RESEARCH ARTICLE

Phylogenetic Analysis of P4b Gene of Fowl Pox Virus in Naturally Infected Backyard Chicken

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Abstract

In the present study, fowl pox was diagnosed in 129 backyard chicken based on gross and histopathological lesions. Fowl pox is manifested by pock nodular lesions on featherless parts of body (cutaneous form) and purulent, cheesy patches in upper respiratory tract (diphtheritic form). Tissue samples revealed Bollinger bodies in epithelial cells on histopathology. Among these, extraction of DNA was carried out from cutaneous and diphtheretic lesions from 29 fowl pox affected chicken. Molecular diagnosis of fowl pox was performed by amplification of P4b gene of fowl pox virus using specific primers and obtained a desired amplicon of 578 bp. Nucleotide sequencing and phylogenetic analysis of four cutaneous fowl pox samples revealed close relation with the FPV isolates from Singapore, North America and USA (KY464130, MH175285 and KC017961 with an identity of 97.28%, 97.21% and 97.16% respectively) when compared to FPVs isolated from Portugal, Brazil, Jammu and India (KM974727, KX863706, MF496042 and HM481406 respectively with an identity of 96.66% to 96.86%).

Key words: Bollinger bodies, Fowl pox virus, P4b gene, Phylogenetic analysis.

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Introduction

Backyard poultry farming has been inevitable part of sustainable livelihood of Indian farmers and it provides not only food security but also employment to the farmers and rural youth (Singh *et al.,* 2021). Fowl pox is one of the important contagious diseases of poultry and is caused by *fowl pox virus* (double stranded DNA virus) belonging to the genus *Avipox virus,* subfamily *Chordopoxvirinae* and family *Poxviridae* (Luschow *et al.*, 2004; Adebajo *et al.*, 2012). Fowl pox disease manifestation involves both mild cutaneous (dry pox) and severe diphtheritic form (wet pox) of which the latter is particularly prevalent in young birds causing high mortality (Sharma *et al.*, 2019).

A presumptive diagnosis of avian pox can be made by the gross lesions on the body, but confirmation of avian pox is accomplished by microscopic examination for the characteristic Bollinger bodies, virus isolation by transmission of the organism via egg inoculation, serological tests and polymerase chain reaction (Hess *et al.,* 2011). Molecular detection of fowl pox virus is usually based on PCR amplification of the highly conserved gene locus, *i.e.,* P4b gene and it is proven to be the most sensitive diagnostic technique. Phylogenetic relationships of avian pox viruses are usually analyzed using the DNA sequences of the P4b core protein-coding gene with molecular weights of 75.2 kDa, which is also a useful and sensitive test to detect the avian pox viruses (Manarolla *et al.,* 2010).Hence the present study was undertaken to analyse the P4b gene of fowl pox virus in naturally infected backyard chicken.

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Materials and Methods

The present study on fowl pox in 129 backyard chicken was conducted at NTR College of Veterinary Science, Gannavaram (India) and the samples from both the cutaneous (102 cases) and diphtheretic (27 cases) form were procured from postmortems conducted at the Department of Veterinary Pathology during May 2022 to December 2022. Backyard chicken from different flocks were suspected for fowl pox and representative tissue samples (102 skin pock lesions, 8 from oesophageal, 7 from oral mucosa and 12 from tracheal tissue) were collected in 10%

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neutral buffered formalin for histopathological studies and 29 samples (21 cutaneous and 8 diphtheretic lesions) were also stored at -20°C for molecular studies.

Histopathology

Formalin-fixed tissues with a thickness of 2-3 mm were processed for the paraffin embedding technique. The tissue samples were washed in running tap water to remove formalin, processed in ascending grades of alcohol for dehydration and were cleared in xylol. The paraffin embedded tissues were sectioned about 3-4 µm thick using a semiautomatic microtome and were stained with routine hematoxylin and eosin as per the standard procedure (Luna, 1968).

