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Research Article

# Sequencing and Phylogenetic Analysis of P4b Gene in Pigeon Poxvirus

**Rawaa Saladdin Jumaa**

*Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.*

**Abstract** | This is the first study in Iraq to look at P4b gene analysis of pigeon poxvirus virus in locally infected pigeons with crust lesions in the eye and nostril cere. The DNA extracted from the collected samples was amplified using conventional PCR by amplification of the P4b gene. The positive samples had been inoculated on chorioallantoic membrane (CAM). Thickening of CAM were seen in the first passage, whereas pock lesion at the second (2nd) and third (3rd) passages. The chicken embryo fibroblast (CEF) cells propagated by the prepared infected CAM have shown the cytopathic effect (CPE) that included rounding, detachment of the cells from the monolayer and aggregation of cells during the first and second tissue culture passages. The results of sequencing analysis of pigeon poxvirus field samples revealed that the gene has some mutations (nonsense and missense) and is highly conserved among pigeon poxvirus isolates. The phylogenetic analysis revealed that the P4b gene of Iraqi isolates was related to the (Egypt strain) (99% similarity). Phylogenetic analysis shown that the P4b gene of an avipoxvirus obtained from an infected pigeon was classified as a pigeon poxvirus. The conclude of this study: The main findings that demonstrate the typical characteristic of poxviridae on tissue culture and embryonated chicken eggs. Also, the phylogenetic tree was confirmed that the avipoxvirus infection observed in local pigeons was caused by pigeon poxvirus.

**Keywords** | Isolation, P4b gene, Sequencing, Pigeon poxvirus, Iraq

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***Correspondence** | Rawaa Saladdin Jumaa, Microbiology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq; **Email:** rawaa.saladdin@covm.uobaghdad.edu.iq
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## INTRODUCTION

Poxvirus virions (poc, known as pustule) are brick shaped with complex symmetry, large size (Abd El-Hafez *et al.*, 2021). The genome is a double stranded DNA molecule that runs in a straight line (Weli and Tryland, 2011). The replication cycle takes place within the cytoplasm (also known as viral factories or viroplasm), and the mature virion or enveloped virsion is released via budding (Abd El-Hafez *et al.*, 2021; Hartati *et al.*, 2021). Pox is a contagious infection that affects all ages, breeds, and sexes of wild and domestic birds. This disease is caused by the poxvirus, which belongs to the subfamily *Chordopoxvirinae* in the *poxviridae* family. Avipoxvirus is made up of different species (Bassiouny *et al.*, 2021). It is a common viral disease that affects both domestic and wild birds and commercial poultry. The virus is divided into three species: Pigeon poxvirus, fowl poxvirus, and canary poxvirus (Elias *et al.*, 2014; Parker *et al.*, 2011). Pigeon poxvirus (PPV), and other avian ex. (fowl and canary) poxviruses are all members of the *Avipoxvirus* genus. The viruses are antigenically besides immunologically distinct from one another, although a cross-relationship for this strain of the virus makes identification difficult (Elias *et al.*, 2014).

Proliferative and nodular lesions in the featherless areas of the skin are indicative of the disease. In addition to the fibronecrotic lesions in the esophagus, mouth, and upper respiratory tract mucous membrane (Hartati *et al.*, 2021).

Viruses are isolated in an embryonated chicken eggs, cell culture (chicken fibroblasts), or by molecular detection of poxviruses for appropriate investigation and diagnosis (Ramesh *et al*., 2018; Rahman *et al*., 2019).

Thus, in this study, an *Avipoxvirus* obtained from a pigeon with cutaneous lesions was concentrated on isolation and molecular detection tool for confirmation the avipox disease and determination the viral species via phylogenetic analysis.

## MATERIALS AND METHODS

### Samples collection

The scab lesions were obtained from (50) infected local pigeons in Baghdad province. Infected birds' clinical signs included the presence of crusts and lesions on their eyelids, legs, nostrils, and other un feathered body parts. These scabs were stored at 4°C in a screw cup container. The diagnosis was made in the laboratory at the Department of Virology in Veterinary Medicine College Baghdad University.

