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Study on Histopathological Changes Induced by Fowl Pox Virus (FPV) in Chorioallantoic Membrane (CAM) of Developing Chicken Embryo and Immunological Characterization of the Egg Adapted and Cell Culture Grown Virus

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Histopathological examination was conducted on the chorioallantoic membrane (CAM) of the embryonic chicken eggs (ECE) infected with a field isolate of the fowl pox virus (FPV). In infected CAM, the ectodermic cells appeared swollen and contour of the cells appeared rounded. Some of the cells revealed large vacuole while, other showed increased granularity of the cytoplasm and blood vessels appeared dilated at 6th passage. Ectoderm layer revealed hydropic degeneration with eccentric deposition of nucleus and formation of acidophilic intracytoplasmic inclusion bodies. At 10th passage mesoderm appeared intensively edematous with heavy infiltration of hetrophils and erythrocytes. Presence of FPV antigen could be easily detected in tissue sections. The antigen was mostly localized in the perinuclear area in the cytoplasm of the infected cells and the area showing intracytoplasmic inclusions were easily appreciable.To confirm the replication of virus in the CAM and cell culture, the 10% (w/v) suspensions of infected CAM and cell culture supernatants were subjected to Enzyme linked immune sorbent assay (ELISA) for detection of FPV antigen using antigen capture ELISA. The comparison of ELISA titers indicates that virus yield was better on CAM as compared to BGM-70 cells.

Key words: Fowlpox virus (FPV), Chorioallantoic membrane (CAM), Pock lesions, Acidophilic intracytoplasmic inclusion bodies, BGM-70 cells.

Avipoxviruses (APVs) are among the largest and most complex viruses known. APVs belong to the *Chordopoxvirinae* subfamily of the *Poxviridae* family[1]. They infect and cause disease in poultry, pet and wild birds of many species which results in economic losses to the poultry industry. Infections have also been reported in a number of endangered species or species in captive breeding recovery programs.[2,3] Latest case report of Fowlpox virus infection is found in Hungarian partridges (Perdixperdix)[4], white-tailed sea eagle (Haliaeetusalbicilla)[5], and Hungarian great tits (Parus major)[6].APVs are transmitted via biting insects and aerosols and are usually named on the basis of the bird species from which the

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virus was first isolated and characterized[3]. Interspecies transmission also occurs of fowlpox virus[7].The avipox virus appears incapable of causing disease in mammals; therefore there appears to be no zoonotic potiential[8]. 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[3,9]. Histopathology and electron microscopy are use to confirm the clinical diagnosis. It is necessary to naturally occurring dual infection of layer chickens with fowlpox virus and gallid herpesvirus 1 (infectious laryngotracheitis virus) which can be diagnose with the help of electron microscopy, PCR and histology[10]. The usefulness of the measurement of antibody titter to FPV by ELISA was evaluated in SPF chickens with or without inoculation with FPV[11,12]. An ELISA for the detection of antibodies to FPV was developed and compared with agar gel diffusion technique to examine serum from vaccinated chickens[13].FPV multiplies in cytoplasm of epithelial cells with the formation of large intracytoplasmic inclusion bodies known as Bollinger bodies that contain smaller elementary bodies (Borrel bodies) during Histopathological examination is characteristic of the disease[14]. The inclusions can be demonstrated in section of cutaneous and diphtheritic lesions by the use of haematoxylin and eosine (H and E), acridine orange or giemsa stain[15].With few exceptions the disease only causes drop in productive performance of birds. Mortality from primary infection of pox virus is uncommon. A recombinant fowlpox virus vaccine expressing key protective Mycoplasma gallisepticum antigens could facilitate in the prevention both of fowlpox virus and M. gallisepticum infections[16]. Pigeon pox virus may be used as first dose in chickens against fowl pox. This is preferred especially for layers to avoid any reaction[17].

MATERIALS AND METHODS

Histopathology of CAM

Infected CAM at each passage were collected on 6 day PI and kept in 10% neutral buffered formalin for 5 days. After 5 days formalin was replaced with 5% solution of neutral buffer formalin and allowed to fix further for a period of 10 days in the same solution. For processing, the membrane were cut in to small pieces and washed in running tape water overnight. The pieces of membrane were then dehydrated in ascending series of ethyl alcohol (50%, 60%, 70%, 80%, 90%, and absolute alcohol). The tissue was then cleaned in xylene and embedded in paraffin wax. Membranes were then sectioned at 5-6µ and 2-3 such sections were lifted on slides. Several sets of such slides were prepared. The slides were then stained with usual Haematoxyline and Eosine (H&E) staining procedure as described by Lillae[18] and mounted with DPX. The slides were then examined under light microscope for cellular changes produced by FPV on CAM.

