Molecular detection and phylogenetic analysis of *Avipoxvirus* strains isolated from different bird species

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Summary

The polymerase chain reaction (PCR) was used to amplify a 578 bp fragment of the poxvirus 4b core protein. *Avipoxvirus* (APV) specific DNA was detected in all 10 different isolates (each of which had been isolated from an epidemic) isolated from chicken; canary and mynah were collected from Tehran province. Sequencing was performed for 2 isolates as representative and the nucleotide sequence showed a similarity of 71-100% with the other sequences in the GenBank. The derived phylogenetic tree showed six distinguishable sequence clusters. 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. Concerning the distance between countries which is the origin of the studied isolates that are situated in the same cluster with our Iranian isolates, nearly the same identity (95-99%) of isolates in this cluster exist, and so potential of infectivity of the isolates in several species and regions, and the import and export of birds from all over the world can likely spread the virus to other countries. Hence, strict quarantine measures should be considered in the entrances of every country. Moreover, this is the first molecular study in *Avipoxviruses* in Iran, especially in exotic birds.

Key words: Avian poxvirus, 4b core protein, Quarantine, PCR

Introduction

Avipoxviruses (APVs) within the family *Poxviridae* contain nearly 300 k 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* (Bolte *et al*., 1999; Murphy *et al*., 1999; Afonso *et al*., 2000). They are presented by sixteen described species, in more than 232 species of birds in 23 orders (Bolte *et al*., 1999; Saif *et al*., 2008). The species include Fowlpox virus (FWPV), Canarypox virus (CNPV), Turkeypox virus (TKPV), Pigeonpox virus (PGPV), Juncopox virus, Mynahpox virus, Starlingpox virus, Psittacinepox virus, Quailpox virus, Crowpox virus, Peacockpox virus, Penguinpox virus, Alalapox virus, Apapanepox virus, Condorpox virus and Sparrowpox virus (Moyer, 2000; Saif *et al*., 2008). The disease is described by coetaneous lesions on the featherless skin around the eyes, beak, nostrils and feet in the coetaneous form and/or as proliferative lesions and diphtheritic membranes on the mouth or upper respiratory mucosa and gastrointestinal system (Tripathy *et al*., 1991).

Disease caused by FWPV is one of the important diseases in commercial poultry production and can produce significant problems when conditions are favorable for transmission, especially by mosquitoes, and the best control of disease is prevention of transmission and by vaccination (Boyle, 2007). The severity of the disease is influenced by the strain of virus, route of infection, and the species of bird (Ensley *et al*., 1978). *Avipoxvirus* infections in poultry are mainly associated with considerable transient drop in egg production, reduced growth in young birds and increased mortality (Luschow *et al*., 2004). Natural disease in wild and caged birds range from dry form and tend to be mild and self limiting to severe disease with high mortality in wet form (diphteritic) (Singh *et al*., 1987; Boyle, 2007). Ordinary diagnosis of avipox infections is carried out by histopathological examination to show the presence of the virus in infected tissue samples, electron microscopy, virus isolation in cell culture or on chorioallantoic membranes (CAM) of embryonated chicken eggs (Manarolla *et al*., 2010).

The 4b core protein gene (p4b) of *Avipoxvirus* that encodes the protein with molecular weights of 75.2 kDa is usually chosen for comparative genetic analysis. On the other hand, amplification of the p4b of *Avipoxvirus* by PCR has often been used as a molecular tool for the detection of avian poxviruses (Manarolla *et al*., 2010) and is one of the most sensitive techniques for the routine diagnosis. Eight cases of epidemics suspected of *Avipoxvirus* infections with nodular coetaneous lesions and 2 cases of epidemics in diphtheric form have been reported. In this study PCR has been carried out for all samples and sequencing and phylogenetic analysis of the 4b core protein genes of 2 different *Avipoxviruses*, as representative to follow up the source of infection.

Materials and Methods

Samples

Ten different samples collected from chicken, canary and mynah were used in this study. Each sample had been isolated from an epidemic, as total affected population was nearly 400 birds. Two were collected from diphtheritic lesions of upper respiratory tracts while 8 were from skin lesions. All samples were obtained from several epidemics during 2010-2011 in Tehran province.

