Fowl Pox outbreak in a layer farm: update data on phylogenetic analysis in Iran, 2018

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Abstract

Fowl pox is an economically significant viral disease in poultry which described in two forms of clinical signs including cutaneous and diphtheritic lesions. Fowl pox can have several adverse effects on flock performance such as decreasing the egg production and growth and increasing the mortality. In winter 2018, an infection suspected to Fowl pox was reported from a Hyline W36 layer farm from Isfahan province. The birds were 38 weeks of age and showed obvious diphtheritic signs in mucous membranes with increases in mortality and decreased egg production. Twenty samples from diphtheritic lesions (Trachea and Esophagus) of infected birds were collected. Polymerase Chain Reaction 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 DNA sequences of the 4b core protein coding protein with molecular weights of 75.2 kDa. For virus detection, specific gene element, and sequencing was performed for one isolate as representative. The result of, nucleotide sequence showed that this isolate (FP\UT-POX-2018) has an identity of 99.53% with the previous Iranian fowl pox isolate (FP\GHPCRLAB.3) sequences in the Gen Bank. Also, there was 100% similarity between current isolate nucleotide sequence and FP\NobilisVarioleW and FP\FPV-VR250. The derived phylogenetic tree showed that these isolates were clustered in A1 subclades. Hence, 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. It is necessary that veterinarians and farmers don’t underestimate fowlpox and know the importance of vaccination against this disease like any other disease care.

Keywords: Fowl pox, PCR, Phylogenetic analysis, layer flock, Iran

Introduction

The Fowlpox virus belongs to the Avipoxvirus genus and Poxviridae family. Avipoxviruses (APVs) infect a wide ranges of domestic and wild birds in more than 232 species and 23 orders which named according to their hosts, including 19 described species: Fowlpox, Turkeypox, Canarypox, Pigeonpox, Quailpox, Sparrowpox, Starlingpox, Psittacine pox, Juncopox, Mynahpox, Peacockpox, Cowpox, Penguinpox, Alalapox, Appaneepox, Condorpox, Pipitpox, Flamingopox and Eaglepox (Bolte et al., 1999; Zimmermann et al., 2011). These viruses are antigenically and immunologically distinguishable from each other, but there are some cross-reactions complicating strain identification (Tripathy ND and MR., 2013). Fowl Pox is a viral disease which described in two forms of clinical signs such as cutaneous and diphtheritic lesions in poultry. The cutaneous form involves skinless area like around the eye, beaks, nostrils, feet, cloaca and diphtheritic form affect mucosal membrane of the larynx and gastrointestinal tract such as mouth, pharynx, and esophagus with proliferative necrotic lesions. Such lesions are due to hyperplasia in epithelial cells of epidermis resulting in proliferative lesions and diphtheritic membrane (Tripathy ND and MR., 2013). Fowl pox is a common and an economically important disease in commercial poultry, and it can have adverse effects on flock performance such as decreasing the egg production and growth, blindness and increasing the mortality (Bolte et al., 1999; Lüscho et al., 2004). This disease spreads slowly. However, it expands so faster if some insects such as mosquitoes 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 protein with molecular weights of 75.2 kDa which is also useful and sensitive test for detection of avian pox viruses by amplification specific gen elements with PCR(Lüscho et al., 2004; Weli et al., 2004; Adams et al., 2005; Jarmin et al., 2006; Manarolla et al., 2010). In Previous studies
Avipoxvirus (APV) had been isolated from chicken; canary and mynah were collected from Tehran province (Nayeri Fasaei et al., 2014) and were detected in the backyard poultry in different parts of western areas in Iran (Gholami-Ahangaran et al., 2014) and was identified in backyard chickens of Khorramabad with clinical in affected flocks in Lorestan province (Norouzian and Farjanikish, 2017).

Based on phylogenetic studies, almost of avian pox isolates are clustered in three major clades which Fowlpox virus, canarypox virus and psittacine pox virus are 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 includes viruses isolated from birds of the order Galliformes such as domestic fowl, blue-eared pheasant, with wide geographic distribution (Jarmin et al., 2006). Subclade A2 was formerly identified as Turkey pox but recently viruses originating from the Columbiformes order like rock doves, oriental turtle doves are described in this sub clade (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 which is a new defined, has a common ancestor with subclade A1, 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 isolates from a wide range of passerine species (Passeriformes), clade C contains exclusively of viruses from psittacine species. Either clade C formed a separate clade, or it was maybe a member of clade B with weak support (Gyuranecz et al., 2013). In this study, an infection suspected of Fowlpox with yellowish diphtheritic lesions in mucosal membranes from a layer flock in Isfahan was reported and PCR technique has been carried out for collecting samples to detection fowl pox virus. Then sequencing and phylogenetic analysis conducted on the 4b core protein genes.