Immunoperoxidase staining:

Serial 3-4 mm thick section of virus infected CAM from each paraffin block were mounted on slides and allowed to dry overnight. The section were deparaffinized on hot plate. Slides of CAM section were kept in xylene I for 15 min followed by xylene II for 15 min. Tissues were rehydrated in a graded series of ethanol solution (100%, 95%, 70%, 50%), and finally washed with deionized water. The rehydrated sections were then placed in a plastic coplin jar containing 0.01 M Sodium citrate buffer (pH 6.0). The tissue slides were dipped in 2.5% trypsin for 1 hr at 37^0C followed 5 min of washing in 0.05 M Tris-buffer-saline (TBS) solution (pH 7.6) containing 0.05% Tween 20 (TTBS), The sections were then incubated in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase. After a brief wash of TTBS, nonspecific binding was blocked by bathing in normal poultry serum in (1: 10 dilution in TBS) for 10 min. The tissues were then incubated with hyper immune serum (1: 3,200 dilutions in TBS) for 30 min. The slides were incubated with conjugate for 30 min. After a brief wash with TTBS, the slides were incubated with substrate for 10 min. After a last wash in TTBS, slides were then rinsed with distilled water and counterstained with haematoxylene for 3 min. The Slide was rinsed in running tap water for 5 min, and mounted in 50% glycerol in PBS and examined under light microscope.

Enzyme linked immune sorbent assay

The FPV antigen was detected in FPV infected CAM suspensions and infected cell

culture supernatants using indirect Enzyme Linked Immune Sorbent Assay (ELISA) as per the method described by Sharma[19].

Before starting the test, appropriate dilutions of hyper immune serum and conjugate were determined by the checker board titration method. The hyper immune serum of FPV was diluted 1:10 and 50µl of diluted hyper immune serum was added to each well of 96 well ELISA plate in triplicate. The plate was kept at 4^0C overnight and then washed thrice with PBS-Tween-20 (PBS-T) for 5 min each and tapped thoroughly. The unreacted sites were blocked by addition of 50µl of 2% Bovine Serum albumin (BSA) solution for 1 hr at 37^0C. The plate was again washed thrice in PBS-T and tapped. The supernatant collect at each passage was serially diluted 10 fold in carbonate-bicarbonate buffer and 50µl quantity of each dilution was added to respective wells and the plates was incubated at 37^0C for 2 hr. The negative control wells received PBS-T in place of antigen sample. The plate was then washed thrice with PBS-T. The 50µl of hyper immune sera was again added in each well and the plate was incubated at 37^0C for 2 hr followed by three washing with PBS-T and tapping. The 50 µl of anti-chicken horse radish peroxidase (HRP) conjugate (Genei) initially diluted to 1:20,000 in PBS-T was added in each well and the plate was incubated at 37^0C for 1 hr. The unbound conjugate was removed by three washing of PBS-T and subsequent tapping. The freshly prepared substrate H_2O_2- TMB (tetra methyl benzidene) prepared in citrate buffer was then added in each well in 50µl quantity and the plate was incubated at 37^0C for 20 min in dark. The reaction was stopped with addition of 50µl of the stopper solution i.e. 1M H_2SO_4, in each well and the absorbance of each well was recorded at 492 nm wave length in ELISA plate reader. The mean values were calculated and double the mean OD value of negative wells was taken as positive cut off point as recommended by Garett[20].

RESULTS

Histopathology of CAM

The infected CAM harvested at different passage levels, were subjected to Histopathological examination following H and E staining. Microscopically the ectodermal cells appeared swollen at many places and the contour of the cells appeared rounded. Some of the cells revealed a large vacuole while other shows increased granularity of the cytoplasm. The blood vessels appeared dilated and congested at 6^{th} passage in the epithelial cells. The ectoderm layers revealed intense hydropic degeneration with eccentric deposition of the nucleus and formation of acidophilic inclusion bodies. Some of the cells were ruptured to leave a large cleft. The mesoderm was moderately edematous and infiltrated with hetrophils and few erythrocytes. The endoderm, formation of vacuoles was evident in the epithelial cells. At the 10^{th} passage mesoderm appeared intensively edematous with heavy infiltration of hetrophils and erythrocytes. The blood vessels were greatly dilated and congested. There was infiltration of mono nucleus cell. The endodermal layer persist moderate degree of degenerative changes with occasional vacuole formation.

