جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة ديالى
كلية الطب البيطري

التحري الجزيئي والتحليل الوراثي والتشخيص النسيجي لفايروس جدري الطيور في محافظة ديالى

رسالة مقدمة الى

مجلس كلية الطب البيطري _جامعة ديالى

وهي جزء من متطلبات نيل درجة الماجستير في علوم الطب البيطري/علم الاحياء المجهرية

من ِقبل

عائشة فيصل مجيد

بإشراف

أ.د. عامرخزعل العزاوي

2024 م

1445 هـ

Republic of Iraq
Ministry of Higher Education & Scientific Research
University of Diyala
College of Veterinary Medicine, Department of Microbiology

Molecular, Phylogenetic Analysis and Histopathological Identification of Avian Pox Virus in Diyala Province

A Thesis

Submitted to the College of Veterinary Medicine, University of Diyala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Microbiology

By

Aisha Faisal Majeed

Supervised by

Prof. Amer Khazaal Saleh Al-Azzawi (Ph. D)

2024 A.D. **1445 A.H.**

Abstract

Fowlpox is an extremely contagious viral disease affecting domestic and wild birds. This study aims to identify the presence of avianpox virus in chickens and pigeons in Diyala Province, Iraq.

In fact, the study uses histopathological examination and molecular detection using PCR to identify the virus. Therefor, the study aims to investigate the genetic sequence and conduct a phylogenetic analysis of the avianpox virus, as well as the relatedness of local Iraqi strains and reference strains from the Gene bank (NCBI). Forty pigeons (Columba livia domestica) of varying ages were gathered for this inquiry from multiple bird markets in various areas of the Diyala Province and forty chickens (Gallus gallus) that were suspected of having pigeon pox and fowl pox respectively. Tissue samples from the affected areas of these birds' vents, eyes, wings, cere, and beak were obtained after euthanization and processed for histological and molecular investigation.

Deoxyribonucleic Acid (DNA) extraction was performed using a specific kit (gSYNC™ kit, Canada) for core protein gene region (pb4). For detection of the selected gene (p4b), two pairs of oligonucleotide primers were designed using NCBI.

These primers were supplied and provided by micro-gene Company (South Korea) F primer (5′AATCTTAGAAAAGACGCAGATGCT3′) and R primer (5′AAGTTTGTTGATTGAAACCTAGTCG3′). DNA was screened to amplify the P4b core protein gene of the avipox vius.

Sequencing was followed to determine the biological diversity of the predominant serotypes from chickens and pigeons in Diyala Province. Within this locus, six samples for sequencing were included in the present study, two samples for fowlpox virus (S2 and S3), and four samples for avipox virus (S1, S4, S5, and S6).

Accordingly, the samples were tested to amplify P4b (core protein) for sequencing of the fowlpox and avipox viruses. The amplified parts were

submitted to sequencing experiments (Sanger) to assess the genetic polymorphism as compared with the references in the NCBI database. Then, the comprehensive tree used to assess the accurate serotyping of the phylogenetic distribution and observed variants.

The results reveals that the clinical signs are characterized by nodular lesions in featherless regions, crusts disseminated around the face, legs, and feet, unpigmented skin around the beak, eyelids, legs, combs, wattles, and wings. The result of the histopathological examination of pigeons infected with the avian pox virus revealed notable changes in the epidermis, including hyperplasia and ballooning degeneration with eosinophilic inclusion bodies. The subcutaneous tissue showed active cell proliferation and infiltration of polymorph mononuclear cells, along with the presence of eosinophilic inclusions (Bollinger bodies) and vacuolation, as well as the occurrence of fibrosis and the proliferation of fibroblasts in certain areas.

The histological analysis of chickens that were infected with the avian pox virus revealed changes in the epidermal tissue that exhibited hyperplasia of stratified squamous cells along with a significant presence of inflammatory exudate-containing cells. The outstanding characteristic observed was the existence of peculiar eosinophilic intracytoplasmic structures known as Bollinger bodies, which manifested as circular inclusions and vacuolation. Molecular results of current studies using the PCR for APV detection, showed that all collected samples gave positive results from pigeons and chickens. On 1.5% agarose gel all samples generated a specific cDNA band of 329 bp.