DNA Isolation and PCR Amplification

The genomic DNA from pock lesions of fowl pox affected backyard chicken collected from different sources was extracted as per the manufacturer's protocol, using the "HiPurA® Mammalian Genomic DNA Purification Kit (Himedia). The forward and reverse primers designated as F (5'CAGCAGGTGCTAAACAACAA3') and R (5'CGGTAGCTTAACGCCG AATA3'), respectively, were used for the amplification of P4b gene of FPV ((Rajasekaran *et al.,* 2019). Initial denaturation was done at 98°C for 1 min followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 53°C for 10 sec, extension at 72°C for 30 sec then final extension was at 72°C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis with 1 kb DNA ladder.

Nucleotide Sequence Analysis and Phylogenetic Analysis

The PCR products of P4b gene of FPV obtained were sent for DNA sequencing. The sequencing was performed on automated sequencer (ABI 3730, Applied Biosystems) using Sangers dideoxy chain termination method at Bar Code Biosciences Pvt. Ltd (Bengaluru). The nucleic acid sequences of the P4b gene of FPV obtained were assembled for analysis using clustal W program as implemented in MEGA 11 and a consensus sequence of each sample was obtained. Consensus sequence thus obtained was submitted to GenBank (Submission I.D: 2658835) and was aligned with various published sequences of the P4b gene in GenBank using MEGA 11 software programme (Tamura *et al.*, 2021). For phylogenetic analysis, published sequences of complete P4b gene of FPV were retrieved from the BLAST search. Neighbour - joining tree was constructed using default option with 1000 bootstrap replications.

Results and Discussion

In the present study, 102 samples from cutaneous form of fowl pox were positive based on histopathology (100%) and 21 cases among these were confirmed by PCR. Only 24 cases (out of 27 suspected) were positive for diphtheritic form of fowl pox based on histopathology (88.8%) and 8 among these were confirmed by PCR. Backyard chicken affected with cutaneous form of fowl pox revealed pock lesions ranging from small nodules to large proliferative wart like projections on eyelids (Fig. 1), beak, comb, wattles, and feet, whereas nodular to purulent, cheesy patches at laryngeal orifice (Fig. 2), trachea, oral cavity and oesophageal tract were noticed in diphtheritic form. The gross lesions observed in present study were similar to findings reported by Biswas *et al.* (2011) and Sultana *et al.* (2019). Histologically, cutaneous pock lesions revealed ballooning degeneration of epidermal cells, hyperplasia of epidermis (acanthosis) in focal areas along with presence of medium to large single or multiple intracytoplasmic eosinophilic inclusion bodies (Bollinger

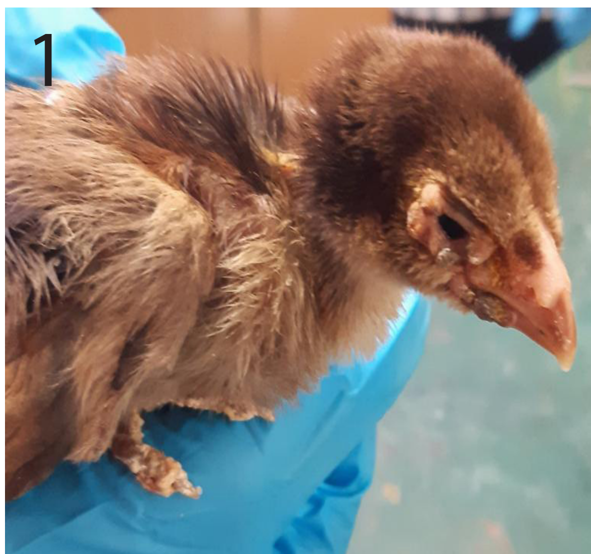

Fig. 1: Small to medium sized nodular pock lesions on face.

Fig. 2: Cheesy yellow plaques in oral cavity and oesophagus.