Virus isolation on CAMs of embryonated chicken eggs

Tissue sample were homogenized and suspended with phosphate-buffered saline containing penicillin (50 IU/ml)/streptomycin (50 µg/ml). After centrifugation (4000 rpm for 15 m), 0.2 ml of the supernatant was inoculated in the CAMs of 9–11-day-old specific-pathogen-free chicken embryos (obtained from Razi Vaccine and Serum Research Institute, Karaj, Iran). Inoculated embryos were incubated at 37°C for 5-7 days and then examined for poxvirus-specific lesions (pock) on the CAM (Luschow *et al*., 2004; (Manarolla *et al*., 2010). The pocks were put in sterile Petri dishes and stored at -20°C until use. Also, small pieces of some clinically important samples were stored at -20°C until use.

DNA extraction

DNA was extracted from samples and infected pocks by Molecular Biological System Transfer (MBST, Iran) DNA extraction kit. Extraction was performed by the protocol supplied by the kit. The pocks were mixed with phosphate-buffered saline and were homogenized. Then, they were centrifuged at 3000 rpm/15 min.

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 a last centrifugation step at 8000 × g for 1 min.

PCR

APV-specific PCR was established with primers described by Lee and Lee (1997) (P1:5′-CAGCAGGTG CTAAACAACAA-3′; P2: 5′-CGGTAGCTTAACGCC GAATA-3′) and based on APV 4b gene sequence (Binns *et al*., 1989). PCR consisted of 25 µl reaction containing 1.5 units of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 6 pmol of each primer, 60 ng DNA extracted from CAM 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 elongation at 72°C. A final extension step was performed for 2 min at 72°C (Manarolla *et al*., 2010).

Then 5 µl of the amplified PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide. PCR products of p4b gene with a specific size were purified by Qiaquick PCR purification kit (Qiagen and Milan, Italy). Negative control includes all the reagents without template. There was no standard positive control, but clinically confirmed fowlpox lesions which generated pockmark in CAM and had positive results in specific PCR and sequencing (SAM-M-R) were employed as positive control in the next PCR reactions.

DNA sequencing and analysis

DNA sequencing was carried out for 2 strains (SAM-M-R, SAM-B-F), which were isolated from mynah and chicken, respectively. SAM-M-R had been selected from coetaneous lesions of mynahs and SAM-B-F from diphtheric form of chickens as representative. Sequencing was performed by using an automatic sequencer (ABI-370, Applied Biosystem), with forward and reverse primers. They were compared with twenty one selected sequences of APV strains in the GenBank DNA and the inferred amino acid sequence of the APV 4b core protein gene. Sequences were selected from isolates from countries as near as possible on the basis of different places and times of reporting. AM050377UK, EF568397U, GU108503, AJ005164 and HM623675 are isolated from chicken and GQ180203 is isolated from canary. Mynah's related sequence is not found in GenBank. Sequence analysis was performed by neighbor-joining method with MEGA5 program.

Results

Virus isolation and molecular detection

All samples generated poxvirus-specific lesions (pock) on the CAM. APV specific DNA was detected in all 10 samples and all the isolates were considered to belong to the genus *Avipoxvirus* of the family *Poxviridae*. Amplified fragments of approximately 578 bp for p4b core protein genes of 10 isolates were obtained (Fig. 1) and were in agreement with the size of 578 bp on the basis of published APV 4b nucleotide sequence (Binns *et al*., 1989).

Sequence analysis

The APVs sequencing of the amplified region of the 4b gene was performed for 2 isolates and the nucleotide sequence similarity with twenty one selected sequences of APV strains in the GenBank DNA, showed a similarity of 71-100%. The derived phylogenetic tree and identity matrix showed six distinguishable sequence clusters (clusters are distinguished and counted in the tree obviously) (Fig. 2, Table 1). Two strains of present study belonged to the first cluster which has several

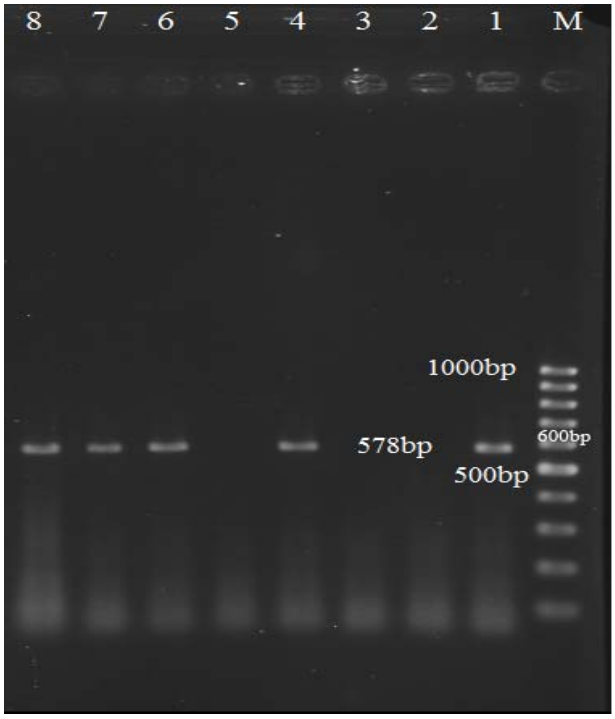

Fig. 1: PCR amplification of the p4b gene from some of the samples of this study. Lane M: DNA size marker (100 bp DNA ladder). Lane 1: Mynah, Lane 4: Canary, Lanes 6-8: Chicken isolates representatives and Lanes 2, 3, 5: Negative results in PCR and are not APV