### Viral DNA extraction

Viral DNA purification Kit (Qiagen, Germany) was used to extract the DNA.

### Viral detection by conventional PCR

Using NCBI, a specific primer design was used to amplify a 558bp fragment of the *Avipoxvirus* P4b gene,
Forward primer: CAGCAGGTGCTAAACAACAA
Reverse primer: CGGTAGCTTAACGCCGAATA

### Preparation of samples and viral isolation

The positive scab sample from infected birds was ground separately in sterilized mortar and pestle before being suspended in sterilized phosphate buffered saline (PBS) to make a 10% suspension. The suspension was centrifuged at 3000 rpm for 15 minutes, and the supernatant was treated for 45 minutes at 37°C with a mixture of penicillin and streptomycin at (10.000IU and 10mg /ml) to reduce bacterial contamination. All of the positive samples by PCR were first inoculated onto the chorioallantoic membrane (CAM) of an embryonated chicken egg at 12 days old, as described in (Puro *et al*., 2017). Following the inoculation, incubation at 37°C was completed and daily checks were performed. CAM was collected and examined for pock lesion after 5-6 days of inoculation.

### Cell culture of chicken embryo fibroblast

The primary fibroblast cell culture was prepared according to (Cunningham, 1966). The harvested charoiallantoic membrane was prepared and propagated into chicken embryo fibroblast cell culture (CEFCC) according to (Youngner, 1954). The inoculated sample was passage through several times. The cytopathic effect was demonstrated after 24-48 h of incubation.

### Sequence analysis

For Sanger sequencing, the amplified P4b gene product was shipped to Korea (Macrogen). Later, the acquired nucleotide sequences were analyzed using Mega 6 software and BLAST (http://blast.ncbi.nlm.nih.gov).

## RESULTS AND DISCUSSION

### Conventional PCR for detection the amplified DNA

Using the P4b gene, gel electrophoresis revealed bands of amplified nucleic acid from pigeon poxvirus (PPV) ten samples out of fifteen samples were positive as in Figure 1.

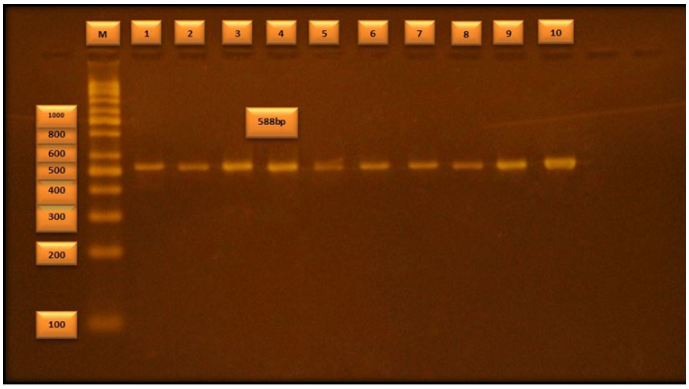


**Figure 1:** The agarose gel electrophoresis pattern demonstrated the conventional PCR for the P4b gene in infected birds (M=leader, lane1-10 samples).

### Inoculation of virus in the embruonated chicken egg (ECE)

Embryonated chicken eggs (ECEs) were inoculated by positive samples at (11-13 age). The charoiallantoic membrane (CAM) was harvested after 5 days post infection. The infected CAM was grossly thickened as seen in the first passage Figure 2B but the pock lesion which showed up round slightly elevated necrotizing points appeared at the second and third passages at the CAM Figure 2C, D. Non-pathogenic CAM (control) was shown in Figure 2A.

