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Original Article

Isolation and Phylogenetic Characterization of Avipoxvirus Causing Outbreaks in Iran, 2019

Fatemeh Sadat Mousavi[1], Arash Ghalyanchilangeroudi[1], Hamed Abdollahi[2], Hossein Hosseini[3], Ali Rajeoni[1], Leila Aghayian[1], Mohammadreza Ghorani[4], Bahram Gholamian[1], Amir Modiri[1], Naser Sadri[1], Zahra Ziafati Kafi[1*]

1. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2. Iranian Veterinary Organization, Tehran, Iran
3. Department of Clinical Sciences, Faculty of Veterinary Medicine, Islamic Azad University, Karaj Branch, Karaj, Iran
4. Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Abstract

Background and Aims: Avian pox infection is a widespread disease with cyclical occurrence in endemic areas, especially in areas with dense poultry production. An outbreak of poxvirus infecting commercial laying herd in Esfahan, Iran, is reported. The current study attempted to realize if new isolate has been emerged and, if so what the difference is and how it has appeared.

Materials and Methods: The chickens were previously vaccinated against pox. Avipoxvirus was isolated by PCR analysis of specimens taken from the infected skin lesions and mucosal tissues (trachea).

Results: Sequencing and alignment of the amplified P4b gene of the avipox showed 100% similarity to the FP/NobilisVariole W (vaccine) and fowlpox virus vaccine strain (FPV-VR250). The Iranian avipoxvirus isolates in this study grouped in clade A1 with other Iranian commercial chicken avipoxviruses.

Conclusions: This research is the latest report of APVs outbreak in a commercial laying herd in Iran. It seems indispensable to investigate vaccines' efficacy, which is being used in Iranian commercial laying herd routinely to illuminate whether these vaccines have appropriate efficacy or not and are they still effective? We intended to update the viral monitoring system as to country livestock and poultry.

Keywords: Avipoxvirus, Phylogenetic analysis, vaccine, PCR, Iran.

Introduction

The pox virus is a member of the Poxviridae family, which the family is subcategorized into the Entomopoxvirinae and Chordopoxvirinae subfamilies infecting insects and chordates, respectively. Avipoxvirus (APV) is the only characterized genus of the Chordopoxvirinae subfamily, which can cause infection to nonmammalian hosts, including more than 230 avian species globally [1, 2]. APV has numerous species such as Fowlpox virus (FWPV), Turkeypox virus (TKPV), Pigeonpox virus (PGPV), Canarypox virus (CNPV), Ostrichpox virus (OSPV), Penguinpox virus (PEPV), Falconpox virus (FLPV), and Sparrowpox virus (SRPV), and many others recently emerged poxviruses in different avian species. According to the available evidence, 232 out of the about 9,000

[*] **Corresponding author:** Zahra Ziafati Kafi, Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, Email: zahra.ziafati@gmail.com, Tel: +98216111-7154.

bird species have been naturally infected with poxvirus or suffer from various forms of poxvirus infection [3].

Fowlpox virus (FWPV) genome size is roughly 288 kbp, which encodes 260 open reading frames, among them 101 are similar to genes with definite function. The genome consists of a two-stranded DNA in which two identical inverted terminal repeat regions encompass a central coding region [4].

The disease caused by FWPV is one of the important diseases in commercial poultry production, as it can lead to significant problems when conditions are favorable for transmission, especially by mosquitoes. The best strategy for the control of the disease is prevention of transmission by vaccination [5]. The coetaneous form of the disease is described as lesions on the featherless skin around the eyes, beak, nostrils, and feet, and/or proliferative lesions and diphtheritic membranes on the mouth or upper respiratory mucosa and gastrointestinal system [6].

Poxvirus can be transmitted mechanically to injured skin or through the bite of mosquitoes or mites. APV infection varies in the incubation period and duration ranging from a few days to several months, but the majority of affected birds with mild lesions, particularly those of wild ones, frequently recover.

Depending on climate, management, and hygiene or the practice of regular vaccination, the incidence rate varies among different areas. The main clinical manifestations are egg production decrease or growth retardation in younger birds [7].

Polymerase Chain Reaction (PCR) is one of the reliable procedures that detected virus genes in tissue samples. PCR along with restriction endonuclease enzyme analysis (REA) followed by sequence analysis of the amplified fragments is used for detection, differentiation and molecular characterization of fowlpox virus isolates [8].

Several researchers have analyzed different APVs from various geographic zones and studied their phylogenetic relationships with each other. For example, the study done in northern Italy, revealed that the majority of 15 isolates belonged to either clade A or clade B, while only one isolate from Japanese quail was classified and clustered within the clade [1, 8].