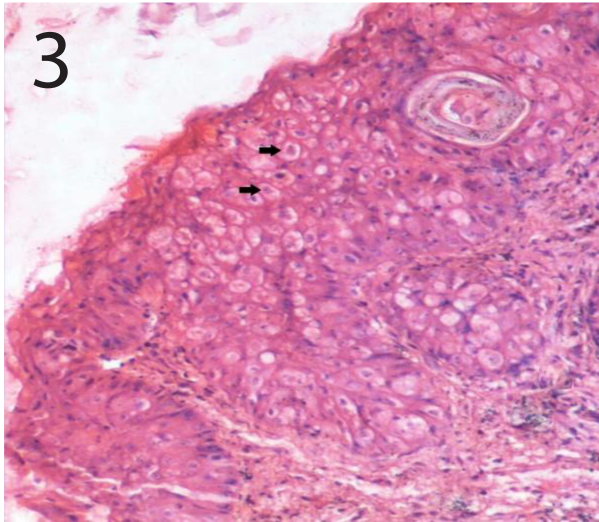

Fig. 3: Ballooning degeneration of epidermal cells along with presence of Bollinger bodies (H&Ex100)

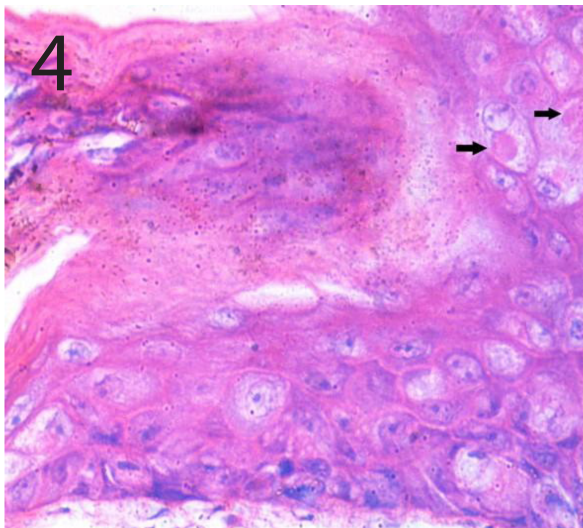

Fig. 4: Cellular swelling of epithelial cells of feather follicles along with presence of Bollinger bodies (H&Ex400).

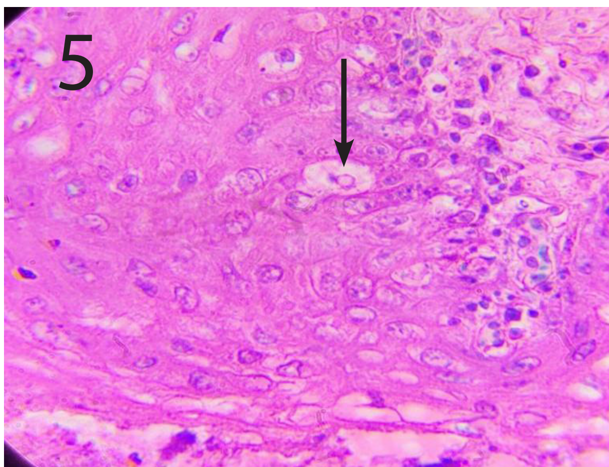

Fig.5. Hyperplasia of epithelium in mucosa along with presence of Bollinger bodies (arrow) in oesophagus (H&Ex400)

bodies, Fig. 3-5). Similar lesions were reported by Moayyedian *et al.* (2008) and Pandiyan *et al.* (2022).

Molecular diagnosis of fowl pox disease in backyard chicken was carried out by using primers specific for P4b gene of fowl pox virus. The genomic DNA extracted from skin , oesophagus and trachea were used for amplification. On electrophoretic analysis, an amplicon was obtained at 578 bp in all the samples confirming presence of fowl pox viral DNA in the samples (Fig. 6). Nayeri Fasaei *et al.* (2014), Ghalyanchilangeroudi *et al.* (2018) and Fallah Mehrabadi *et al.* (2020) obtained similar amplicon from skin and diphtheritic pock lesions.

