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Original Research Article

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AVIAN POX VIRUS ISOLATED FROM PET BIRDS AND COMMERCIAL FLOCKS, IN IRAN

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Abstract: Avian pox (AP) is a viral disease with a wide host range. The aim of the study was molecular identification and characterization of field isolated pox virus from pet birds and commercial flocks in Iran, by polymerase chain reaction (PCR). Scab materials of skin and mucosal lesions were collected from five clinically affected cases. PCR was used to amplify a 578 bp fragment of the poxvirus 4b core protein. In order to determine the genetic relationships among the viruses, this conserved poxvirus genetic region was sequenced and analyzed. The Iranian Avipoxvirus isolates in this study grouped in clade A1 (commercial chicken and turkey flocks) and B1 (canary). Further studies on a larger scale need to be developed to have a better understanding of the molecular characterization of the Iranian APV strains.

Key words: avian pox; phylogenetic analysis; molecular characterization; Iran

Introduction

Avian Pox (AP) is a viral well-known disease in hens, turkeys and many other birds (278 species from 70 families and 23 orders), characterized by cutaneous lesions on the feather-less skin and/or diphtheritic lesions of mucous coats of the upper alimentary and respiratory tract. Moreover, concurrent systemic infection causing high mortality often occurs in canaries. AP lesions, however, may compromise vision, the ability to feed or lead to secondary bacterial or fungal infection leaving wild birds vulnerable to predation.The poxviruses which infect birds belong to the genus Avipoxvirus of the Poxviridae family. Avipoxviruses (APVs) within the family Poxviridae contain nearly 300-kilo base pair (kbp) of double-stranded DNA that replicate in the cytoplasm of infected cells and the members of the genus Avipoxvirus in the subfamily chordopoxvirinae (1). Pox infection usually occurs through the mechanical transmission of the virus to injured skin and also the bite of mosquitoes or mites. The incubation period and duration of APV infection is variable (from a few days to many months), but affected birds with mild lesions frequently recover and this is considered to be the most common

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situation in wild birds. Its incidence is variable in different areas because of differences in climate, management and hygiene or the practice of regular vaccination. It can cause drops in egg production, or retarded growth in younger birds (2).

The conventional laboratory diagnosis of APV is carried out by histopathological examination, electron microscopy, virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs or cell cultures, and serologic methods (2). The 4b core protein gene (p4b) of APV that encodes the protein with molecular weights of 75.2 kDa is usually chosen for comparative genetic analysis (3,4,5). Also, amplification of the p4b of APV by PCR has often been used as a molecular tool for the detection of APVs (6). PCR in combination with restriction endonuclease enzyme analysis (REA) followed by sequence analysis of the amplified fragments is used for detection, differentiation and molecular characterization of fowl pox virus isolates (7). Even considering the decrease in problems caused by poultry production, avian pox is still a significant pathogen which can have serious effects on wild Galliformes. The incidence of AP in Iran is high in pet birds and also in fewer levels in commercial farms. In recent years, some outbreaks of skin lesions suspected to be avian pox were observed in the backyard poultry in different parts of western areas in Iran. Generally, the number of reports concerning incidence and characterization of avian pox viruses in Iran is very low. Gholami-Ahangaran et al. performed a survey on 328 backyard poultries with suspected signs of avian pox virus infection. Their results showed 217 and 265 out of 328 samples were positive for avian pox virus on histopathological and PCR examination, respectively (8). In the study of Fasaei et al. (2014), Avipoxvirus specific DNA was detected in all 10 different isolates from chicken, canary and mynah that were collected from Tehran province (3). The aim of this study was a characterization of AFPv isolates from canary, chicken and turkey flocks by PCR.

Materials and methods

Sampling

Samples (cutaneous scrub and caseous lesions) were collected from different species with characteristic clinical signs (5 samples from chicken, canary, turkey) had been submitted to PCR Veterinary Diagnostic Laboratory (Tehran, Iran), during 2012- 2014. The data of samples are available in table 1.

