Occurrence and phylogenetic analysis of avipoxvirus isolated from birds around Jammu

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Abstract Domesticated fowls, pigeons and turkey birds were screened for avipoxvirus infection from different areas in Jammu region. Based on typical pox lesions the overall occurrence in fowl was found to be 18.52%, 17.03% in pigeons and 57.14% in turkeys. Mortality recorded in chicks was 41.96%, 45.36% in squabs, 100% in poults, and 20.00% in adult turkeys. Both cutaneous and diphtheritic forms of the disease was observed of which the latter was particularly prevalent in young birds. One sample of putative fowlpox virus (FWPV) from skin lesions of a fowl, and two samples of putative pigeonpox virus (PGPV) from skin and diphtheritic lesions each were inoculated on chorio-allantoic membrane (CAM) of

10–12 days old chicken embryonated eggs. A confirmatory diagnosis was made by PCR amplification of a highly conserved *P4b* gene locus detected in tissue samples from skin, diphtheritic membrane and virus inoculated CAM yielding a predicted 578 bp product. Phylogenetic analysis based on the same *P4b* gene locus revealed FWPV and turkeypox virus (TKPV) to be 99% related and belonging to clade 1, while PGPV was found to belong to clade 2. All three isolates illustrate considerable heterogeneity within the conserved *P4b* gene locus. The study indicates that the closely related FWPV and TKPV isolates may have the potential of cross infection between fowls and turkeys and therefore cross transmission studies are suggested.

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Introduction

Avian pox is a cutaneous viral disease caused by the DNA virus of the genus *Avipoxvirus* under family *Poxviridae* and subfamily *Chordopoxvirinae*. It is characterized by the formation of proliferative lesions, scabs on the skin, and diphtheritic lesions in the upper digestive and respiratory tracts. The geographic distribution of avian poxviruses is worldwide [21].

The lesions are fairly diagnostic and can be readily confirmed by histological observations or electron microscopy [OIE. Fowlpox. In: OIE Terrestrial Manual. 2008. pp. 531–537]. Avipoxvirus can be grown on the chorioallantoic membrane (CAM) of embryonated chicken egg causing its thickening and typical focal and diffuse lesions and is one of the identification tools of choice [4]. Molecular diagnosis using Polymerase Chain Reaction

(PCR) have proven to be the most sensitive technique for diagnosis of APV infection, which is mostly based on the amplification of a 578 bp *P4b* gene of APVs and is being increasingly used during the last few years [14].

Avipoxvirus infections have been observed in an extensive range of wild, captive and domesticated avian hosts, yet little is known about the genome diversity and host-range specificity of the causative agent(s). Antigenic relationship exists among avipoxvirus, and some avian poxviruses are more closely related than others. However, there is evidence of considerable heterogeneity among species of avian poxviruses. Serology has revealed cross-reactivity among several of the viral species, whereas genomic and antigenic characterizations have been only moderately useful for identifying differences among strains.

In India, avian pox infection has been regarded as a re-emerging disease [18] causing high mortality and morbidity in many domestic and wild birds. Amongst the domesticated birds, the disease has been recorded in fowls [5, 17] with reports of vaccination failure [7–12]. Molecular characterization of fowlpox strains isolated from backyard chicken also reveals integration of reticuloendotheliosis virus sequences [3]. Similarly, avipox virus has also been isolated from turkey, pigeons and ducks [17, 19, 1] and many wild birds [18].

In Jammu, Ahamad et al. [2] reported Turkey pox amongst farmed turkeys, while Mehmood [15] observed the prevalence of Pigeon pox in the region. Apart from these known infrequent incidences, the infection status of avipoxvirus in this region is largely unknown and unreported. It was therefore intended to know about the occurrence of different avipoxvirus amongst the domesticated avian population and possible virus heterogeneity. To the authors knowledge this is probably the first characterization of avipoxvirus from the state of Jammu and Kashmir.

Materials and methods

Virus isolates

A commercial live attenuated vaccine (Venkateshwar Hatcheries Pvt. Ltd., Ventri Biological, Vaccine Division-Batch no. FP15-037) was used as reference avipoxvirus (APV) isolate. Field/wild type virus isolates were obtained from affected fowls, pigeons and turkeys and designated as *Fowlpox virus* (FWPV), *Pigeonpox virus* (PGPV) and *Turkeypox virus* (TKPV).

A total of 610 backyard fowls, 998 domesticated pigeons and 70 turkey birds were screened (categorized as young and adult birds) for avian pox lesions in Rajouri,

Jammu and Poonch areas of Jammu province from April 2016 to May 2017, and some formalin-fixed archival tissues of a previous outbreak in 2008–2009 amongst turkeys were also included. Clinical materials included scabs, skin biopsies, or lesions from oral membranes from affected birds.