### Isolation of the virus at chicken embryo fibroblast (CEF) cells

The prepared samples of charoiallantoic membrane were propagated in CEF cell culture for two passages, the cytopathic effect was observed with the first passage, which started showing rounded cells. At the second passage, the signs became more severe, including rounding cells as grape clusters with signs of degenerative changes, particularly at 24 h (P.I) and excessive cell detachment from the monolayer, which appeared at 48 h as shown in Figure 3.

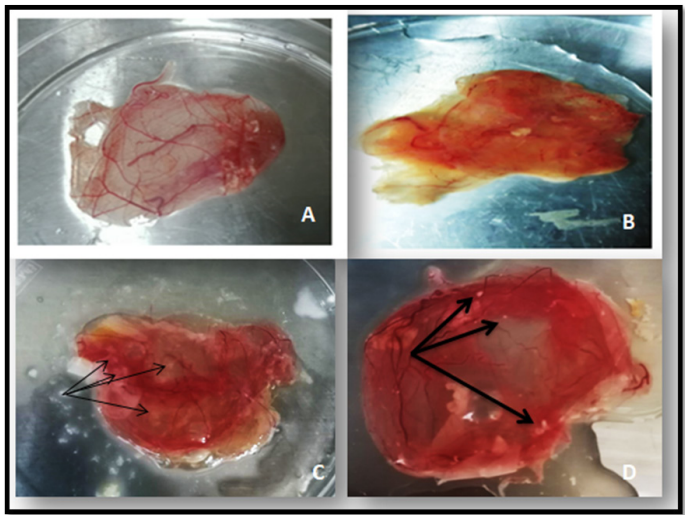


**Figure 2:** (A) Non-pathogenic CAM (Control); (B) Infected CAM with thickened and hemorrhagic at 1st passage; (C), (D) Infected CAM with Pock lesions at 2nd and 3rd passages.

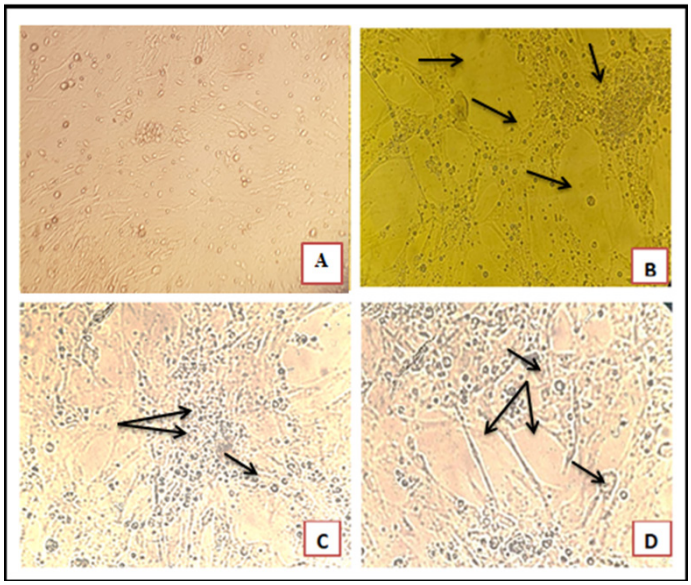


**Figure 3:** Cytopathic effect of the virus when propagated in embryonated chicken eggs; (A) Uninfected cell culture (control); (B) cell rounding, aggregation and sloughing of it at 1st passage after 48h; (C) and (D) cell aggregation and sloughing after 24h and 48h of 2nd passage.

## Sequencing analysis of P4b gene

Mega 6 software was used to analyze the sequences of PCR products for the P4b gene, and the results of nucleotide sequence alignment of our isolate's 558 bp P4b gene revealed a close relationship (99%) with the majority of PPVs in GenBank are shown in the Table 1. Substitutions in P4b genes were found in pigeon poxvirus in this study. The P4b gene of pigeon poxvirus isolates showed substitutions: Glutamine, Leucine and Proline in P4b gene are nonsense mutation, while (Isoleucine to Threonine), (Threonine to Alanine), (Tyrosine to Cysteine) (Cysteine to Serine), (Leucine to Proline), (Asparagine to Serine), (Proline to Arginine), (Isoleucine to Asparagine), (Phenylalanine to Serine), (Aspartic acid to Histidine) and (Leucine to Serine) in P4b gene is missense mutation.

