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Identification of FPV by PCR amplification of p4b gene in infected cell culture and Chorioallantoic membrane

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Five local fowl were brought for postmortem examination with a history of sudden death in the area of Kumarganj, Faizabad (U.P.). The gross examination of birds revealed multiple light whitish nodules around the eye, on the skin at the level of hock joint, on the anterior part of tracheal mucosa, congested lung and pallor liver. Impression smears from nodules revealed numerous heterophils, red blood cells, necrotic epithelial cells and bacterial colonies. Histopathological examination of nodules revealed eosinophilic intracytoplasmic inclusions (Bollinger bodies) in keratinocytes, epidermal hyperplasia and necrosis with ballooning degeneration, and bacterial colonies. The virus was isolated and infection was produced on both chorioallantoic membrane and BGM-70. Polymerase chain reaction was carried out and primer set designed from the 4b core protein gene of fowl poxvirus revealed amplification at 576bp.

Key words: Avianpox virus, Polymerase chain reaction (PCR), BGM-70, Chorioallantoic membrane.

The largest known virus of terrestrial and marine mammals belongs to the *Chordopoxvirinae* subfamily of the *Poxviridae*family[1]. The Poxviridae is divided into two subfamilies: Entomopoxvirinae, that comprises insect poxviruses, and Chordopoxvirinae, that includes all poxviruses of vertebrates[2].The family Poxviridae possess non-infectious, double stranded DNA genomes that range in size from 130-380 kbp and replicate almost exclusively in the cell cytoplasm[2].Poxviruses are highly adapted viruses infecting a large number of hosts, including insects, reptiles, birds, over 30 mammalian species and in a number of endangered species or species in captive breeding recovery programs[3,4]. Latest case report of Fowlpox virus infection is found in Hungarian partridges (Perdixperdix)[5], white-tailed sea eagle (Haliaeetusalbicilla)[6], and Hungarian great tits (Parus major)[7].APVs are transmitted viabiting insects and aerosols and are usually named virus was first isolated and characterized[8]. Interspecies transmission also occurs of fowlpox virus[9].The avipox virus appears incapable of causing disease in mammals; therefore there appears to be no zoonotic potiential[10]. The disease characterized by proliferative lesions of the skin and diphtheric membranes of the respiratory tract, mouth and esophagus, has been described in avian species[8]. Large intracytoplasmic inclusion bodies known as Bollinger bodies that contain smaller elementary bodies (Borrel bodies) is formed when FPV multiplies in cytoplasm of epithelium ischaracteristicof the disease and can be seen in histopathological examination[11].

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APV are increasingly being detected and characterized by PCR, Restriction fragment length polymorphism (RFLP), Southern blot hybridization, and cycle sequencing, directed at specific genes such as the 4b core protein gene[12,13]. Prior to PCRtechnology, viruses were isolated in cell culture before any further analyses could occur[14]. Identification and differentiation of viruses before the implementation ofPCR relied on less specific serologic assays such as, virus neutralization, hemagglutination and immuno fluorescence assays, later complemented by restriction endonuclease profiles of viral DNA resolved in agarose or poly acrylamide gels[15,16,17,18,2]. A recombinant fowlpox virus vaccine expressing key protective *Mycoplasma gallisepticum* antigens could facilitate in the prevention both of fowlpox virus and *M. gallisepticum* infections[19]. Pigeon pox virus maybe used as first dose in chickens against fowl pox.This is preferred especially for layers to avoidany reaction *Ganguli*[20].

Consideringpoxviruses in particular, PCR and genome sequencing has meant the evolution from sometimes vague histopathologic and electron microscopic (EM) diagnoses to muchmore definitive genetic assays for poxvirus infection[21,22,23]. Naturally occurringdual infection of layer chickens with fowlpox virus and gallid herpesvirus 1 (infectious laryngotracheitis virus) can be diagnosewith the help of electron microscopy, PCR andhistology[24].Because of this common poxvirus morphology, it is difficult to discernbetween genera of poxviruses when using techniques like histopathology and EM.However, PCR and sequencing methods reveal not only the genus, but in most cases, species of the virus being examined[21,25,23]. The advent of PCR allowed for the direct amplification of viral DNAand rapid genome sequencing[2]). Thesesequencing advances have allowed for a better ability to define and understand theevolutionary relationships between the different poxvirus genera. Comparing genes thathave been identified as highly conserved can aid in new virus characterization andcomparison. Previous studies have elected phylogeneticanalysis to be the best tool available for characterizing poxviruses known to date[26,25,27]. PCR allows for sensitive and specific detection of viral nucleic acids and has been shown to increase the diagnostic sensitivity for many viral pathogens when compared to culture. Detection by realtime PCR has been used to identify recombinant APV from individual plaques[28].

MATERIALS AND METHOD

Detection of FPV using polymerase chain reaction

The FPV specific viral DNA was amplified and detected in FPV infected CAM as well as BGM-70 cell solution samples.

Extraction of viral DNA

The method described by Eo.[29] was used for extraction of viral DNA from FPV infected CAM suspensions.

