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Case Study

Fowlpox Outbreak in a laying farm: Up to Date Data on Phylogenetic Analysis in Iran, 2018

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Abstract

Fowlpox is an economically significant viral disease in poultry, characterized by two forms of clinical signs, including cutaneous and diphtheritic lesions. This infection can have several adverse effects on flock performance, such as a reduction in egg production and growth and an increase in mortality. In winter 2018, an infection suspected to fowlpox was reported from a Hy-line W-36 laying farm in Isfahan province, Iran. The birds were 38 weeks of age and showed obvious diphtheritic signs in mucous membranes with increased mortality and reduced egg production. In total, 20 samples were collected from diphtheritic lesions (Trachea and Esophagus) of infected birds. The Polymerase Chain Reaction method was used to amplify a 578 bp fragment of the poxvirus *4b* core protein gene. Phylogenetic relationships of avian poxviruses are usually analyzed using the 4b core protein-coding gene sequences with molecular weights of 75.2 kDa. The major elements had the fowlpox genome, and sequencing was performed for one isolate as representative. The nucleotide sequence result showed that this isolate (FP\UT-POX-2018) had a similarity rate of 99.53% with the previous Iranian fowlpox isolate (FP\GHPCRLAB.3) sequenced in the GenBank.Moreover, there was a 100% similarity among the current isolate nucleotide sequence, FP/NobilisVarioleW, and FP/FPV-VR250. The derived phylogenetic tree showed that these isolates were clustered in A1 subclades. Therefore, Iranian isolates of fowlpox virus have remained in the same subclade of phylogenetic classification (subclade A1), and they show high genomic similarity with previous isolates of Iran. Veterinarians and farmers must not underestimate fowlpox. However, they should consider the importance of vaccination against this disease like any other disease care.

Keywords: Fowlpox, Iran, Laying flock, PCR, Phylogenetic analysis

Epidémie de Variole Aviaire Dans une Ferme de Ponte: Données à Jour sur l'analyse Phylogénétique en Iran, 2018

Résumé: La variole aviaire est une maladie virale ayant un impact économique significatif chez la volaille, caractérisée par des signes cliniques sous formes de lésions cutanées et diphtériques. Cette infection peut avoir plusieurs effets néfastes sur les performances du troupeau, comme une réduction de la production et de la croissance des œufs et une augmentation de la mortalité. En hiver 2018, une infection suspecte de variole aviaire a été signalée dans une ferme de ponte Hy-line W-36 dans la province d'Ispahan, en Iran. Les oiseaux étaient âgés de 38 semaines et présentaient des signes diphtériques évidents dans les muqueuses avec une mortalité accrue et une production d'œufs réduite. Au total, 20 échantillons ont été prélevés sur des lésions diphtériques (trachée et œsophage) d'oiseaux infectés. La méthode de réaction en chaîne par polymérase a été utilisée pour

amplifier un fragment de 578 pb du gène de la protéine centrale de la poxviridae 4b. Les relations phylogénétiques des poxvirides aviaires ont été analysées en utilisant les séquences de gènes codant pour la protéine de base 4b avec un poids moléculaire de 75,2 kDa. Les principaux éléments avaient le génome de la variole aviaire, et le séquençage a été effectué pour un isolat comme représentatif. Le résultat de la séquence nucléotidique a montré que cet isolat (FP\UT-POX-2018) avait un taux de similitude de 99,53% avec le précédent isolat de variole aviaire iranien (FP\GHPCRLAB.3) séquencé dans la GenBank. De plus, il y avait un taux de 100% similitude entre la séquence nucléotidique de l'isolat actuel, FP/NobilisVarioleW et FP/FPV-VR250. L'arbre phylogénétique dérivé a montré que ces isolats étaient regroupés en sous-clades A1. Par conséquent, les isolats iraniens du virus de la variole aviaire sont restés dans la même sous-clade de la classification phylogénétique (sous-clade A1), montrant donc une similitude génomique élevée avec les isolats précédents iraniens. Les vétérinaires et les éleveurs ne doivent pas sous-estimer la variole aviaire. Cependant, ils devraient considérer l'importance de la vaccination contre cette maladie comme tout autre traitement contre les maladies.

