indicates the widespread presence of pigeon pox virus variants in different regions, as observed in a study by Yeo *et al*. (2019). We did not observe any genetic mutation or any differences between the isolates recorded in this study and the pigeonpox virus strains isolated and recorded in the gene bank. The reason may be that the causative agent of pox is the fowl pox virus, which has deoxyribonucleic acid DNA, and most viruses of this type are more stable and less susceptible to genetic mutations. Unlike viruses that contain RNA, which are susceptible to genetic mutations, may be attributed that to the polymerases of DNA viruses has 3′ exonuclease proofreading activity and hence are less error-prone than those of RNA viruses also, the ability of DNA viruses to correct DNA mismatches by proofreading and/or post-replicative repair (Sanjuán and Domingo-Calap, 2016). The present finding corroborates the previous report where no difference was found among the global circulating strains of pigeon pox virus (Gyuranecz *et al*., 2013). No genetic diversity between the sequences might be due to the highly conserved nature of P4b gene among the all avian pox viruses and very low rate of mutation in pox virus (Saito *et al*., 2009). Furthermore, this remarkable similarity may be due to the importation of birds from infected countries into Iraq, or the possibility that the pigeonpox virus was transmitted to Iraq by migratory wild birds or through the live bird trade.

The results of this study confirmed the prevalence of pigeon pox virus in Babil Governorate in central Iraq. This finding has been recorded in various regions of Iraq, including northern, central, and southern Iraq (Manswr, 2024; Jumaa, 2024; Faisal and Al-Azzawi, 2024; Khaleefah *et al*., 2024). Also, the five strains of pigeonpox virus detected in this study are closely related to the strains isolated from pigeons infected with the cutaneous form of pigeonpox in the capital, Baghdad (Jumaa, 2024).

CONCLUSIONS AND RECOMMENDATIONS

This study concluded that the pigeon pox virus (PPV) strain detected in infected pigeons in Babylon Province is closely related to globally circulating strains. The findings underscore the importance of genetic analysis in understanding the evolutionary characteristics of avian poxviruses. Successfully identifying and characterizing the local PPV strain provides a foundation for developing targeted strategies to control and prevent future outbreaks. Based on the outcomes of this study, efforts will be directed toward the development of vaccines derived from these locally identified strains, with the aim of enhancing disease control in the pigeon population. Currently, there are no specific antiviral treatments available for pigeon pox, and existing management relies primarily on supportive care. Therefore, this research also paves the way for future intensive studies focused on the discovery and development of effective antiviral therapies against avian poxvirus infections.

ACKNOWLEDGMENTS

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NOVELTY STATEMENTS

The current study provides the first partial diagnosis of PPV in Babylon, Iraq, contributing unique genetic data to global databases.

AUTHOR'S CONTRIBUTIONS

Khitam Hassan Salih Alaarajy contributed in the molecular diagnosis while Hayder Abd AL-Emier Almremdhy contributed in pathology and writing of the manuscript.

Conflict of Interest

The authors have declared no conflict of interest.

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