strains from different countries and birds. However, one of the isolates (SAM-M-R) has a distance from the root in this group.

Discussion

The established PCR technique that can be used to detect very low copy numbers of viral nucleic acids is a sensitive and valuable diagnostic tool for characterization and for epidemiological study at the molecular level. In this study, isolates of APVs from clinical cases of 10 different avian (each of which had been isolated from an epidemic, as total affected population was nearly 400 birds) were detected by PCR and two of them, as representative, were analysed by sequencing and finally phylogenetic tree was generated to increase the knowledge about the relationship between these viruses and the other strains isolated from other regions in GenBank. This is the first study in molecular level about *Avipoxviruses* in Iran, especially in exotic birds.

The other established PCR for 39-KD protein gene of FPV is able to detect only FPV and turkey poxvirus but not pigeon poxvirus, canary poxvirus and different avian

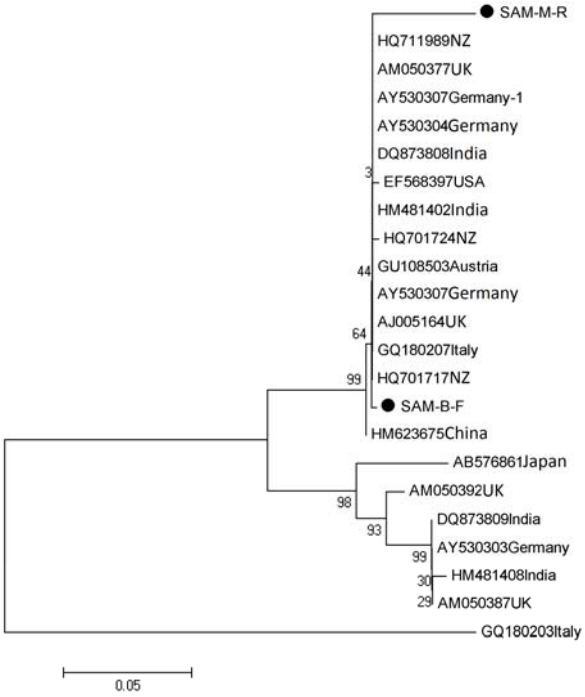

Fig. 2: Phylogenetic tree of nucleotide sequence of the 4b core protein gene PCR fragment of different APVs tested and of the published sequence in GenBank constructed by neighbor-joining method with MEGA5 program. Name of the origin country of each isolate has been added to the end of its name in the tree. Values at the branches and clusters are bootstrap value and bar indicates distance scale from the roots

Table 1: Identity matrix and distances of isolates of this study with compared sequences from GenBank. SAM-M-R and SAM-B-F are isolates of this study

SAM-M-R																							
SAM-B-F	0.043																						
DQ873808 India	0.040	0.002																					
HM481402 India	0.040	0.002	0.000																				
GU108503 Austria	0.040	0.002	0.000	0.000																			
AY530304 Germany	0.040	0.002	0.000	0.000	0.000																		
AY530307Germany	0.040	0.002	0.000	0.000	0.000	0.000																	
AY530307Germany-1	0.040	0.002	0.000	0.000	0.000	0.000	0.000																
EF568397 USA	0.043	0.005	0.002	0.002	0.002	0.002	0.002	0.002															
AJ005164 UK	0.040	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002														
HM623675 China	0.043	0.005	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.002													
AM050377 UK	0.040	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.002												
GQ180207 Italy	0.040	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000											
HQ711989 NZ	0.040	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.000										
HQ701717 NZ	0.040	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.000									
HQ701724 NZ	0.043	0.005	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.002	0.005	0.002	0.002	0.002	0.002								
HM481408 India	0.164	0.114	0.111	0.111	0.111	0.111	0.111	0.111	0.114	0.111	0.108	0.111	0.111	0.111	0.111	0.114							
DQ873809 India	0.157	0.108	0.105	0.105	0.105	0.105	0.105	0.105	0.108	0.105	0.102	0.105	0.105	0.105	0.105	0.108	0.005						
AY530303 Germany	0.157	0.108	0.105	0.105	0.105	0.105	0.105	0.105	0.108	0.105	0.102	0.105	0.105	0.105	0.105	0.108	0.005	0.000					
AM050387 UK	0.157	0.108	0.105	0.105	0.105	0.105	0.105	0.105	0.108	0.105	0.102	0.105	0.105	0.105	0.105	0.108	0.005	0.000	0.000				
AM050392 UK	0.144	0.096	0.093	0.093	0.093	0.093	0.093	0.093	0.096	0.093	0.090	0.093	0.093	0.093	0.093	0.096	0.030	0.025	0.025	0.025			
AB576861 Japan	0.159	0.113	0.110	0.110	0.110	0.110	0.110	0.110	0.113	0.110	0.107	0.110	0.110	0.110	0.110	0.113	0.074	0.068	0.068	0.068	0.051		
GQ180203 Italy	0.389	0.326	0.325	0.325	0.325	0.325	0.325	0.325	0.329	0.325	0.325	0.325	0.325	0.325	0.325	0.330	0.332	0.332	0.332	0.332	0.349	0.360	