Virus inoculation in chicken embryonated eggs (CEE)

A 10–20% solution of crushed scab/skin tissue samples in PBS was prepared from affected chickens and pigeons, and clarified by centrifugation at 3000×g for 15 min. The supernatant was then passed through a 0.45 µm syringe filter (Millipore) and incubated with streptomycin, penicillin antibiotic solution (@ 200 IU of penicillin and 200 µg of streptomycin per ml suspension) for 1 h at 37 °C. Processed samples were used for virus inoculation in chorioallantoic membrane (CAM) in 10–12 days old chicken embryonated eggs (CEE) with no history of Fowlpox infection as described previously [4].

PCR amplification

For diagnosis and confirmation of avipoxvirus, the *P4b* gene encoding for 4b core protein of the virus was targeted using primer sequences (*P4b* F 5'-CAGCAGGTGCTAAACAACAA-3'; *P4b* R 5'-CGGTAGCTTAACGCCGAATA-3') and protocol as previously described [13]. Viral DNA was isolated using DNeasy Blood & Tissue Kit (QIAGEN, USA, #69504) from affected tissues and virus inoculated CAM. Amplification was performed using 5 µl 10 × PCR buffer, 1.5 µl MgCl₂ (25 mM), 0.5 µl dNTPs (10 mM), 0.3 µl *Taq* (5 U/µl), 1 µl each of primers (10 pmol), 2 µl DNA template and nuclease free water up to 25 µl in PCR tubes (Eppendorf, Germany). Cycling conditions were set at 94 °C for 5 min for initial denaturation; followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min and held at 4 °C. Products were electrophoresed in 1.2% w/v agarose and visualized in a Gel Documentation System (BioDoc Analyze, Biometra, Germany).

Partial gene sequencing

Sequencing of PCR products were done commercially for FWPV and PGPV at Chromous Biotech Lab., Bangaluru, while TKPV was sent to SciGenome Lab. Pvt. Ltd., Kerala. Nucleotide sequences corresponding to VACV *fpv 167 locus* (P4b), was aligned with published sequences obtained from GenBank, and phylogenetic relationships

were determined based on these alignments using Clustal W program of Mega 5 by neighbor joining method.

Results

Occurrence and pathology

The detailed occurrence and mortality of different birds affected with avipoxvirus presenting typical cutaneous/diphtheritic pox lesions is represented in Fig. 1. The occurrence of avian pox in adult fowl was 10.28% and 45.45% amongst young ones with an overall occurrence of 18.52% with mortality of 41.96% in chicks only. In pigeons, an occurrence of 45.36% and 10.20% were recorded in young and adult birds respectively, with a mortality of 45.36% seen amongst the squabs only. Overall occurrence in turkey was 57.14%, affecting all the young and 40% of the adults, amongst which all the young birds died and a mortality of 20.00% was recorded amongst the adult birds. The clinical signalment varied depending on the stage of infection, clinical form of disease, and the age of the birds affected. Amongst the young birds-respiratory distress, a general malaise, anorexia, dehydration, weight loss, emaciation and stunted growth was notable. Some birds showed ocular involvement with blepharitis and conjunctivitis, often leading to blindness. In severe cases, significant mortality was observed. Signs in the adults included alopecia, decrease in egg production and a general lack of thriftiness depending on severity of infection. The cutaneous form was manifested by external proliferative lesions characterized by small solitary, or multiple nodules or typical pock-like cratered lesions on the skin

Diphtheritic form of pox was seen in young chicks, pigeons and poults that typically appeared as opaque-white to yellowish, raised, diphtheritic patches on the mucosa of the oral cavity.

Diagnosis

Chicken embryonated egg inoculation (CEE)

Out of various field samples, putative FWPV and PGPV were adopted on CAM of 10-12 days old chicken embryonated eggs through three passage levels, candled daily and opened on 5 dpi. CAM thickening and pock lesions were discernible.

Molecular diagnosis and avipoxvirus confirmation

All samples processed for PCR analysis (Table 1) showed specific bands of 578 bp in 1.2% agarose gel electrophoresis. No band was observed in negative control.

Molecular characterization and phylogenetic analysis of different avipoxviruses

It was observed that the sequences of the *P4b* gene shared homology with other APVs reported from different parts of the world. Different published sequences in GenBank library were compared with the isolates of present study.