The incidence of APV in Iran is high and relatively high in pet birds and commercial farms, respectively. Recently, some suspected outbreaks of skin lesions were reported from the backyard poultry in different parts of western areas in Iran [9]. The aim of this study was the characterization of AFPV isolates from chicken flocks by PCR.

Methods

Virus isolation and DNA extraction. All of the five samples were collected from a commercial laying herd in Esfahan, Iran. The samples were referred to the microbiology and immunology lab of the faculty of Veterinary Medicine, University of Tehran. The infected skin lesions from the chickens were screened for the presence of poxviruses. The DNA was extracted from the affected skin and mucosal tissues as well as lyophilized live fowl pox vaccine (Razi Vaccine And Serum Research Institute, Iran), as positive control, by SinPure DNA extraction kit (Sinaclon, Iran).

Furthermore, DNA was extracted from epithelial and mucosal tissues of healthy chickens as negative controls in each species. Extraction was performed based on the manufacturer's recommendations.

PCR. APV-specific PCR was conducted using primers as described by Lee et al. (1997) [10] (P1:5′ CAGCAGGTGCTAAACAACAA-3′; P2: 5′-CGGTAGCTTAACGCCGAATA-3′) and based on APV P4b gene sequence [11].

These primers amplify a fragment of about 578 bp. PCR Master Mix 2x (Sinaclon, Iran) were used, 6 pmol of each primer, 60 ng DNA sample, and nuclease-free water up to 25 µl were added.

The PCR parameters for amplification were: initial DNA denaturation over 2 min at 94 °C followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min elongation at 72°C and terminated with a final extension step at 72 °C for 2 min [1].

DNA sequencing and analysis. PCR products were sent for sequencing (Bioneer Co., Korea) with PCR primers for both forward and

reversed directions. The sequence results were downloaded and analyzed using Chromas (Technelysium Pry Ltd., Australia). Phylogenetic analysis was performed by analyzing the obtained data and comparing to those of other FPVs sequences. DNA sequencing was carried out for 25 strains. An automatic sequencer (ABI-370, Applied Biosystem), and both forward and reverse primers were used for sequencing. Sequences were selected from isolates of countries as near as possible based on different places and times of reporting. Sequence analysis was performed by neighbor-joining method (Tamura-Nei model) with MEGA7 program. The robustness of the phylogenetic trees was assessed by 1,000 bootstrap replicates with values higher than 50. The FPV sequences tested in this study were deposited in GenBank under accession numbers IVO-UT-POX2019.

Results

Molecular detection. The detected APV specific DNAs in all of the five samples were found to belong to the genus Avipoxvirus of the family Poxviridae. The obtained amplified fragments of approximately 578 bp for the P4b gene were noticed to be consistent with the size of 578 bp based on published APV P4b nucleotide sequence.

Sequence analysis. The amplified region of the P4b gene was sequenced for one isolate, and as depicted in table 1, the nucleotide sequence similarity to 25 selected sequences of APV strains in the GenBank showed a similarity of 57/87-100%. The generated phylogenetic tree and identity matrix showed four distinguishable sequence clusters. The strain of the present study belonged to the first cluster which has four strains from Iranian commercial laying herds and several strains from different countries and birds (figure 1). Sequencing of the partial fpv167 gene coding for the P4b core protein of Avipox and sequence alignment revealed 99.53% similarity to the FP/NobilisVariole W (vaccine) and fowlpox virus vaccine strain (FPV-VR250).

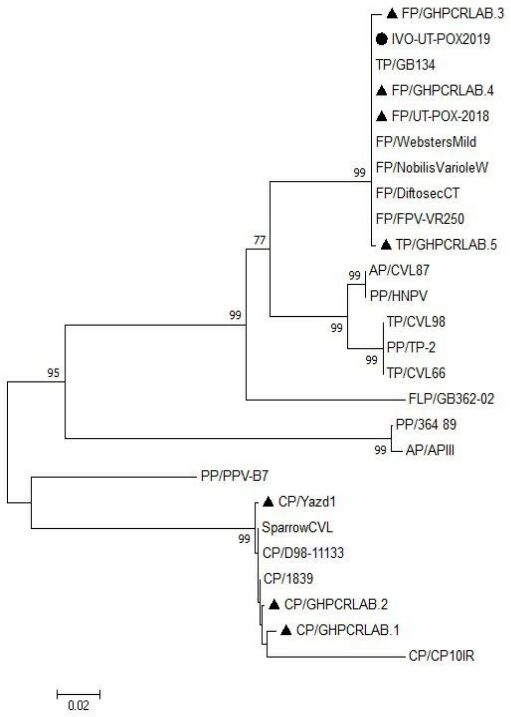

Fig. 1. Phylogenetic tree of nucleotide sequence of the 4b core protein gene PCR fragment of different APVs tested and of the published sequence in GenBank constructed by the neighbor-joining method with MEGA7 program. The name of the origin country of each isolate has been added to the end of its name in the tree. Values at the branches and clusters are bootstrap value, and the bar indicates distance scale from the roots.