The FPV infected CAM was triturated in TE buffer (10 mMTris and 1mM EDTA, pH 8.0) and centrifuged at 150xg at 4^0C for 10 min. In 2 ml appendroff tube, to 400µl suspensionof each sample, equal volume of extraction buffer was added and tubes were incubated at 55^0C in water Bath for 2 hr. Equal volume of Ph:Cl (48:2) solution was then added to the mixture and tubes were vortexed followed by centrifugation at 13000 rpm for 10min. The aqueous phase was collected in another appendroff tube and the step was repeated once. Following centrifugation at 13000rpm for 10 min, the aqueous phase was collected and to this, equal volume of chloroform was added and tubes were vortexed. The suspension was then centrifuged at 13000 rpm for 10 min and aqueous phase was collected. The step was repeated once with chloroform and to the aqueous phase, 1/10 volume of 3M Sodium Acetate and 2 volume of absolute ethanol was added. The mixture was kept overnight at -70^0C.The mixture was then centrifuged at 13000 rpm for 30 min at 4^0C, supernatant was removed and again 700µl of 70% ethanol was added, mixed properly and centrifuged at 13000 rpm for 10 min. After removing the supernatant, tubes were allowed to dry. To the product, 50µl of TE buffer was added after drying the tube and it was stored at -20^0C till further use.

Primers

PCR was performed to amplify the p4b core protein gene of avian pox virus. The published primers, forward (CP1, 5'-CAGCAGGTGCTAAA CAACAA-3') and reverse (CP2, 5'-CGGTAGCTTAACGCCGAATA-3') specific to the p4b core protein gene (Weil[13]*et al.*, 2004) of avian pox virus were used

Composition of Reaction mixture for PCR

Reaction mixture was prepared in 50µl volume for one PCR reaction as given below:

Table 1.

Master Mix (10mM)	25.o µl
Forward Primer (50.0 picomol)	5.0 µl
Reverse Primer (50.0 picomol)	5.0 µl
Nuclease free water	10 µl
DNA Template	5.0 µl
Total	50.0 µl

Steps and conditions of thermal cycling

Following cycling conditions were used as described by Eo[29]

Table 2.

Parameters	Temperature	Time
Heat lid	105^0C	
Initial denaturation	94^0C	5 minutes
No. of cycle	35	
Denaturation	94^0C	20 second
Annealing	53^0C	30 second
Extension	72^0C	40 second
Final Extension	72^0C	5 minutes
Final hold	04^0C	

Confirmation of PCR Products

The amplified PCR products were confirmed for their expected size in 1.5% agarose gel in 0.5X TBE buffer using horizontal submarine electrophoresis apparatus. A 1.5 percent agarose gel prepared in 0.5X TBE buffer was boiled for 2 min and allowed to cool down to 50^0C, Ethidium bromide was added @ 2µl/50 ml and mix thoroughly. The gel casting platform was placed on a leveled surface and the open slides were sealed with adhesive tape. The gel comb was then placed across the gel casting platform, so that the teeth of comb remained 1 mm above the base of platform. The molten agarose was then poured onto the gel casting platform and it was kept undisturbed until the gel was properly solidified. The comb was taken out and adhesive tape was removed. The set gel with gel casting platform was then submerged in the sufficient quantity (about 1 mm above the gel level) of electrophoresis buffer keeping the wells towards cathode end.

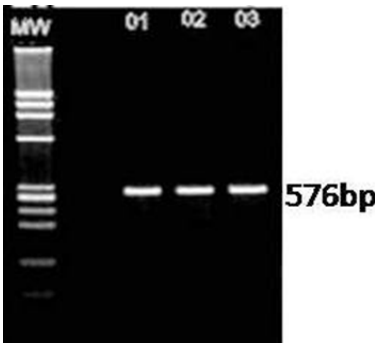
Eight µl of PCR products and molecular weight markers (100bp DNA ladder) were mixed with 2µl of Bromophenol blue dye (6X) and loaded into the respective wells. Electrophoresis was performed at 5 volt/cm current and the progress of motility was monitored by the migration of dye. At the end of electrophoresis, the gel was visualized under Gel documentation system (UV Tech.) for the amplified product of desired length.

RESULTS

On PCR analysis using P4b gene specific primer set, the P4b gene could be amplified in both CAM as well as BGM-70 cell infected with the local isolate of the FPV with an amplification size of 576 bps.

DISCUSSION

In present study P4b gene was successfully amplified in the CAM as well as BGM-70 cells infected with the field isolate of FPV with an amplicon size of 576 bps. PCR and sequencing



Lane-MW: 100 bp DNA leader,
Lane 01: FPV vaccine virus
Lane 02: CAM grown FPV
Lane 3: Cell culture grown virus.
PCR amplification of P4b core protein gene

Fig. 1. Fowl Pox Virus in Avian.

are reported to be the methods of choice for rapid identification and differentiation of poxviruses up to the species level[30,31,32]. The P4b gene was targeted by many workers for identification of Avipox viruses in infected tissue because P4b is a conserved 752 kDavirion core protein found in all poxviruses[33,34]. Weil and Eo[13,29] also used PCR to amplify the P4b core protein gene to identify avian pox virus and could get a 576 bp product. Williams[35] also used PCR on nucleic acid extracted from Vectormune FP-LT (fowl pox virus-laryngotracheitis) vaccine strain and a live attenuated fowl pox vaccine and from paraffin embedded lung tissues from FPV-LT vaccinated birds. PCR-based diagnosis is considered superior to other techniques in terms of sensitivity and speed[30].PCR amplification of the 4b corepoxvirus gene sequence was successful in four of five individuals resultingin products that averaged 575 bp[36].Polymerase chain reaction was carried out and primer set designed from the 4b core protein gene of fowl poxVirus revealed amplification at 578 bp[37].

Summary

On PCR analysis using P4b gene specific set, the P4b gene could be amplified in both CAM as well as BGM-70 infected with the local isolate of FPV with an amplification size of 576bps.

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