## Conformation of P4b gene of Iraqi pigeon poxvirus

The result appeared 3D secondary structure, ramachandran plots for amino acid in general and local quality estimation with Z score of P4b gene for pigeon poxvirus, the model prediction 3D of protein as in Figure 4 which composed of alpha helix, beta sheet and coil (A). Model evaluation revealed the amino acid of query protein (B). The model validation includes the local quality estimation with Z score (C and D).

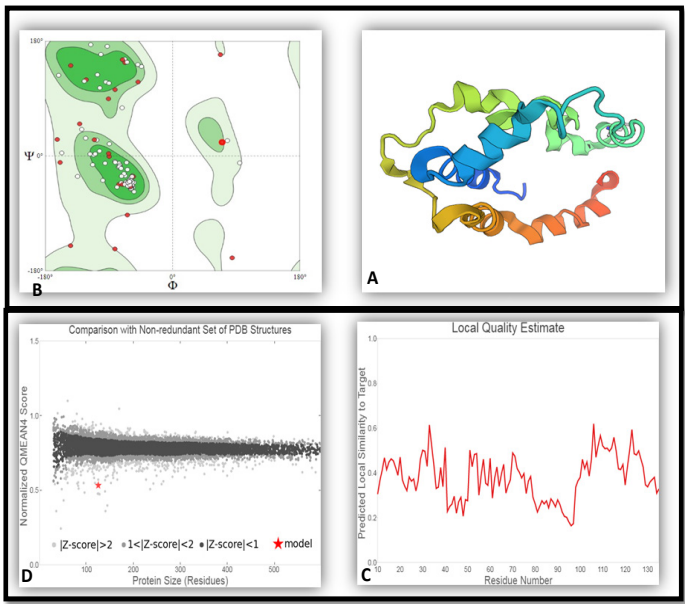


**Figure 4:** Three dimensional models of P4b gene for pigeon poxvirus (A), Model evaluation (Ramachandran plot) of P4b gene for pigeon poxvirus (B), Model validation (local estimation and Z score) of P4b gene for pigeon poxvirus (C and D).

## Phylogenetic analysis P4b gene for pigeon poxvirus

The phylogenic analysis of the P4b gene of Iraqi pigeon poxvirus, which was deposited in NCBI under the accession number and studied in comparison to other registered types regarding the P4b gene, revealed that the Iraqi PPV was 99 percent homologous to the Egypt strain (MN892361.1), as shown in Table 2 and Figure 5.

Avipoxvirus is a contagious disease that affects different wild and domestic of birds. The current research concentrated on the pigeon poxvirus (PPV) isolation in Iraq. As well as, the results of viral isolation are confirmed via PCR diagnosis and sequencing of P4b gene of pigeon poxvirus.