Nucleotide Sequencing and Phylogenetic Analysis

The PCR amplicons of P4b gene of FPV obtained from cutaneous fowl pox samples (4) were sequenced by Sangers dideoxy chain termination method at Barcode Biosciences Pvt. Ltd, Bengaluru. All the sequences were aligned using clustal W program as implemented in MEGA 11. The sequence was submitted to the GenBank (Submission ID: 2658835). For phylogenetic analysis, published sequences of P4b gene of FPV were retrieved from BLAST search. The first sequence of the FPV published was regarding P4b gene and so the researchers designed primers depending on this gene as a conservative gene (Lee and Lee, 1997)

Fig. 6: Fowl pox virus- Agarose gel electrophoresis of amplified product of P4b gene, Lane 1- 100 bp DNA ladder, Lane 2 to 6- Cutaneous pock samples, Lane 7- Oesophageal pock sample, Lane 8,9 - Tracheal pock samples, Lane 10- Template control.

The BLASTn analysis of the present samples revealed close relation with the FPV isolates from Singapore, North America and USA (KY464130, MH175285 and KC017961 with an identity of 97.28%, 97.21% and 97.16%, respectively) when compared to FPVs isolated from Portugal, Brazil, Jammu Kashmir, India (KM974727, KX863706, MF496042 and HM481406, respectively, with an identity of 96.66% to 96.86%, 96.69 and 96.66 % respectively. The phylogenetic

analysis of partial sequences of P4b gene of FPVs obtained from four cutaneous fowl pox samples revealed that they were slightly divergent from FPV isolates in India (MF496042 and HM481406) available in the Database (Fig. 7). Nucleotide sequencing and phylogenetic analysis of P4b gene (*fpv* 167) could classify avipox viruses into three major clades as A (Fowl pox like viruses), B (Canary pox like viruses) and C (Psittacine pox like viruses) according to Jarmin *et al.* (2006) and Manarolla *et al.* (2010).

The nucleotide sequence of P4b gene of fowl pox isolates collected from West Bengal reported by Das *et al.* (2013) was found to be cognate (99% similarity) with fowl pox isolates from wild birds in India and Egypt (HM481404.1 and JQ665838.1). Meseko *et al.* (2017) found 100% similarity between the FPV isolates from Nigeria (KP987207 - KP987214) with vaccine virus strain (Baudette), whereas Sharma *et al.* (2019) found 99% relation between FWPV and TKPV based on phylogenetic analysis of P4b gene locus.

The *fpv140* gene sequence and phylogenetic analysis of (Sharkia2017/VSVRI) isolate confirmed that it was highly conserved and located within clade A 1 (Fowl pox viruses) according to Aboul Soud *et al*. (2020). Similarly, Hasan *et al.* (2021) found 100% similarity of aligned sequences of *fpv*167 loci of P4b gene (MN971579) with Iran and Egypt FPV isolates (KU522209.1 and JQ665838.1). Phylogenetic analysis of P4b gene of fowl pox isolates collected from Nagpur, India was closely related to fowl pox strains isolated from wild birds in India (Pandiyan *et al.,* 2022).

Conclusion

Histopathological studies of fowl pox lesion revealed characteristic intracytoplasmic inclusion bodies and fowl pox can be rapidly diagnosed by amplification of highly conserved P4b gene of fowl pox virus using specific primer. Nucelotide sequencing and phylogenetic analysis of P4b gene of FPV from the fowl pox isolates collected in and around Gannavaram showed close relation with FPV isolates from Singapore, North America and USA.