DNA extraction

DNA was extracted from the skin or pulmonary lesions of the clinical cases and lyophilized vaccines (as a positive control) by QIAamp DNAMini Kit (Qiagen) following the manufacturer's guidelines. DNA samples were stored at -20°C until analysis.

Amplification of 4b Gene

The AVP-specific PCR was performed using primer pairs described based on FPV 4b core protein (P4b) gene sequence of Fowl pox virus strain HP444 previously (9). The sequence of the primers was as follows: forward primer: 5′-CAGCAGGTGCTAAACAACAA and reverse primer: 5′- CGGTAGCTTAACGCCGAATA. PCR consisted of 25 µl reaction containing1.5 units of *Taq* DNA polymerase, 1.5 mM MgCl2, 200µM of each deoxynucleoside triphosphate, 6 pmol of each primer, DNA extracted from clinical samples and nuclease free water up to 25 µl. Amplification was performed after initial denaturation for 2 min at 94°C for 35 cycles and consisted of 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C. In this study, live fowl pox vaccine (Razi Vaccine and Serum Research Institute, Iran) was provided and used as positive control.Negative control includes all the reagents without a template.

Sequencing and Phylogenetic analysis

The positive PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized bye GelRed ™ (Biotium , USA) staining and ultraviolet transillumination. The PCR products were purified by the PCR AccuPrep® PCR Purification Kit (Bioneer Co., Korea) and purified PCR products were sent for sequencing (Source Bioscience, UK) with PCR primers for in a forward and in a reverse direction. Sequencing reactions were run on an ABI Prism 310 Genetic Analyzer. The sequence results were downloaded and analyzed using Chromas (Technelysium Pry Ltd., Australia). Phylogenetic analysis was carried out by analyzing the data obtained here with those of other sequences of FPVs

from the GenBank database. The phylogenetic analysis was performed with the MEGA5 (Phylogeny Inference Package) software, version5. Distance-based neighbor-joining trees were constructed using the Tamura–Nei model (10). The robustness of the phylogenetic trees was assessed by 1,000 bootstrap replicates. Bootstrap values lower than 50 were omitted. The FPV sequences tested in this study were deposited in GenBank under accession numbers KT003286 –KT003290.

Results

Because of the highly conserved nature of the analyzed genes, nucleotide sequences rather than amino acid sequences were used to determine divergence. Clades and sub clades have been named according to previous APV phylogenetic studies based on the P4b (11). The strains were placed in A1 and B1 sub clades. The homology between isolates was 67.3%-100% (Table 3).

Table 1: Description of fowl pox virus strains investigated in this study

Strain name	Nam in Tree	Species	Type of Lesion	Year	Province	Accession No.
IR/Canary poxvirus/ H364/12	CP/H364/12	Canary	Cutaneous	2012	Alborz	KT003286
IR/Canary poxvirus/ H913/14	CP/H913/14	Canary	Cutaneous	2014	Kurdistan	KT003287
IR/Fowl pox virus/H252/12	FP/H252/12	Chicken	Caseous	2012	Isfahan	KT003288
IR/Fowl pox virus/H756/13	FP/H756/13	Chicken	Caseous	2013	Tehran	KT003289
IR/Turkey pox virus/ H335/12	TP/H335/12	Turkey	Cutaneous	2012	Alborz	KT003290