This study, two GenBank accession numbers were obtained (OR619724 and OR619725) to represent the fowlpox viral samples (S2 and S3) respectively. Also, four GenBank accession numbers were obtained (OR670580, OR670581, OR670582, and OR670583) to represent the pigeon pox viral samples (S1, S4, S5 and S6) respectively. Concerning the 329 bp amplicons of the avipox viruses, the NCBI BLAST demonstrated that 99% sequence likely between the sample and the reference. One nucleic acid identified in both fowlpox and avipox viral

samples namely (205C>G and 204T>C), resulting in missense and silent ((p.101Leu>Val and p.108Pro) effects on specific proteins respectively.

The constructed cladogram organized the incorporated local sequences into two main phylogenetic clades representing fowl pox viruses and pigeon pox viruses. Phylogenetic analysis revealed that chicken and pigeon samples were suited in the close proximity isolates from other geographic regions. The fowlpox viruses were positioned close to the root of the tree, indicating their ancestral relationship. However, the investigated S2 and S3 samples were suited in the vicinity of variable strains and showed similarity to strains from Iraq, Egypt, Iran, Brazil, India, and the USA for fowlpox. Whereas the pigeon samples S1, S4, S5, and S6 were closely associated with variable strains isolated from Australia. Based on the information provided, it can be concluded that both domestic and wild birds are vulnerable to avian pox, a viral disease.

Finally, histological and molecular studies have shown that this infection can cause varying degrees of severity, leading to significant pathological changes in the cutaneous regions, particularly affecting the epidermal tissues of pigeons and chickens. Molecular detection showed that the constructed cladogram categorized the included local sample sequences into two primary phylogenetic clades, representing fowl pox viruses and pigeon pox viruses, respectively. Two out-group clades were observed, containing other viral sequences.

Chapter One

Introduction

1.1. Introduction

Fowl pox (FWP) is a viral infection that affects both domestic and wild birds. It is caused by one of the larger DNA virus groups replicated in cytoplasim and is distinguished by lesions (mild to severe) with a high rate of morbidity and mortality (Zhao *et al.*, 2014).

The characteristic features of this illness in birds include the presence of unique, rapidly growing abnormalities on the head, toes, mucous membranes, and legs, systemic infections may also occure. There are several species and strains of avipoxviruses in this subgroup, and they differ in terms of their pathogenicity and host specificity (Tripathy and Reed, 2003; van Riper and Forrester, 2007 and Williams *et al.,* 2023).

The Poxviridae family has two subfamilies: Chordopoxvirinae have (8) genuses, and Entomopoxvirinae have (3) genera. The fowl pox virus (FWPV) is a virus with an oval form and a broad envelope. It is classified in Avipoxvirus, Poxviridae (family), and Chordopoxvirinae (subfamily) (Weli and Tryland, 2011). The avian poxvirus (APV) genus, which belongs to chordopoxvirinae, impacts 232 species of birds from 23 distinct orders (Tripathy and Reed, 2013; Gyuranecz *et al*., 2013). Birds infected with APV exhibit the growth of excessive skin lesions on their external and internal membranes, resulting in injury to the birds (Kleindorfer 2006, Pendl and Schmidt 2024).

Their DNA consists of dSDNA (250–365 kb) in a linear configuration (Diallo *et al*., 2010). The core structure contains genes that are shared by all poxviruses and are important to the fundamental replication processes, and create proteins implicated in host (Brennan, Greg, *et al*., 2023).

The viral particle has a size range of 270-350nm and consists of a centrally positioned core with two peripheral bodies that are electron dense (Weli and Tryland, 2011). The viral particle dimension of 270 × 350 nm and consists of an electron-dense core positioned centrally, along with two side bodies (Weli and Tryland, 2001).

Nearly all types and varieties of chicken are exposed to the fowl pox virus. Due to the larger surface area of these parts exposed to skin wounds, birds with large wattles and large combs appear more prone to developing pox lesions. Fowl pox is a wide spread disease in the chickens and birds. It is characterized by dry and crusty skin lesions that typically appear on areas of the body without feathers, such as the comb, wattle, and face. This information is supported by studies conducted by Skinner *et al*. in 2005, Skinner and Laidlaw in 2009, and Delhon in 2022.