Table 1. Percentage of 4b core protein sequence identity of APV isolated in this study and some selected APV isolates from GenBank

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	FP/UT-POX-2018													
2	IVO-UT2019	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	SparrowCVL	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	

Furthermore, the gene had 99.53% identity with the field isolate to the Iranian Avipox virus strain FP/UT-POX-2018 which is the latest strain isolated from Iran before this study (Figure1). These results indicate the rigidity of the 4b protein-encoding gene to mutations, probably because these viruses have a double-stranded DNA [12].

Discussion

Several avian species and commercial poultries have been identified to be infected with avian poxviruses. The probability of Poxvirus infection is very high when there are proliferative skin and oral and tracheal lesions. The genomes of APVs are extremely conserved [5, 13, 14], and because of containing two-stranded DNA, the rate of mutation is relatively low in Poxviridae [15].

The P4b is a conserved region of the fowlpox genome, which is less likely to undergo frequent mutation, and this may explain the high degree of homology observed in the clustering on the phylogenetic tree. Our study also corroborates similar observations by Carulei et al. (2009) [13] and Murphy et al. (1999) [15], suggesting that the PCR amplification of the P4b gene is a valuable diagnostic method for fowlpox infection.

The conventional diagnosis of APVs is based on histopathological examination, electron microscopy, virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs or cell-cultured, and serological methods [16]. The aforementioned techniques are usually complex, and it takes longer to perform them [17, 18], but PCR is a reliable method and easier than other procedures for detection of APVs.

In the recent study by Ghalyanchi et al. (2012) APVs on clinical cases of affected commercial chickens, turkeys, and canary were identified and characterized by molecular methods to determine the etiology of APV in Iran [9].

Fasaei et al. (2014) carried out phylogenetic analysis of Avipoxvirus strains isolated from different Iranian bird species and detected the similarity to be 71-100% to those of other sequences in GenBank, but the sequences can to GenBank, and the clade of isolates was not determined [19]. The research was done by Gholami-Ahangaran et al. (2014) on avian pox of backyard poultry in Iran indicated that 66.1% and 80.7% of samples were positive for the virus using histopathological and PCR methods, respectively [20].

Avian pox infection is a widespread disease with cyclical occurrence in endemic areas, especially in areas with dense poultry production [21]. Some factors may be relevant in identifying the source of an outbreak; for example it occurs in a limited number of birds, with high morbidity but less mortality.

Aligning the sequences of avipoxvirus gene, we demonstrated that the virus does not stem from wild birds or passerines [22].

This research is the latest report of the APVs outbreak in a commercial laying herd in Iran. The current study attempted to realize if new isolate has been emerged and, if so what the difference is and how it has appeared.

In this study, the isolates shared 57/87-100% homology with the selected sequences from GenBank and clustered on the phylogenetic tree in clade A, subclade A1 with other strains from Iranian commercial herds fowlpox virus (Figure 1).

Genomic and antigenic variation in circulating field and vaccine strains could lower vaccine efficacy requiring close monitoring, especially where outbreaks in vaccinated flocks are observed as reported by Nie et al. (2000) [23] and Odoya et al. (2006) [24].

Fowlpox (FP) vaccine is used in Iranian poultry industry in layer and breeder farms, although specific live avian pox vaccines have yielded better results in commercial poultry farms. Most of FP vaccine manufacturers are searching for new vaccine candidates as the routine ones may not prove up to the mark.

With this aspect, one of the possible alternatives may be the evaluation of FP isolates from a vaccinated flock. However, the problem of contamination with other interacting viral pathogens is yet to be solved.

Therefore, the search for suitable APV as a candidate vaccine strain could be attempted as it has less possibility of contamination but induces full immunogenicity [25].

The literature also reveals reports on fowlpox vaccination failure. Thus the need for efficient and improved vaccines is being felt. It seems indispensable to Investigate vaccines' efficacy, which is used in Iranian commercial laying herds routinely to illuminate whether these vaccines have appropriate efficacy or not and are they still effective? We intended to update the viral monitoring system as to country livestock and poultry.

Finally, the phylogenetic tree was generated to increase the knowledge of the relationship between this virus and the other strains isolated from other regions in GenBank. In this study, there were 4 clusters, and phylogenetic analysis showed that the strain of our study (Iranian strain) belonged to the first cluster with 99 and 95% identity to the other members of this group. It means our strain has not changed as compared to the previous strain isolated from Iran.

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