**Table 1:** Sequencing analysis of P4b gene in pigeon poxvirus.

| Source | Identities | Score | Sequence ID | Predicted effect | Amino acid change | Nucleotide change | Nucleotide | Location | Type of substitution | No. of sample |
|---|---|---|---|---|---|---|---|---|---|---|
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 734 | ID: MG787227.1 | Missense | Isoleucine> Threonine | ATT>ACC | T>C | 68 | Transition | 1 |
| | | | | Missense | Isoleucine> Threonine | ATT>ACC | T>C | 69 | Transition | |
| | | | | Missense | Threonine> Alanine | ACT>GCT | A>G | 94 | Transition | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 738 | ID: MG787227.1 | Missense | Tyrosine> Cysteine | TAT>TGT | A>G | 146 | Transition | 2 |
| | | | | Missense | Cysteine> Serine | TGT>TCT | G>C | 164 | Transvertion | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 734 | ID: MG787227.1 | Missense | Leucine> Proline | CTT>CCT | T>C | 193 | Transition | 3 |
| | | | | Missense | Asparagine> Serine | ATT>AGT | A>G | 218 | Transition | |
| | | | | Nonsense | Glutamine> Glutamine | CAA>CAG | A>G | 240 | Transition | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 734 | ID: MG787227.1 | Nonsense | Leucine> Leucine | CTA>CTT | A>T | 258 | Transvertion | 4 |
| | | | | Missense | Leucine> Proline | CTT>CCT | T>C | 278 | Transition | |
| | | | | Missense | Proline> Arginine | CCA>CGA | C>G | 314 | Transvertion | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 738 | ID: MG787227.1 | Missense | Isoleucine> Asparagine | ATT>AAT | T>A | 326 | Transvertion | 5 |
| | | | | Nonsense | Leucine> Leucine | TTA>CTA | T>C | 361 | Transition | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 743 | ID: MG787227.1 | Missense | Phenylalanine > Serine | TTT>TCT | T>C | 440 | Transition | 6 |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 734 | ID: MG787227.1 | Missense | Isoleucine> Threonine | ATT>ACC | T>C | 68 | Transition | 7 |
| | | | | Missense | Isoleucine> Threonine | ATT>ACC | T>C | 69 | Transition | |
| | | | | Missense | Threonine> Alanine | ACT>GCT | A>G | 94 | Transition | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 734 | ID: MG787227.1 | Nonsense | Leucine> Leucine | CTA>CTT | A>T | 258 | Transvertion | 8 |
| | | | | Missense | Leucine> Proline | CTT>CCT | T>C | 278 | Transition | |
| | | | | Missense | Proline> Arginine | CCA>CGA | C>G | 314 | Transvertion | |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 743 | ID: MG787227.1 | Missense | Aspartic acid > Histidine | GAT>CAT | G>C | 385 | Transvertion | 9 |
| Pigeonpoxvirus isolate PiPVIR18 P4b (p4b) gene | 99% | 738 | ID: MG787227.1 | Missense | Leucine> Serine | TTA>TCA | T>C | 41 | Transition | 10 |
| | | | | Nonsense | Proline> Proline | CCA>CCG | A>G | 315 | Transition | |

**Table 2:** Iraqi pigeon poxvirus compared with other registered global of P4b gene.

| S. | Accession | Country | Source | Compatibility |
|---|---|---|---|---|
| 1 | ID: MN892361.1 | Egypt | Pigeonpoxvirus (p4b) gene | 99% |
| 2 | ID: MG787227.1 | Iran | Pigeonpoxvirus (p4b) gene | 99% |
| 3 | ID: MH721412.1 | India | Pigeonpoxvirus (p4b) gene | 99% |
| 4 | ID: MH365477.1 | India | Pigeonpoxvirus (p4b) gene | 99% |
| 5 | ID: MH175237.1 | Canada: Ontario | Pigeonpoxvirus (p4b) gene | 99% |
| 6 | ID: MF496043.1 | India | Pigeonpoxvirus (p4b) gene | 99% |
| 7 | ID: MF102271.1 | Iran | Pigeonpoxvirus (p4b) gene | 99% |
| 8 | ID: MF102270.1 | Iran | Pigeonpoxvirus (p4b) gene | 99% |
| 9 | ID: MF102269.1 | Iran | Pigeonpoxvirus (p4b) gene | 99% |
| 10 | ID: KJ913659.1 | Tanzania | Pigeonpoxvirus (p4b) gene | 99% |
| 11 | ID: KJ801920.1 | South Africa | Pigeonpoxvirus (p4b) gene | 99% |
| 12 | ID: JQ665840.1 | Egypt | Pigeonpoxvirus (p4b) gene | 99% |
| 13 | ID: DQ873811.1 | India | Pigeonpoxvirus (p4b) gene | 99% |
| 14 | ID: AY530303.1 | Germany | Pigeonpoxvirus (p4b) gene | 99% |
| 15 | ID: MH721417.1 | India | Pigeonpoxvirus (p4b) gene | 99% |
| 16 | ID: MH721413.1 | India | Pigeonpoxvirus (p4b) gene | 99% |