Mots-clés: Variole aviaire, l'Iran, Poules pondeuse, PCR, Analyse phylogénétique

Introduction

The fowlpox virus belongs to the avipoxvirus (APVs) genus and poxviridae family. The APVs infect a wide range of domestic and wild birds in more than 232 species and 23 orders which named according to their hosts, including 19 described species, such as Fowlpox, Turkeypox, Canarypox, Pigeonpox, Quailpox, Sparrowpox, Starlingpox, Psittacine pox, Juncopox, Mynahpox, Peacockpox, Cowpox, Penguinpox, Alalapox, Apapanepox, Condorpox, Pipitpox, Flamingopox, and Eaglepox (Bolte et al., 1999; Zimmermann et al., 2011). These viruses are antigenically and immunologically distinguishable from each other; however, there are some cross-reactions complicating strain identification (Tripathy ND and MR., 2013). Fowlpox is a viral disease described in two forms of clinical signs, such as cutaneous and diphtheritic lesions in poultry.

The cutaneous form involves the skinless area around the eye, beaks, nostrils, feet, and cloaca. On the other hand, diphtheritic form affects the larynx and gastrointestinal tract's mucosal membrane, such as mouth, pharynx, and esophagus with proliferative necrotic lesions. Such lesions are due to hyperplasia in the epidermis' epithelial cells, resulting in proliferative lesions and diphtheritic membrane (Tripathy ND and MR., 2013).

Fowlpox is a common and economically important disease in commercial poultry, and it can have adverse effects on flock performance, such as a decrease in the egg production and growth, blindness, as well as an increase in the mortality (Bolte et al., 1999; Lüschow et al., 2004). This disease spreads slowly; however, it expands so faster if some insects, such as mosquitos and mites, are present in the flock (Tripathy ND and MR., 2013). Diagnosis of pox is based on clinical signs, histopathological examinations, electron microscopy, molecular tests, and virus isolation (Manarolla et al., 2010). Phylogenetic relationships of avian poxviruses are usually analyzed using the DNA sequences of the *4b* core protein-coding gene with molecular weights of 75.2 kDa, which is also a useful and sensitive test to detect the avian poxviruses by amplification of specific gen elements with PCR (Lüschow et al., 2004; Weli et al., 2004; Adams et al., 2005; Jarmin et al., 2006; Manarolla et al., 2010).

In previous studies, the APV had been isolated from chicken. Moreover, canary and mynah were collected from Tehran province, Iran (Nayeri Fasaei et al., 2014). The APV was also detected in the backyard poultry in western areas of Iran (Gholami-Ahangaran et al., 2014) and identified in the backyard chickens in Khorramabad, Iran and affected flocks in Lorestan province, Iran (Norouzian and Farjanikish, 2017).

Based on phylogenetic studies, avian pox isolates were clustered in three major clades in which fowlpox virus, canarypox virus, and psittacine poxvirus were placed in clades A, B, and C, respectively (Weli et al., 2004; Jarmin et al., 2006). Clade A contains seven subclades (A1-A7). Subclade A1 was formed by fowlpox virus in the narrowest sense and included viruses isolated from birds of the order Galliformes, such as domestic fowl and Blue-eared pheasant with wide geographic distribution (Jarmin et al., 2006).

Subclade A2 was formerly identified as Turkey pox; however, viruses recently originated from the Columbiformes order, such as rock doves and oriental turtle doves, are described in this subclade (Jarmin et al., 2006). Subclade A3 consists of an albatross virus, a falcon virus, and isolates from other seabirds. Subclade A4 still has an outlier and contains viruses from peregrine falcon and red-footed falcon.

Subclade A5, a newly defined subclade, has a common ancestor with subclade A1 that includes isolates from Anseriformes. Subclades A6 and A7 share a common ancestor with subclades A2 and A3. Clade B contains three subclades (B1-B3). Formerly reported subclade B1 consists of isolates from a wide range of passerine species (Passeriformes), and clade C contains viruses exclusively from psittacine species (Gyuranecz et al., 2013). This study evaluated an infection suspected of fowlpox with yellowish diphtheritic lesions in mucosal membranes from a laying flock in Isfahan, Iran. The Polymerase Chain Reaction (PCR) technique was used to detect the fowlpox virus. Subsequently, sequencing and phylogenetic analysis were conducted on *4b* core protein genes.

Material and Methods

Samples. In winter 2018, an infection suspected to fowlpox was reported from a Hy-line W-36 laying farm in Isfahan, Iran. The birds aged 38 weeks and vaccinated at 16 weeks. They showed obvious diphtheritic signs in mucous membranes with increased mortality (~8%) and decreased egg production (~10%). A total of 20 samples were collected from diphtheritic lesions (Trachea and Esophagus) of the infected birds.