Table 2: Details of poxvirus sequences obtained from GenBank

Isolate name	Abbreviation (Tree)	Host	Country	Accession Number	Clades
Fowlpox virus isolate FPV-VR250	FP/FPV-VR250	Chicken	Norway	AY453172	A1
FowlpoxvirusNobilis Variole W (Intervet)	FP/ Nobilis Variole W	Chicken	United Kingdom	AM050379	A1
Fowlpox Mild (Websters; Fort Dodge)	FP/Websters Mild	Chicken	United Kingdom	AM050378	A1
Avipoxvirus isolate GB 134/01	TP/ GB 134	Turkey	Germany	AY530304	A1
FWPVD Diftosec CT (Merial)	FP/ Diftosec CT	Chicken	United Kingdom	AM050380	A1
pigeonpox PGPV TP-2	PP/ TP-2	Pigeon	Germany	AY530303	A2
Avipoxvirus isolate CVL 2/11/66	TP/ CVL 66	Turkey	United Kingdom	AM050387	A2
Avipoxvirus isolate CVL 10/12/98	TP/ CVL 98	Turkey	United Kingdom	AM050388	A2
Avipoxvirus HNPV/NZL/2002	PP/ HNPV	Pigeon	New Zealand	HQ701713	A3
AvipoxvirusCVL 353/87	AP/CVL 87	Albatross	United Kingdom	AM050392	A3
Falconpox FLPV GB362-02	FLP/ GB362-02	Falcon	Germany	AY530306	A4
Canarypox virus isolate CP10IR	CP/CP10IR	Canary	Iran	KC193679	B1
Canarypox virus isolate Yazd1	CP/Yazd1	Canary	Iran	KF673397	B1
Canarypox virus strain AT_Canarypox/1839/2009	CP/1839	Canary	Austria	GU108510	B1
anarypox virus isolate D98-11133	CP/ D98-11133	Canary	Canada	GQ487567	B1
Avipoxvirus CVL	SP/ CVL	Sparrow	United Kingdom	AM05038	B1
Pigeonpox PPV-B7	PP/PPV-B7	Pigeon	Norway	AY453177	B2
Parrot pox 364/89	PP/364/89	Parrot	United Kingdom	AM050383	C
Avipoxvirus isolate APIII	AP/ APIII	Agapornis	Germany	AY530311	C