**Materials and Methods**

**Samples**

In winter 2018, an infection suspected to Fowl pox was reported from a Hyline W36 layer farm from Isfahan province. The birds were 38 weeks of age (vaccinated at 16 wks.) and showed obvious diphtheritic signs in mucous membranes with increases in mortality (~8%) and
decreased egg production (~10%). Twenty samples from diphtheritic lesions (Trachea and Esophagus) of infected birds were collected.

**Virus Isolation**

Tissue samples were homogenized with PBS solution containing Penicillin (50 IU/ml) and Streptomycin (50 µg/ml) and incubated for 1 hour at 37 °C to ensure eliminating bacterial contamination. Then, the antibiotic-treated solution centrifuged for 15 minutes at 4000 rpm, 0.2 ml if supernatant inoculated to the CAMs of 9-day old SPF embryonated chicken eggs (VenKý's, India). Inoculated embryonated eggs were incubated at 37 °C for seven days. After seven days, all inoculated eggs examined for specific lesions of 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 protocol provided by the manufacturing factory. The pocks were mixed and homogenized with PBS and centrifuged for 15 minutes 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 by addition of 70 µl elution buffer, incubation for 3 min, and at last centrifugation step at 8000 × g for 1 min.

**Polymerase Chain Reaction (PCR)**

PCR was done on the pocks to confirm fowlpox virus in the samples. The primers established based on a previous study(Lee and Lee, 1997) to target 4b gene sequence of avian pox virus (P1:5’-CAGCAGGTGCTAAACAACAA-3’, P2:5’-CGGTAGCTTAACGCGGAATA-3’). The amplified PCR products were separated by 1.2% agarose gel and visualized by ethidium bromide. PCR products of p4b gene purified based on their specific size by Qiaquick PCR purification kit (Qiagen, Italy).
DNA sequencing and analysis

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

Results

Virus isolation and molecular detection
All 20 samples showed the pock on chorioallantoic membrane (CAM) which represent 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 fowlpox virus genus (fig. 1).

Sequence analysis
The sequencing of the amplified region of the 4b gene of one fowlpox isolate was performed, and nucleotide sequence showed 66.05-100% identity with 12 selected sequences of APVs strains in the GenBank DNA. Also, nucleotide sequences similarity of 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 Subclade of A1 (Fig. 2).

Discussion

Fowl pox is a common and economically important disease of the backyard and commercial poultries. The causative agent of the disease is a double-stranded DNA virus belongs to the genus Avipoxivirus, within 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 layers that lead to decreased egg production and even mortality (Tripathy ND and MR., 2013). Using widespread vaccination in layer farms of Iran and controlling external parasites lead to the occurrence of the disease became sporadically (Nayeri Fasaei et al., 2014). Vaccination is the most effective way to prevent infection of a flock. The low incidence of fowl pox in most parts of Iran in recent years has led to that the farmers suppose that the disease is eradicated, so, underestimate the importance of vaccination against this disease, and some flocks may not be vaccinated. In February 2018, a commercial Hyline
layer farm in Isfahan province experienced a sudden decrease in egg laying and increase in mortality rate. The birds were not vaccinated against fowlpox. Poxvirus isolated from diphtheritic lesions of the infected birds. To investigate possible changes in recent fowlpox virus genome and compare phylogenetic of the isolated strain with previous strains, the 4b gene of the isolated strain sequenced. Because of having 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, it is the same clade and subclade that previous Iranian isolates belong. The isolate has 66 to 100% similarity with 12 compared isolates from GenBank. The similarity between recent isolate (FP/UT-POX-2018) and the previous Iranian isolate (FP/GHPCRLAB.3) was 99.53%. This level of similarity indicates cross-protection and that if vaccination has done properly, the disease will successfully prevent.

In a study in western areas of Iran revealed that frequency of avian pox virus infection in backyard poultry in this areas was high (Gholami-Ahangaran et al., 2014).

In a study that was done by Norouzian and Farjanikish (2017) the isolated fowl poxvirus was classified in a different subclade far from other Iranian isolates and near to isolates from Tanzania, Egypt and Germany (Norouzian and Farjanikish, 2017). But in another study the sequence analysis reveals that the Iranian isolates are within the cluster with highly conserved p4b core protein in different countries and species of birds (Nayeri Fasaei et al., 2014).

The results of this study indicate that circulating FPV isolates belongs to subclade A1 of clade A and they show high genomic similarity with previous isolates of Iran. It is necessary that veterinarians and farmers don’t underestimate fowlpox and know the importance of vaccination against this disease like any other disease care.

Conflict of Interest

The authors declared no conflict of interest.
References:


Figure 1: The gel electrophoresis for detection fowl pox; size band: 573 bp (Ladder 100 bp, CP: Control Positive, Sample)
Fig. 2: Phylogenetic tree of the nucleotide sequence of the 4b core protein gene PCR fragment of some Avian Pox Viruses (APVs) tested and of 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 bar indicates 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. FP/UT-POX-2018 is the isolate of this study and FP/GHPCRLAB3, and CP/GHPCRLAB2 are the previous Iranian APVs isolates.

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