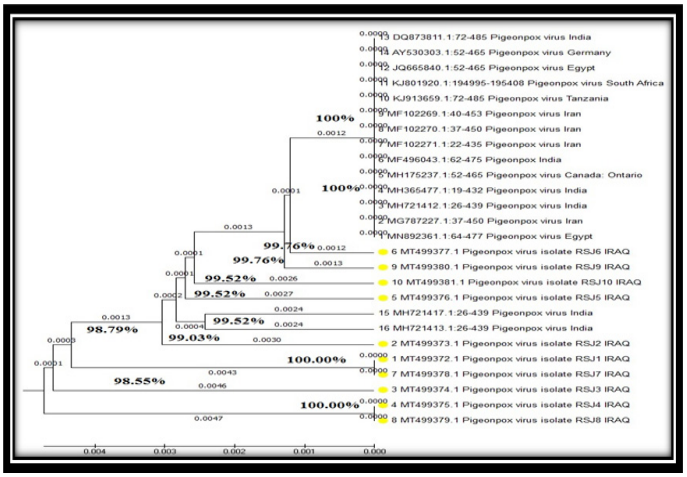


**Figure 5:** Phylogenic tree of Iraqi sample that submitted to NCBI under the accession number (MT499377.1, MT499380.1, MT499381.1, MT499376.1, MT499372.1, MT499373.1, MT499378.1, MT499374.1, MT499375.1 and MT499379.1) using Mega 6 software for P4b.

The current study's findings support the findings of (Bayati, 2017; Ghalyanchilangeroudi *et al*., 2018; Audarya *et al*., 2018; Sultana *et al*., 2019), who reported CAM thickening after PPV inoculation and development of Pock lesions. Bayati (2017) demonstrated that these isolates produced pocks lesions at the 3rd and 4th passages, whereas thickening and necrosis of CAM were only seen at the first passage. Ghalyanchilangeroudi *et al*. (2018) and other researchers described the pock lesions on CAM of chicken embryos that inoculated with pigeon poxvirus. However, in the current study, there was thickening and pock lesion on the CAM, which is in line with the findings of (Ghalyanchilangeroudi *et al*., 2018; Sultana *et al*., 2019) reported CAM thickening. In addition, in the current study, there was cytopathic effect on fibroblast embryonated chicken eggs which characterized with rounding, aggregation and detachment of cells from monolayer which showed after 24h and 48h of 1st and 2nd passage respectively, which is consistent with the results of (Ghalyanchilangeroudi *et al*., 2018; Audarya *et al*., 2018) revealed that the virus had the same impact on these kinds of cells. Sultana *et al*. (2019) showed the appearance of cytopathic effect which include rounding, necrotic lesions, degeneration and clumping of cells beside the formation of giant cell that observed due to fowl poxvirus and pigeon poxvirus at chicken embryos fibroblast. Bayati (2017) revealed the cytopathic effect of viral inoculated at fibroblast cell culture which included aggregation and detachment of cells from monolayer at 3rd and 4th passages.