species. For this reason, the next PCR was carried out with primers that are designed for a conserved region within APV 4b core protein gene of 39-KD protein gene (Lee and Lee, 1997).

Results showed that the selected primers allowed amplification of DNA from all 10 isolates that had been obtained from different avian species from several epidemics, all of which had occurred in Tehran and were diagnosed during routine diagnostic activity, even if the sequence analysis showed a similarity in nucleotide of 71-100% between APVs (Table 1).

Our histopathological results pointed out that all 10 strains did grow in embryonated eggs after one passage, which showed their possible ability to adapt to the embryos.

Because of having two stranded DNA, the rate of mutation is relatively low in *Poxviridae* (Murphy *et al*., 1999). In this study, there were six clusters. Phylogenetic analysis showed that two strains of our study (Iranian strains) belonged to the first cluster, SAM-B-F isolated from chicken and SAM-M-R from mynah have 99 and 95% identity with the other members of this group, respectively, but SAM-M-R has a distance from the root.

PCR results for 4b core protein in fowlpox performed by Luschow *et al*. (2004) revealed no recognizable differences in size of amplified fragments among the different APVs. Nucleotide sequence analysis showed similarity of 72-100% among the different species, which is in agreement with our study (Luschow *et al*., 2004). Other scientists studied about 15 strains of avipox virus from different avian species. PCR reaction of highly conserved p4b gene was positive for all cases. Sequencing confirmed most strains clustered either with Fowlpox virus or with Canarypox virus (Manarolla *et al*., 2010). Others confirmed the avian poxvirus was isolated from oriental turtle-doves by PCR using primers specific to the 4b core protein gene of avian poxvirus (Eo *et al*., 2011). In addition, Pawar in 2011 studied fpv167 and fpv140 and amplified them by PCR using DNA from viruses isolated from eight Indian wild birds. Blast and phylogenetic analysis indicated that (FWPV) was the nearest phylogenetic neighbor to the viral isolates, from two Indian peacocks, two golden pheasants, one silver pheasant and one sparrow (Pawar, 2011). Finally, analysis of the 4b core protein gene by Literak *et al*. in 2010 showed identical DNA sequence in six isolates and the acidophilic type inclusion was documented by electron microscopy in cells from lesions on great tits.

This study showed that the viruses conserve their characteristic nucleotide sequence of p4b core protein in different geographical areas. Also, in accordance with different regions and species origin of strains situated in the same cluster in the tree, one strain is probably able to infect several species in several regions.

In the present study, the sequence analysis reveals that the Iranian isolates are within the group with highly conserved in p4b core protein in different countries (with identity percent more than 99%) and species of birds, while there is not a great deal of information about Asian isolates except for India, China and Japan in GenBank (Table 1). Since our two isolates belonged to the common cluster in the tree and isolates from several species and regions existed in this cluster, it can be concluded that there is no direct relation and specificity between sequences and region of isolation or species, hence the isolates are capable of causing infection in different species and regions.

Concerning distance between countries which are the origin of the studied isolates with our Iranian isolates, the same identity of isolates in the tree exist, so the potential of infectivity of the isolates in several species and regions, and the import and export of the birds from all over the world can likely spread the virus to other countries and so strict quarantine measures should be considered in the entrances of every country.

Also, in this study, the specific DNA of avian pox virus with PCR was shown, which is a valuable and sensitive diagnostic system for study of epidemiology at the molecular levels.

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