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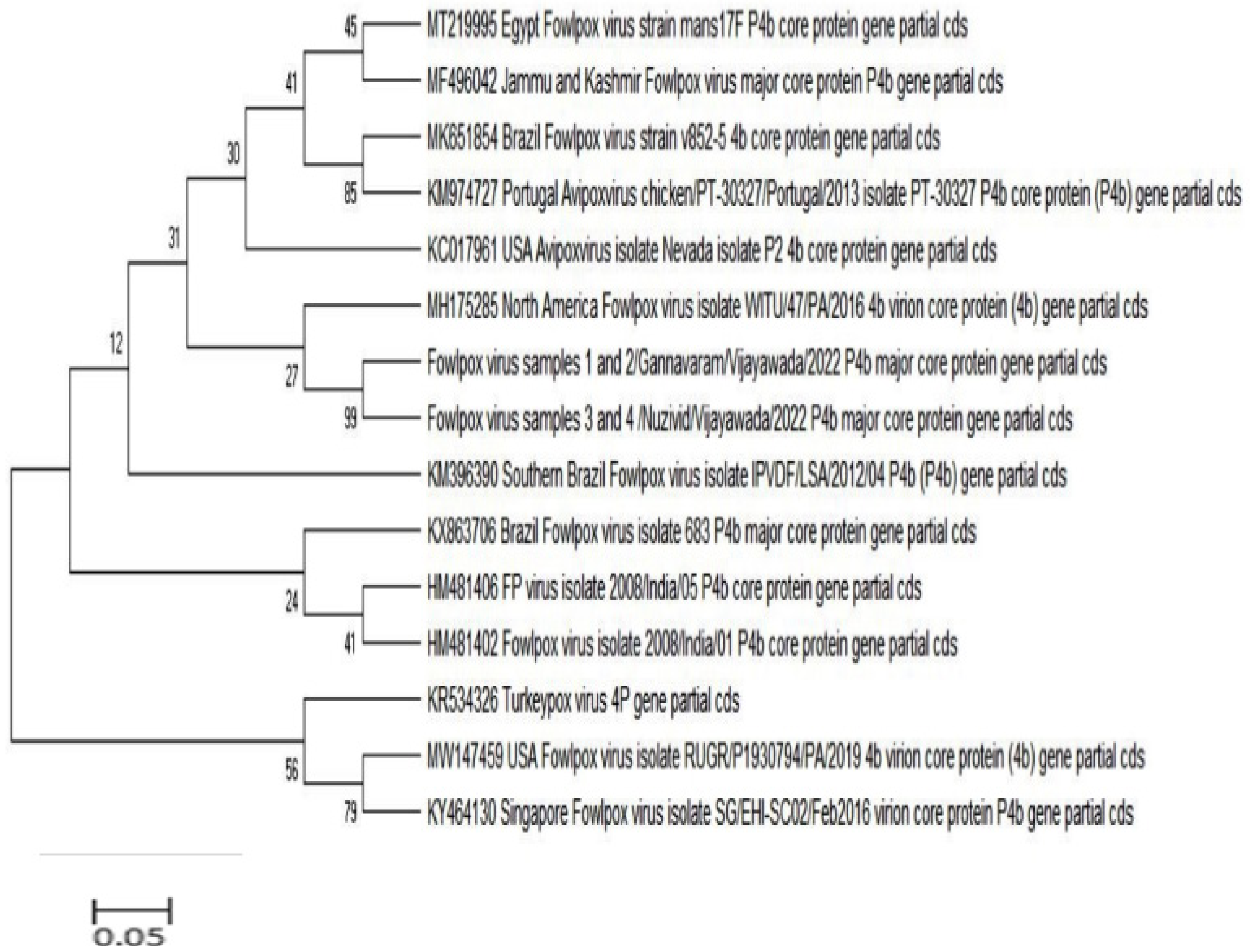

Fig.7: Phylogenetic analysis of nucleotide sequences of P4b gene of FPV samples (Bootstrapped neighbor-joining tree). Bootstrap values for 100 iterations as shown on branches.

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