On phylogenetic analysis (Fig. 2a) it was observed that Fowlpox and Turkeypox virus isolates of APV in the present study clustered under recognized clade A1 along with other *Fowlpoxvirus* strains reported from India and other

Fig. 1 Age-wise occurrence of avian pox infection amongst various birds in select areas of Jammu province

Table 1 Molecular confirmation of avipoxvirus isolates from different samples by PCR amplification of *P4b* core protein gene

Species (putative Avipox isolate)	Tissue	PCR positive/no. of samples	Sequencing GenBank accession number
Chicken fowl (FWPV)	Skin biopsy	3/3	
	CAM (3rd passage) inoculated with FWPV from skin lesion	1/1	MF496042
Pigeon (PGPV)	Skin biopsy	4/4	
	Diphtheritic membrane	2/2	
	CAM (3rd passage) inoculated with PGPV from skin lesion	1/1	MF496043
Turkey (TKPV)	Formalin fixed skin tissue	1/1	MF496044
Vaccine virus (FWPV)	–	1/1	

FWPV, *Fowlpox virus*; PGPV, *Pigeonpox virus*; TKPV, *Turkeypox virus*; CAM, Chorio-Allantoic Membrane of chicken embryonated egg (CEE)

Fig. 2 Phylogenetic analysis and divergence of different Avipox viruses isolated from Jammu: **a** Phylogenetic tree based on the *P4b* gene nucleotide sequence—*Fowlpox virus* (FWPV) and *Turkeypox virus* (TKPV) isolates align with major clade A1 and *Pigeonpox virus* (PGPV) align with major clade A2, using Clustal Method; **b** Pair-wise sequence comparison of nucleotide of the *P4b* gene from *Fowlpox virus*, *Pigeonpox virus* and *Turkeypox virus* using CLUSTAL method

parts of world. However virus from Pigeonpox isolate clustered under established clade A2 where majority of *Pigeonpoxvirus* have been reported.

The divergence and relatedness analysis of the different avipoxvirus isolated from Jammu in the study are depicted

in Fig. 2b. Fowlpox virus and Pigeonpox virus showed 90% identity, while Fowlpox virus and Turkeypox virus showed 99% identity.

Discussion

Apart from the known infrequent incidences, the infection status of the avipoxvirus in this region is largely unreported. The disease was seen amongst backward, free-range chicken only, and none amongst the commercial broiler birds. Similarly amongst pigeons, the disease causes significant losses to the pigeon fanciers particularly in the form of mortality in squabs. Disease dissemination in pigeon is more likely since the birds fly to other lofts as a part of a sport for luring opponent's birds by the fanciers. Besides, the birds are within a few kilometres across the International Indo-Pakistan border, for possible cross transmission across boundaries. As in the case of the backyard poultry, resident pigeons have never been vaccinated against avian pox. Although farming turkey in the region is still in its infancy, its rearing was limited for experimental or recreational purpose. The source of infection amongst turkeys was unknown, but the susceptibility of these turkeys to pox infection is likely to discourage their farming.

The mortality pattern with avian pox was highest amongst young fowls, squabs and poults, of which the latter was found to be most susceptible. Similar mortality rates have been described in the literature for young turkeys [11, 8] particularly when lesions on the mucous membranes of the mouth and upper air passages are present [6]. A preponderance of lesions in eyes and mouth of turkeys have also been cited by Forrester and Spalding [8] leading to blindness and starvation; a verity observed in the present study. Susceptibility of young birds could be attributed to their primal active immunity and especially when the maternal antibody levels may be dwindling. The severity of the diphtheritic form of pox over cutaneous lesions, resulting in higher mortality has been reported earlier [20, 16]. It was observed in the present study, that a differential diagnosis of the diphtheritic form in pigeons from Trichomoniasis or canker was essential because of similar lesions, a fact that has also been suggested by Tripathy and Reed [20]. Adult birds with cutaneous lesions could spontaneously recover from mild infection within a few weeks.

In the present study the *P4b* gene has proved to be useful to diagnose all suspected samples of avipoxvirus and establishes its utility to detect across all avipoxvirus and the signifies the highly conserved gene region. Jarmin et al. [10] opined that the divergence among avipoxviruses is very great and therefore it is difficult to identify PCR primers, whether pan-genus or species-specific; as a consequence, the only PCR locus used until now has been the *P4b* locus.

It was observed from the present study that the Fowlpox and Turkeypox isolates are related, with 99% identity and corresponding to clade A1. On the other hand, Pigeonpox isolate grouped under clade A2 along with other *Pigeonpoxvirus*. It is however important to note as Jarmin et al. [10] opined, that there could be a problem with the host species-based approach to the taxonomy of avipoxviruses, in that, sequences of isolates taken from particular species can be found in different subclades (e.g. FLPV) or even different clades (e.g. PGPV).

In conclusion, it was successfully established that FWPV, PGPV and TKPV are prevalent in this region and confirmed by PCR for the first time. This list however, may not be exclusive. The viruses illustrate considerable heterogeneity in their genetic makeup within the highly conserved *P4b* gene locus. Since the FWPV and TKPV were closely related, the possibility of cross infection between fowls and turkeys cannot be ruled out. Regular vaccination against avian pox should be an important strategy, owing particularly to production losses, and more importantly the mortality of the young, which undoubtedly remain a cause of serious concern for the purpose of establishing any poultry enterprise.