Virus Isolation. Tissue samples were homogenized with phosphate-buffered saline (PBS) solution containing Penicillin (50 IU/ml) and Streptomycin (50 µg/ml) and incubated for 1 h at 37˚C to ensure the elimination of bacterial contamination. Subsequently, the antibiotic-treated solution was centrifuged for 15 min at 4000 rpm, and 0.2 ml of supernatant inoculated to the chorioallantoic membranes (CAMs) of 9-day old SPF embryonated chicken eggs (Venky's, India). Inoculated embryonated eggs were incubated at 37˚C for seven days. After seven days, all inoculated eggs were examined for specific poxvirus (Pock) on the CAM (5). (Manarolla et al., 2010). Harvested pocks were put in sterile Petri dishes and stored at -20˚C for further use.

DNA extraction. DNA of harvested pocks was extracted using Molecular Biological System Transfer (MBST, Iran) extraction kit based on the manufacturing factory's protocol. The pocks were mixed and homogenized with PBS and centrifuged for 15 min at 3000 rpm. Briefly, 200 µl of supernatant was mixed with 20 µl proteinase K and 200 µl lysis buffer. After incubation for 10 min at 55˚C, followed by 10 min incubation at 70˚C with 360 µl binding buffer, 270 µl ethanol was added to the sample. The whole mixture was put on a spin column and centrifuged at 8000×g for 1 min. The spin column was washed with 500 µl washing buffer and centrifuged as before. After a second washing step with 500 µl wash buffer and centrifugation at 8000 g for 3 min, the DNA was eluted from the column using 70 µl elution buffer, incubation for 3 and at last centrifugation step at 8000×g for 1 min.

Polymerase Chain Reaction. The PCR was performed on the pocks to confirm the fowlpox virus in the samples. The primers were established based on a previous study (Lee and Lee, 1997) to target the *4b* gene sequence of avian pox virus (P1:5′-

CAGCAGGTGCTAAACAACAA-3′, P2:5′-CGGTAGCTTAACGCCGAATA-3′). The amplified PCR products were separated by 1.2% agarose gel and visualized by ethidium bromide. The PCR products of the *p4b* gene purified based on their specific size by Qiaquick PCR purification kit (Qiagen, Italy).

DNA sequencing and analysis. Since all isolates were from the same flock, DNA sequencing was performed on one of the isolates (UT-POX-2018). Furthermore, sequencing was performed using an automatic sequencer (ABI-370, Applied Biosystem) with forward and reverse primers. The achieved result was compared with sequences from 25 other isolates in the GenBank. Also, the compared strains were selected based on their origin so that they were previously isolated from Iran or neighboring countries. Sequences were analyzed by the neighbor-joining method in MEGA7 software.

Results

Virus isolation and molecular detection. All 20 samples showed the pock on CAM, which represents poxvirus-specific lesion. Moreover, all of these isolates were positive for fowlpox-specific gene elements (578 bp) in the PCR technique and were considered to belong to the fowlpox virus genus (Figure 1).

Sequence analysis. The sequencing of the amplified region of the *4b* gene of one fowlpox isolate was performed, and the nucleotide sequence showed 66.05%-100% identity with 12 selected sequences of APVs strains in the GenBank DNA. Moreover, the nucleotide sequence similarity of the current isolate and previous Iranian fowlpox isolate (FP\GHPCRLAB.3) was 99.53% (Table 1). Based on phylogenetic analysis, this fowlpox isolate belongs to a first clade (A) in the subclade of A1 (Figure 2).

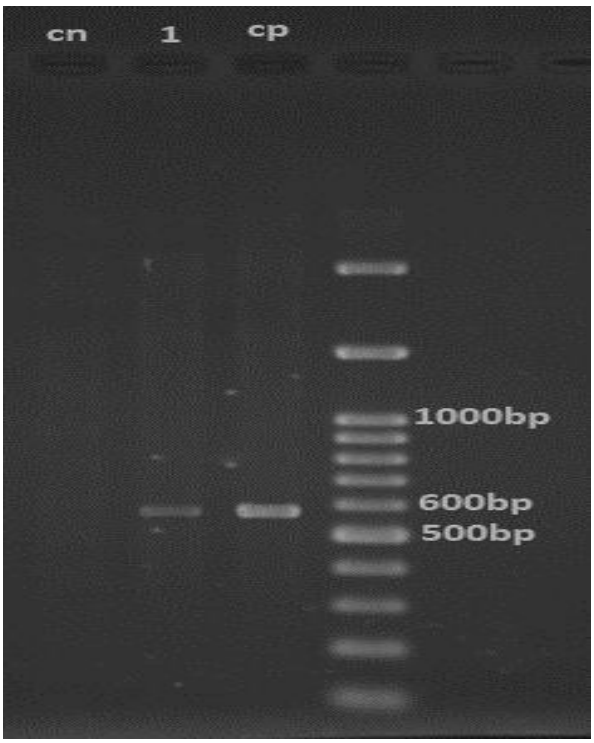

Figure 1: Gel electrophoresis to detect fowlpox; size band: 573 bp (Ladder 100 bp, CP: Control Positive, Sample)