The illness can manifest itself cutaneously, diphtheric/pharyngeally, or in both ways. Additionally, tumorous alterations may form in several bird species. Rarely is a peracute form encountered, which is linked to abrupt death. The cutaneous or dry type is common form; it causes scabs, nodules, and papules on parts of the free-feathers area such as eyelids, comb, wattles, and beak and sometimes on the wings and legs. These lesions can turn into ulcers and progress to impair or interfere with mobility, eating, and vision. After the skin lesions have recovered and healed, no obvious scarring has occurred. Generally, if the cutaneous form predominates, mortality rates are modest (Parker *et al*., 2011; MacLachlan and Dubovi, 2017; Rebeca *et al*. 2019).

In many bird species, the diphtheric type mostly affects the digestive tracts and the upper respiratory tracts. The cutaneous and diphtheric forms may be present at the same time. In comparison to the cutaneous and the diphtheric form may be occur together and leading into higher fatality percentage. However, the aforementioned predisposing factors are also a factor in mortality. Most of the time, but not always, poultry mortality is low. The losses can range from 80 to 100% in flocks of canaries and other finches (Delhon, 2022). Despite good immunization, avian pox regularly reemerges as a result of variant or more virulent strains that are currently sweeping the globe (Dash *et al*., 2005, Sherpa *et al*.,2023). This causes high mortality rates as well as huge economic losses. Although this virus has been controlled in most countries where the industry of

chicken raising occurs, it still affects the majority of impoverished countries due to inadequate hygiene, contaminated tools and surfaces, and vector transmission via mosquitoes (Dash *et al*., 2005).

Diseases can be avoided by immunizing against live chicken poxvirus or pigeon poxvirus (Tripathy and Reed, 2008). There is evidence of infections in some flocks that have received immunizations, despite the fact that vaccines are effective and routinely used to reduce morbidity and mortality, according to recent reports of outbreaks in flocks that have received vaccinations in a variety of nations (Odoya *et al*., 2006).

Histological analysis and virus isolation through viral particle propagation into the chorioallantoic membrane or avian cell cultures of the chicken embryo as a definitive diagnosis of the avian poxvirus is the most frequently used techniques for diagnosing the cutaneous and diphtheritic types of FWPV (MacLachlan and Dubovi, 2017). The cytopathic effect (CPE) of FWPV manifests as proliferative thickening and white opaque pock lesions on the chorioallantoic membrane of the embryonated chicken eggs within (4-6) days after infection. This is because FWPV replicates easily in the chorioallantoic membrane of developing chicken embryos that are 9 to 12 days old (MacLachlan and Dubovi, 2017; Umar *et al*., 2021).

Sequencing and PCR-based amplification of highly conserved regions of the genome have also been employed for confirmatory diagnosis (Akanbi et *al*,.2022). Real-time (RT)-PCR (OIE 2016) and other molecular methods like classical PCR, are being utilized to compare wild and vaccine-derived FWPV strains, or sporadically in routine diagnostics which consider a recent technique and provide an opportunity for rapid identification of avian poxvirus (Audarya *et al*., 2018; Rebeca *et al*., 2019). An electron microscope is used to identify inclusion bodies and check for viral particles with the typical morphology of a pox virus as part of a direct analysis of FWPV (Sarker and Raidal 2023). Despite the avian pox virus being endemic throughout the world, particularly in

laying hens and Pigeon, there is little research on the avian pox virus, the genetic diversity of viruses, and the genetic mutations that go along with endemic infections of fields in the country (Minhas *et al*,.2023). Even though the immunizations used to prevent this disease are effective and widely used to minimize infection and mortality rates, some flocks that had received the vaccine also displayed disease symptoms more frequently (Tripathy and Reed, 2020).

1.2. Aims of the Study:

In Diyala Province, no research was done to define associated histopathology findings and FWPV molecular features. As a result, this study aims to:

1. Molecular detection and Phylogenetic analysis of identified strain of FWPV in chicken and pigeon in Diyala to point out the relatedness of Diyala identified strains with references strain from NCBI (GenBank).
2. Histopathological examination of the birds infected with FWPV in Diyala Province.