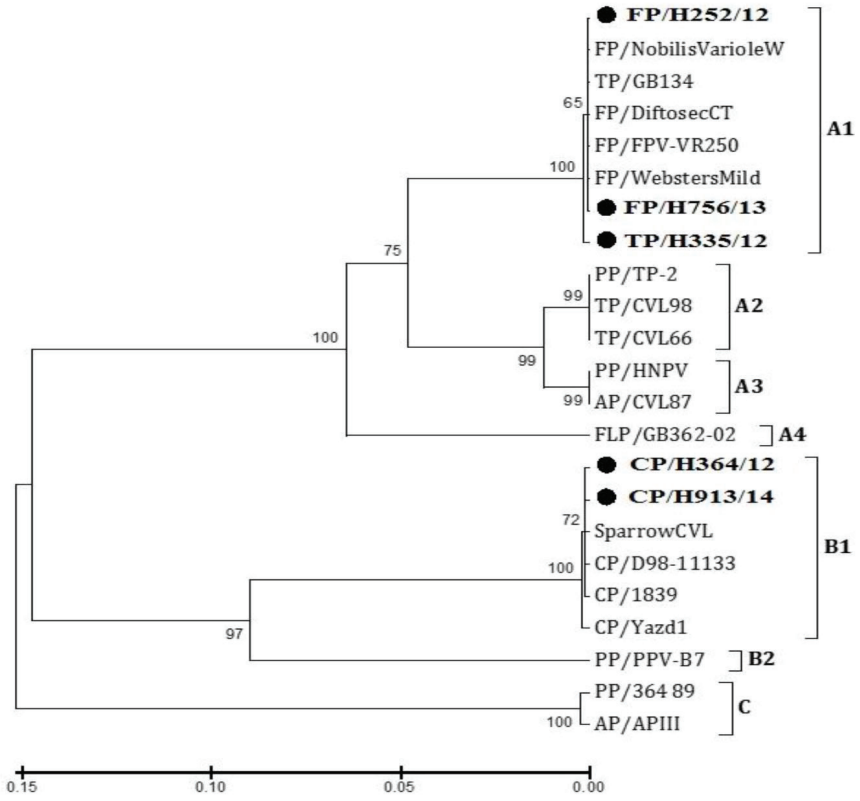

Figure 1: Phylogenetic tree of 578 bp nucleotide sequences of the 4b core protein gene of APV isolated in this study (marked with a black circle), reference APV sequences. The tree was obtained by the neighbour-joining method. Bootstrap testing of phylogeny was performed with 1000 replications and values equal to or greater than 70 are indicated on the branches (as a percentage).The length of each bar indicates the amount of evolution along the horizontal branches as measured by substitution per site. APV clades A–C and sub clades are labelled. You could find the details of viruses in tables 1 &2

Table 3: 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	14	15	16	17	18	19	20	21	22	23	24
1	CP/GHPCRLAB.1																								
2	CP/GHPCRLAB.2	99.04																							
3	FP/GHPCRLAB.3	70.26	70.91																						
4	FP/GHPCRLAB.4	70.67	71.32	99.52																					
5	TP/GHPCRLAB.5	71.02	71.66	99.28	99.76																				
6	TP/GB134	70.67	71.32	99.52	100.00	99.76																			
7	FP/WebstersMild	70.67	71.32	99.52	100.00	99.76	100.00																		
8	FP/NobilisVarioleW	70.67	71.32	99.52	100.00	99.76	100.00	100.00																	
9	TP/CVL98	70.18	70.53	89.44	90.00	89.73	90.00	90.00	90.00																
10	FP/DiftosecCT	70.67	71.32	99.52	100.00	99.76	100.00	100.00	100.00	90.00															
11	FP/FPV-VR250	70.67	71.32	99.52	100.00	99.76	100.00	100.00	100.00	90.00	100.00														
12	PP/TP-2	70.18	70.53	89.44	90.00	89.73	90.00	90.00	90.00	100.00	90.00	90.00													
13	TP/CVL66	70.18	70.53	89.44	90.00	89.73	90.00	90.00	90.00	100.00	90.00	90.00	100.00												
14	AP/CVL87	69.11	69.37	90.54	91.09	90.82	91.09	91.09	91.09	97.58	91.09	91.09	97.58	97.58											
15	PP/HNPV	69.11	69.37	90.54	91.09	90.82	91.09	91.09	91.09	97.58	91.09	91.09	97.58	97.58	100.00										
16	FLP/GB362-02	70.91	71.99	86.28	86.86	86.58	86.86	86.86	86.86	87.65	86.86	86.86	87.65	87.65	87.10	87.10									
17	SparrowCVL	99.28	99.76	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62								
18	CP/D98-11133	99.28	99.76	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62	100.00							
19	CP/1839	99.28	99.76	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62	100.00	100.00						
20	CP/Yazd1	99.04	99.52	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62	99.76	99.76	99.76					
21	CP/CP10IR	93.28	93.54	64.31	64.77	65.13	64.77	64.77	64.77	63.98	64.77	64.77	63.98	63.98	62.72	62.72	64.27	93.80	93.80	93.80	93.54				
22	PP/PPV-B7	81.26	82.15	73.29	73.69	73.36	73.69	73.69	73.69	75.15	73.69	73.69	75.15	75.15	75.15	75.15	75.88	82.15	82.15	82.15	81.83	75.12			
23	PP/364_89	71.66	71.62	70.77	71.16	71.14	71.16	71.16	71.16	69.95	71.16	71.16	69.95	69.95	69.26	69.26	67.17	71.99	71.99	71.99	71.62	65.17	72.37		
24	AP/APIII	70.91	70.87	70.39	70.80	70.77	70.80	70.80	70.80	69.57	70.80	70.80	69.57	69.57	68.87	68.87	66.36	71.25	71.25	71.25	70.87	65.17	72.37	99.52	

Discussion

Avian pox viruses have been isolated from a wide range of avian species including commercial poultry, wild and pet birds. Poxvirus infection is suspected when proliferative skin and/or oral and tracheal lesions are observed. In such cases, a diagnosis is made by histopathology examination of the lesions. Fowl pox vaccine is used in Iranian poultry industry in layer and breeder farms. We don't have any specific vaccine for pet birds. Canary pox has been known as the disease that can result in high losses in a short time, as a re-emerging disease that has not been present during recent years in canary flocks in Iran (12).