Immunoperoxidase staining

The presence of FPV antigen could be easily detected in the tissue sections in all the passages. However the distribution was more

Table 1. ELISA and Infectivity titers of FPV in CAM and BGM-70 cell line

Passage No.	CAM of ECE		BGM-70 Cells	
	ELISA Titers (log_{10}/50µl)	Infectivity titers (EID_{50}/0.1 ml)	ELISA Titers (log_{10}/50µl)	Infectivity titers ($TCID_{50}$/0.1 ml)
P_3	3.94	$10^{2.43}$	3.81	$10^{3.0}$
P_5	6.82	$10^{3.30}$	7.76	$10^{3.6}$
P_8	8.33	$10^{3.50}$	ND	ND
P_{10}	10.67	$10^{4.10}$	ND	ND

ND - Not done.

pronounced with the increase in number of passages. The antigen was mostly localized in the particular area in the cytoplasm of the infected cell and the area showing intracytoplasmic inclusions were easily appreciable.

Enzyme linked immunosorbent assay (ELISA)

To confirm the replication of virus in the CAM and cell culture, the 10% (w/v) suspensions of infected CAM and cell culture supernatants were subjected to ELISA for detection of FPV antigen using antigen capture ELISA. The double of the mean value of negative wells OD was taken as positive cut off value which was calculated to be 0.231 for this study. The ELISA antigens titers of infected CAM was recorded to be 3.94 $\log_{10}$/50µl at 3rd passage followed by, 6.82, 8.33 and 10.67 $\log_{10}$/50µl at 5th , 8th, and 10th passage respectively (Table-1).

In cell culture supernatants the mean antigen titer was found to be 3.81 $\log_{10}$/50µl and 7.76 $\log_{10}$/50µl at 3rd and 5th passage respectively.

DISCUSSION

The Histopathological examination of paraffin embedded sections of infected CAM stained with H and E staining, ectodermal cells appeared swollen with rounded contour of the cells. The blood vessels appeared dilated and congested. The ectoderm layers revealed intense hydropic degeneration with eccentric deposition of the nucleus and formation of acidophilic inclusion bodies. Some of the cells were ruptured to leave a large cleft. The mesoderm appeared intensively edematous with heavy infiltration of hetrophils and erythrocytes. The blood vessels were greatly dilated and congested. There was infiltration of mono nucleus cell. The endodermal layer persist moderate degree of degenerative changes with occasional vacuole formation. The changes observed in present study are in conformity with those reported by Tripathy[3] who also found marked cellular hyperplasia and eosinophilic intracytoplasmic inclusion bodies within enlarged cells in H and E stained preparations of infected CAM. In a similar study Buddingh[21], also reported marked hyperplasia of the epithelial cells of the ectoderm and presence of characteristic inclusion bodies in the swollen cells. Yoshikkawa& Alam[22]and Beytut& Haligur[23]reported that on histological examination revealing the presence of Bollinger bodies, confirmed the suspicion of fowlpox, which are characteristic of this viral infection. These inclusion bodies cause cytoplasmic distention, resulting in cell necrosis.

The growth and replication of FPV isolates on CAM was further confirmed by detection of virus specific antigen in the sections of FPV infected CAM by immune peroxidase staining. The immuno peroxidase staining has been extensively used for detection of FPV antigen in the infected tissue sections byWilliams[24]and Tripathy[15]. Sinclair[25] reported that the immuno peroxidase staining offers several advantages over immunoflorscence in localization of tissue antigen. These include use of microscope with the conventional light source, simultaneous visualization of tissue antigens and tissue structures, the possibility of retrospective examination and permanency of preparations. In present study, the presence of FPV antigen could

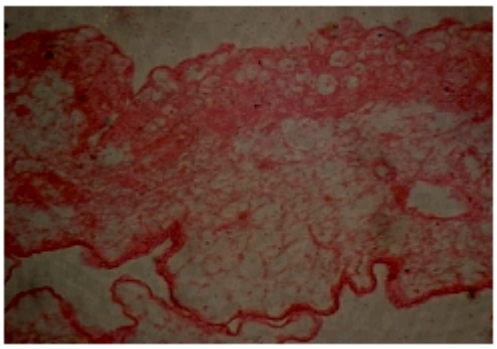

Fig. 1. Histopathological changes induced by FPV in CAM (H and E X 400 X)

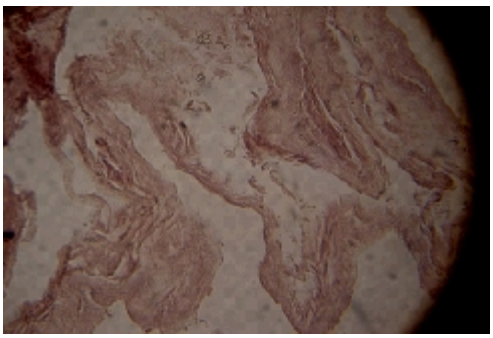

Fig. 2. Immunoperoxidase staining of paraffin embedded FPV infected CAM sections.