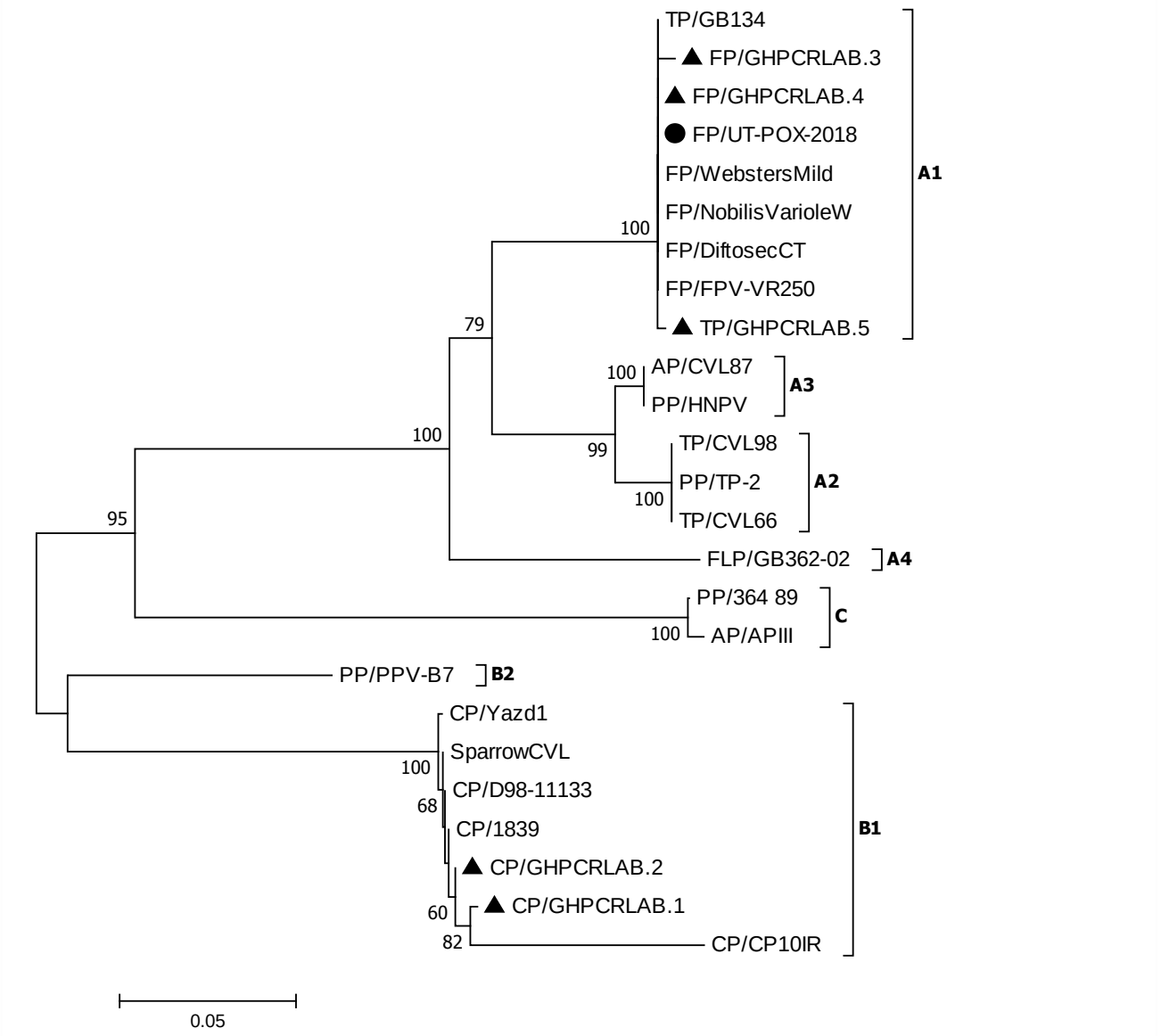

Figure 2: Phylogenetic tree of the nucleotide sequence of the *4b* core protein gene PCR fragment of some Avian Pox Viruses (APVs) tested in this study and the published sequence in GenBank created by a neighbor-joining method with the MEGA7 program.
Values at the branches and clusters are bootstrap value, and the bar indicates the distance scale from the roots.