Most of our knowledge about the situation of APVs in Iran was obtained from very few reports concerning the epidemiology and the infection biology of the virus. In this study, APVs in clinical cases of affected commercial chickens, turkeys, and canary were identified and characterized by molecular methods to determine the etiology of APV in Iran, 2012. As for the molecular biological analysis, gene P4b amplification products of the expected size were obtained for all the strains of this study, thus confirming that PCR is an extremely valuable diagnostic method for APV infections. Phylogenetic relationships of Avipoxviruses have been analyzed based on the gene corresponding to *vaccinia* virus (VACV) P4b (fpv167, VACV A3L), indicating that all Avipoxvirus strains cluster into 3 major clades, namely, A (Fowl pox (FWPV)-like), B (Canary pox (CNPV)-like) and C (Psittacine). Clade A can be further divided into seven sub clades (A1-A7) and Clade B is comprised of three sub clades (B1-B3) (14). Based on the phylogenetic analysis of four conserved regions, the viruses characterized from Iranian columbiformes cluster into two groups. The viruses from turkey and commercial chickens grouped in sub clade A1 and the viruses from canary grouped in sub clade B1. Conversely, it has also been shown that the same viruses can infect different birds. Therefore, in this study as well as in others, APVs from the same species of bird are classified in different sub clades (4,13,14). Fasaei *et al* (2014) in phylogenetic analysis of Avipoxvirus strains isolated from different bird species in Iran showed that a similarity of 71-100% with the other sequences in the GenBank but they didn't submit their sequences in GenBank and didn't determine the clade of isolates (3). The research which did by Gholami-Ahangaran (2014) on avian pox of backyard poultry in Iran indicated that 66.1% and 80.7% of samples were positive for avian pox virus on histopathological and PCR examination, respectively (8).

The data presented in this research provide novel insights into the molecular characterization of avian pox viruses collected from the broad host range outbreaks in different geographical parts of Iran on particular period time.

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MOLEKULARNA KARAKTERIZACIJA IN FILOGENETSKA ANALIZA AVIARNEGA VIRUSA POX, IZOLIRANEGA PRI PETIH VRSTAH PTIC V KOMERICIALNIH JATAH V IRANU

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Povzetek: Osepnice ptic (AP, iz angl. avian pox) so virusna bolezen, ki lahko okužijo veliko različnih vrst ptic. Cilj predstavljene raziskave je bila molekularna identifikacija in karakterizacija izoliranih virusov pox, pridobljenih iz krast ljubiteljskih ptic in farmskih ptic v Iranu, s pomočjo metode PCR. Vzorci krast s področja sprememb na koži in sluznicah je bil zbran pri petih pacientih s kliničnimi znaki bolezni. S pomočjo metode PCR je bil pomnožen 578 baznih parov dolg odsek gena poxvirusa 4b. To ohranjeno območje poxvirusa je pomembno za določitev genskih razmerij med virusi, zato je bilo določeno zaporedje DNK za nadaljne analize. Iranska izolata Avipoxvirusa objavljena v tej študiji sta bila razvrščena v razred A1 (komercialne piščančje in puranje jate) in B1 (kanarčki). Za boljše razumevanje molekularne karakterizacije iranskih sevov virusa AP bo potrebno opraviti nadaljnje študije.

Ključne besede: osepnice ptic; filogenetska analiza; molekularna karakterizacija; Iran