be detected in the paraffin embedded tissue sections. The antigen was mostly found localized in the perinuclear area in the cytoplasm of the infected cells. The area showing intracytoplasmic inclusion bodies were easily appreciable along with other histopathological changes. Tripathy[15] used immune peroxidase technique for the detection of FPV antigen in the formalin fixed and paraffin embedded sections of FPV lesions. Similarly, Williams[24]also detected presence of FPV antigen within the histiolymphocytic inflammatory cells in the formalin fixed, paraffin embedded sections of CAM inoculated with FPV.

The quantitative estimation of FPV antigen was done in the infected CAM homogenates and infected BGM-70 cell supernatants at different passages using liquid phase indirect capture ELISA. The ELISA titers were recorded to be 3.94 $\log_{10}$/50µl at 3rd passage, 6.82 $\log_{10}$/50µl at 5th passage, 8.33 $\log_{10}$/50µl at 8th passage and 10.67 $\log_{10}$/50µl at 10th passage in CAM while it was 3.81at 3rd passage and 7.76 at 8th passage in BGM-70 cells. The comparison of ELISA titers indicates that virus yield was better on CAM as compared to BGM-70 cells. ELISA was reported to be 400-800 times more sensitive than AGPT described by Zhang[13]. Weil[26] also used ELISA for measuring the antibody response in the chicken inoculated with Avipox virus isolated from wild birds. Li[27] used an indirect ELISA using this anti-FPV140 polyclonal antibody was capable of distinguishing avian FPV isolates from other common avian pathogens such as mycoplasma gallisepticum, infectious laryngotracheitis virus, avian influenza virus, infectious bursal disease virus, and avian infectious bronchitis virus. This ELISA will serve as a useful diagnostic tool for the detection of FPV in clinical samples. An indirect-enzyme linked immunosorbent assay (ELISA) was used by Pásztor[28] to determine the levels of antibody specific to Culex quinquefasciatus mosquitoes (Cxq) and FPV proteins.

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REFERENCES

1. Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U. and Ball, L.A. Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses". Elsevier Academic Press.2005.
2. Bohls, R. L., Linares, J. A., Gross, S. L., Ferro, P. J., Silvy, N. J. Collisson, E.W. Phylogenetic analyses indicate little variation among reticuloendotheliosis viruses infecting avian species, including the endangered Attwater's prairie chicken. *Virus Res*., 2006; **119**:187-94.
3. Tripathy, D. N., Schnitzlein W. M., Morris, P. J., Janssen D. L., Zuba, J. K., Massey, G. Atkinson, T. Characterization of poxviruses from forest birds in Hawaii. *J. Wild L. Dis*., 2000;**36**: 225-30.
4. Brower, A.I., Cigel, F., Radi, C., Toohey-Kurth, K. Beak necrosis in Hungarian partridges (Perdixperdix) associated with beak-bits and avian poxvirus infection**.** *Avian Pathol*., 2010; **39:**223-5.
5. Saito K, Kodama A, Yamaguchi T, Gotoh Y, Sakai H, Fukushi H, Masegi T, Yanai T: Avian poxvirus infection in a white-tailed sea eagle (Haliaeetusalbicilla) in Japan**.** *Avian Pathol*.,2009;**38:**485-9
6. Palade E.A, Biro N, Dobos-Kovács M, Demeter Z, Mándoki M, Rusvai M: Poxvirus infection in Hungarian great tits (Parus major): case report.*Acta. Vet. Hung*., 2008, **56:**539-46
7. Terasaki, T., Kaneko, M., Mase, M. Avian poxvirus infection in flamingos (Phoenicopterusroseus) in a zoo in Japan. *Avian Dis.*, 2010;54:955-7.
8. Jarmin, S., Ruth,M., Richard,E.G., Stephen,M.L.,Michael,A.S. Avian poxvirus Phylogenetics: Identification of a PCR length polymorphism thatdiscriminates between the two major clades. Journal of General Virology, Central Veterinary Laboratories: Weybridge, UK. 2006; 87: 2191-2201.
9. Bollinger, O. UeberEpitheliomacontagiosumbeimhavshuhn und die Sogenanntenpocken des Geflugels. *Archive fur pathologischeAnatomie und Physiologie und fur klinishemedizin*., 1873; pp 349-361.
10. Diallo, I.S., Taylor, J., Gibson, J., Hoad, J., De Jong, A., Hewitson, G., Corney, B.G. Rodwell, B.J. Diagnosis of a naturally occurring dual infection of layer chickens with fowlpox virus and gallidherpesvirus 1 (infectious laryngotracheitis virus).*Avian Pathol.*, 2010; **39:**25-30.
11. Iritani, Y., Sawaguchi, K., Measurement of

antibody titre to fowl pox virus by enzyme immunosorbent assay. *J. Vet. Med. Sci*., 1994; **56**(6): 1191-1193.