Table 1. Percent identity of nucleotide sequences of the *4b* gene of a present isolate with compared sequences from GenBank. The FP/UT-POX-2018 is the isolate in this study, and FP/GHPCRLAB3 and CP\GHPCRLAB2 are the previous Iranian Avian Pox Virus isolates.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	FP/UT-POX-2018													
2	FP/GHPCRLAB.3	99.53												
3	FP/NobilisVarioleW	100.00	99.53											
4	FP/FPV-VR250	100.00	99.53	100.00										
5	TP/CVL98	89.92	89.34	89.92	89.92									
6	AP/CVL87	91.05	90.49	91.05	91.05	97.60								
7	FLP/GB362-02	86.73	86.11	86.73	86.73	87.76	87.10							
8	Sparrow CVL	67.63	67.34	67.63	67.63	67.08	65.82	67.88						
9	CP/Yazd1	67.63	67.34	67.63	67.63	67.08	65.82	67.88	99.77					
10	CP/GHPCRLAB.2	67.26	66.96	67.26	67.26	66.70	65.44	68.25	99.77	99.53				
11	CP/CP10IR	57.87	57.56	57.87	57.87	56.98	55.47	57.86	93.14	92.87	92.88			
12	PP/PPV-B7	70.47	70.18	70.47	70.47	71.96	71.93	72.54	81.49	81.16	81.49	72.23		
13	PP/364_89	66.05	65.75	66.05	66.05	65.02	63.94	62.15	67.72	67.30	67.34	57.18	67.94	

Discussion

Fowlpox is a common and economically important disease of the backyard and commercial poultries. The disease's causative agent is a double-stranded DNA virus that belongs to the genus *Avipoxvirus* within the *Poxviridae* family (Afonso et al., 2000). Disease caused by this agent is one of the most important diseases in commercial poultry farming and can induce cutaneous and diphtheritic lesions in the laying flock that leads to decreased egg production and even mortality (Tripathy ND and MR., 2013). The utilization of widespread vaccination in Iranian lying farms and external parasite control led the disease to become sporadic (Nayeri Fasaei et al., 2014). Vaccination is the most effective way to prevent infection of a flock. The low incidence of fowlpox in most parts of Iran in recent years has led farmers to suppose that the disease is eradicated. Therefore, they underestimate the importance of vaccination against this disease, and some flocks may not be vaccinated. In February 2018, a commercial Hyline laying farm in Isfahan province experienced a sudden decrease in egg-laying followed by an increase in the mortality rate. The birds were not vaccinated against fowlpox, and the poxvirus was isolated from the diphtheritic lesions of the infected birds.

The *4b* gene of the isolated strain was sequenced to investigate possible changes in the recent fowlpox virus genome and compare the phylogenetic of the isolated strain with previous strains. Due to the presence of a double-stranded DNA genome, mutations occur relatively slow in *Poxviridae* (Murphy et al., 1999). The results show that the recent isolate belongs to clade A and subclade of A1, which are the same clade and subclade that previous Iranian isolates belong to. The isolate has 66%-100% similarity, with 12 compared isolates from the GenBank. Moreover, the similarity between the recent isolate (FP/UT-POX-2018) and the previous Iranian isolate (FP/GHPCRLAB.3) was 99.53%. This similarity level indicates cross-protection, and if vaccination is performed properly, the disease will successfully prevent it.

According to a study conducted in the western parts of Iran, the frequency of avian poxvirus infection was high in backyard poultry (Gholami-Ahangaran et al., 2014).

Furthermore, Norouzian and Farjanikish (2017) classified the isolated fowlpox virus in a different subclade far from other Iranian isolates but near to isolates from Tanzania, Egypt, and Germany. However, in another study, the sequence analysis revealed that the Iranian isolates were within the cluster with highly

conserved *p4b* core protein in different countries and birds (Nayeri Fasaei et al., 2014).

This study indicates that circulating Feline Parvovirus isolates belongs to subclade A1 of clade A, and they show high genomic similarity with previous isolates of Iran. With this background in mind, veterinarians and farmers must not underestimate fowlpox. However, they should consider the importance of vaccination against this disease like any other disease care.

Authors' Contribution

Study concept and design: Ar. Gh. and M. H. F. M.
Acquisition of data: M. H. F. M., T. Z. and S. Ch.
Analysis and interpretation of data: H. H. and A. Gh.
Drafting of the manuscript: A. Sh. and R. E. D.
Critical revision of the manuscript for important intellectual content: S. Ch. and A. Gh.
Statistical analysis: M. H. F. M. and H. H.
Administrative, technical, and material support: L. A.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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