References

1. Ahamad DB, Punniamurthy N, Sivaseelan S, Ranganathan V. Pathomorphology and ethno-veterinary herbal intervention in an outbreak of turkey pox. *Indian J Vet Pathol*. 2013;37(1):18–21.
2. Ahamad DB, Nashiruddullah N, Azmi S, Qureshi SA, Khan A, Koul A, Katoch R, Taku AK. An outbreak of Turkey Pox in Jammu region. In: National symposium on pathobiology of Poultry and Laboratory Animal Diseases, held at Veterinary College and Research Institute, Namakkal, Tamil Nadu from 5–6 March 2010. Abstract No. 6.1.26; 2010.
3. Biswas SK, Jana C, Chand K, Rehman W, Mondal B. Detection of fowlpox virus integrated with reticuloendotheliosis virus sequences from an outbreak in backyard chickens in India. *Vet Ital*. 2011;47(2):147–53.
4. Cunningham CH. A laboratory guide in virology. 7th ed. Minneapolis: Burgess Publishing Co.; 1973.
5. Das P, Joardar SN, Samanta I, Das PK, Jana C, Isore DP, Sadhukhan TK. Isolation and molecular confirmation of Avipoxvirus in backyard chicken in West Bengal. *Indian J Comp Microbiol Immunol Infect Dis*. 2013;34(1):8–10.
6. Davidson WR, Kellogg FE, Doster GL. An epornitic of avian pox in wild Bobwhite quail. *J Wildlife Dis*. 1980;16:293–8.
7. Dhanesar NS, Sharma SN, Malik BS. Fowlpox outbreaks in vaccinated flocks and isolation of virus strains. *Indian Vet Med J*. 1980;4:144–8.
8. Forrester DJ, Spalding MG. Parasites and diseases of wild birds in Florida. Gainesville: University Press of Florida; 2003.
9. Garg SK, Joshi HC, Shukla HC. Vaccination failure in fowlpox. *Indian J Vet Med*. 1984;4:60–1.
10. Jarmin S, Manvell R, Gough RE, Laidlaw SM, Skinner MA. Avipoxvirus phylogenetics: identification of a PCR length

- polymorphism that discriminates between the two major clades. *J Gen Virol.* 2006;87:2191–201.
11. Jenkins CD, Temple SA, van Riper III C, Hansen WR. Disease related aspects of conserving the endangered Hawaiian Crow. In: Cooper JE (ed) *Disease and management of threatened bird populations*. ICBP technical publication series no. 10, Cambridge, England; 1989, p. 77–87.
 12. Joshi RK, Shakya S, Gupta N. Isolation of fowl pox virus from fowl pox vaccinated layer flock. *Indian J Comp Microbiol Immunol Infect Dis.* 1995;16:139–40.
 13. Lee LH, Lee KH. Application of the polymerase chain reaction for the diagnosis of fowl poxvirus infection. *J Virol Methods.* 1997;63:113–9.
 14. Luschow D, Hoffmann T, Hafez HM. Differentiation of avian poxvirus strains on the basis of nucleotide sequences of 4b gene fragment. *Avian Dis.* 2004;48:453–62.
 15. Mehmood S. Pathological conditions affecting the domestic pigeons (*Columba livia*) in Jammu. M.V.Sc. thesis, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu, Jammu, India; 2017.
 16. Okwor EC, Eze DC, Chah KF. Recurring outbreaks of Fowl Pox in a poultry farm in Nsukka, Southeast Nigeria. *IOSR J Agric Vet Sci.* 2014;7:104–8.
 17. Pathak N. Prevalence, pathology and molecular diagnosis of pox in domestic birds. Ph.D. thesis submitted to Assam Agricultural University, Khanapara, Guwahati, India; 2016.
 18. Pawar RM, Bhusan SS, Poornachandar A, Lakshmikantan U, Shivaji S. Avian pox infection in different wild birds in India. *Eur J Wildl Res.* 2011;57:785–93.
 19. Singh A, Dash BB, Kataria JM, Dandapat S, Dhama K. Characterisation of an Indian isolate of turkey pox virus. *Indian J Comp Microbiol Immunol Infect Dis.* 2003;24(2):149–52.
 20. Tripathy DN, Reed WM. Pox. In: Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne SE, editors. *Diseases of poultry*, 11th edn. Iowa State Press, Ames; 2003, p. 253–269.
 21. van Riper III C, Forrester DJ. Fowl Pox. In: Thomas NJ, Hunter DB, Atkinson CT, editors. *Infectious diseases of wild birds*. Blackwell Publishing; 2008. p. 131–176.

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