Substitutions in P4b genes were found in pigeon poxvirus in this study. The P4b gene of pigeon poxvirus isolates showed substitutions: Glutamine, Leucine and Proline in P4b gene are nonsense mutation, while (Isoleucine to Threonine), (Threonine to Alanine), (Tyrosine to Cysteine) (Cysteine to Serine), (Leucine to Proline), (Asparagine to Serine), (Proline to Arginine), (Isoleucine to Asparagine), (Phenylalanine to Serine), (Aspartic acid to Histidine) and (Leucine to Serine) in P4b gene is missense mutation. In general, these P4b mutations may provide a high degree of immune system resistance and may work in tandem to increase the virulence of the pigeon poxvirus, or vice versa. The nucleotide sequence alignment of our isolate's 558 bp P4b gene demonstrated a strong (99%) correlation with the majority of PPVs in GenBank, suggesting a high degree of conservation of the P4b gene in PPV isolates. Additionally, the PPV isolate from Iraqi strain was found to be clustered in the same branch as PPVs from Egypt and India, according to a phylogenetic tree relied on the nucleotide sequences of the P4b gene and corresponding reference sequences. According to Masola *et al*. (2014), shown that these segments' high degree of conservation may help to explain why the nucleotide sequences of the majority of our isolates and those previously isolated in other nations are comparable. These results are agreement with Zarifi *et al*. (2019) indicated that avipoxvirus conservation was high. Additional aspects were also looked into, such as polymorphisms in the highly conserved P4b gene of poxviruses that led to disparities in clinical outcomes and partial protection induced by employing the improper vaccine strains.

The results of protein conformation analysis revealed that P4b protein's secondary structure is made up of coil or ribbon, sheet, and helical models. This secondary structure conservation is an important parameter in evaluating the structural model of viral proteins. Furthermore, the Rose *et al*. (2017) revealed the secondary structure was created as a result of the hydrogen bond formation between hydrogen and oxygen atoms in order to preserve their spatial stabilities. Street *et al*. (2000) illustrated the secondary structure include two substructures was observed in folded chains: alpha-helices, a spiral-like structure, and beta-strands, which, depending on the orientation of the amino acid sequences, can be parallel or antiparallel. Loops, which are mainly present on a protein's surface, are what connect them. Loops contain polar and charged residues. Loops, beta-strands, and alpha helices arrange the secondary structural elements.

Furthermore, the Ramachandran plot reveals the presence of α-helices and β-sheets of the secondary structure in local protein structure. Ramachandran and Sasisekharan (1968) showed the conformation of protein that include alpha helices and beta sheets also who observed the conformation of amino acids and polypeptide. In addition, the QMEAN analysis was used in this study for calculation the plot of local quality and the Comparison plot or Z score. Abbass (2018) reveled the QMEAN analysis was utilized in the model's evaluation and validation. Also, the

Street *et al*. (2000) illustrated. The overall energy departure of the structure from an energy distribution produced from random conformations is quantified by the Z-score. Benkert *et al*. (2011) said the scores were indicated an extremely accurate structure which fall comfortably within the range of values that can be discovered for similar-sized proteins. The energy plot uses knowledge-based energies as a function of amino acid sequence position to illustrate the quality of the local model (Abbass, 2018).

The phylogenic analysis of the P4b gene of Iraqi pigeon poxvirus, which was placed in the NCBI with the accession number (MT499377.1, MT499380.1, MT499381.1, MT499376.1, MT499372.1, MT499373.1, MT499378.1, MT499374.1, MT499375.1 and MT499379.1) and studied in comparison to other registered types regarding the P4b gene, revealed that the Iraqi PPV was 99 % homologous to the Egypt strain (MN892361.1). Felsenstein (2004) showed the branching diagram called a phylogenetic or evolutionary tree was used to illustrate the links between various biological species based on the similarities and differences in their physical or genetic traits.

## CONCLUSIONS AND RECOMMENDATIONS

The main findings depicted the typical manifestation of poxviridae on chicken embryos eggs and fibroblast cell culture. The sequences of the P4b genes showed is highly conserved among pigeon poxvirus isolates. The P4b gene of an avipoxvirus obtained from an infected pigeon was classified as a pigeon poxvirus based on phylogenetic analysis. This proved that the avipoxvirus infection in local pigeons was caused by pigeon poxvirus.

## ACKNOWLEDGEMENT

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## NOVELTY STATEMENT

The novelty of the study is focus on isolation of pigeon poxvirus and sequencing of P4b gene. As well, the evolutionary tree was illustrated the registered types.

### Conflict of interest

The author has declared no conflict of interest.

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