12. Isa, G., Pyister, K., Kaaden, O. R. Czerny, C. P. Development of a monoclonal blocking ELISA for the detection of antibodies against fowl pox virus. *J. Vet. Med. Ser. B*., 2002; **49**(1): 21-23.
13. Zhang, Tiyin., Li, Junwei., Sun, Lei., Sun, Xuetlui., Zhang, Rukuani Liu, Xiufan. Development of indirect ELISA for the detcction of antibodies to fowl pox virus. *Chinse J. Vet. Sci*., 2005; **25**(2): 128-130.
14. Alehegn, E., Chanie, M., Desalegne.,Mengesha, D. A Systematic Review of Serological and Clinicopathological Features and Associated Risk Factors of Avian Pox*Brit. J. Poult Sci.*, 2014; 3 (3): 78-87.
15. Tripathy, D. N., Hanson, L. E., Killinger. A. H. Immunolperoxide technique for detection of fowl pox antigen. *Avian Dis.*, 1973; **17**: 274-278.
16. Zhang, G.Z., Zhang, R., Zhao, H.L., Wang, X.T., Zhang, S.P., Li, X.J., Qin, C.Z., Lv, C.M., Zhao, J.X., Zhou, J.F.A safety assessment of a fowlpox-vectored Mycoplasma gallisepticum vaccine in chickens.*Poult Sci*., 2010;89:1301-6.
17. *Ganguli, S.*Developing Trends in Livestock and Poultry Vaccines:*A Rev ResJ.Ani. Vet. Fish. Sci.*, 2013 1(3), 25-27
18. Lillie, R. D. Histopathological technique and practical histochemistry. *The blakinston Co., Inc., Newyork, USA.*1965.
19. Sharma, B., Negi, B. S., Yadav, M. P., Shankar, H. Pandey, A. B. Application of ELISA in the detection of goat pox antigen and antibodies. *Acta.Virol*., 1988;**32**: 65-69.
20. Garrett, J.K., Davis, R.B., Ragland,W.L., Enzyme-linked immunosorbent assay for detection of antibody to avian encephalomyelitis virus in chickens. *Avian Dis*., 1984; 28:117–130.
21. Buddingh, G. J. (1938). A study of behavior of fowl pox virus modified by intracerebral passage. *J. Exp. Med*., **67**:921.
22. Yoshikkawa MGT, Alam J. Histopathological studies of fowl pox in bantams. *Inter. J. Poult. Sci..*, 2002; 1(6):197- 199.
23. Beytut E, Haligur M. Pathological, immunochemical, and electron microscopic findings in the respiratory tract and skin of chickens naturally infected with avipoxvirus. *Turk. J. Vet. Ani. Sci.*, 2007; 31(1):1-7.
24. Williams, S. M., Smith, J. A., Garcia, M., Brinson, D., Kiupel, M. andHofacre, C. Severe histiolymphocytic and heterophilic bronchopneumonia as a vaccination in broiler chick's reaction to in ovofowlpox. *Vet. Pathol., 2010;***47**:177.
25. Sinclair, R. A., Burns, J. and Dunnill, M. S. Immunoperoxidase staining of formalin-fixed,paraffin-embedded, human renal biopsies with a comparison of the peroxidase-antiperoxidase (PAP)and indirect methods. *J. Clin. Pathol*.,1981; **34**:859-865.
26. Weli, S. C., Nilssen, O., Traavik, T. Morphogenesis of fowlpox virus in a baby hamster kidney cell line. *Med Electron Microsc.*, 2004; 37:225-235
27. Li, G., Hong, Q., Ren, Y., Lillehoj, H.S., He, C., Ren, X. Development of FPV140 antigen-specific ELISA differentiating fowlpox virus isolates from all other viral pathogens of avian origin. *Poult Sci.*, 2012;91(10):2507-11.
28. Pásztor, J.M. Avian Antibody Responses to Fowl pox virus and a Mosquito Vector. Thesis Department of Animal Sciences College of Agriculture, Human, and Natural Resource Sciences Washington State University 2009; pp 6-20.