JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2015. Vol. **9**(1), p. 169-174

Propagation of a Field Isolate of Avian Pox Virus on Chorioallantoic Membrane of Developing Chicken Embryo and in BGM-70 Cell Line

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(Received: 06 April 2014; accepted: 19 August 2014)

A field isolate of Fowl pox virus (FPV) was grown successfully on chorioallantoic membrane (CAM) of developing chicken embryo and Baby Gravid Monkey Kidney cell line (BGM-70 cells), as evident by different degree of Cytopathic effects (CPE). Studies were conducted on the pathogenicity of these viruses in these different host systems which produced pock lesions in CAM infected with FPV with extensive congestion and foci of hemorrhages covering entire inoculated site. The cytopathic effects in infected BGM-70 cell monolayers consisted of rounding and clumping of cells, vacuolation, macro and micro plaques and intracytoplasmic inclusion bodies. The cytopathic effects were seen in both unstained as well as MGG stained monolayers of BGM-70 cells

Key words: Fowlpox virus (FPV), Chorioallantoic membrane (CAM) Pock lesions, Cytopathic effects (CPE), BGM-70.

Avian pox is an infectious, slow spreading viral disease that has been reported in numerous species of birds including poultry, wild and caged birds[1, 2.] Avian pox viruses (APVs) are the members of genus *Avipoxvirus* under the subfamily *Chordopoxvirinae* of family *Poxviridae*. There is four strains of avipoxviruses (APVs) viz. FPV (Fowlpox Virus), TPV (Turkey pox virus), QPV (Quail pox virus) and PPV (Pigeon pox virus). Fowlpox is a serious disease of poultry that has occurred worldwide for centuries. Most commonly reported in songbirds, upland game birds, marine birds and birds of prey[3]. Recent phylogenetic studies of APV isolates based on this locus[4,5] indicated that most isolates clustered around either CNPV or FPV, while another study based on the same locus demonstrated a third cluster, from psittacine birds[6]. Avipoxviruses are

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large, oval-shaped enveloped viruses with a genome consisting of double stranded DNA ranging in size from 260 to 365 kb[7]. There are two form of Fowlpox, the first is cutaneous form and other is diphtheritic form[8]. Cutaneous form of Fowlpox seldom presents a diagnostic problem. The diphtheritic form is more difficult to diagnose because it can occur in the absence of skin lesions and may be confused with vitamin A deficiency and several other respiratory diseases caused by viruses. Histopathology and electron microscopy are use to confirm the clinical diagnosis. The virus can be isolated by the inoculation of avian cell cultures or the chorioallantoic membrane of embryonated eggs. It is necessary to naturally occurring dual infection of layer chickens with fowlpox virus and gallidherpesvirus 1 (infectious laryngotracheitis virus) which can be diagnose with the help of electron microscopy, PCR and histopathology[9]. A recombinant fowlpox virus vaccine expressing key protective Mycoplasma gallisepticum antigens could facilitate in the prevention both of fowlpox virus and M. gallisepticum infections[10]. Pigeon pox virus may be used as first dose in chickens against fowl pox. This is preferred especially for layers to avoid any reaction[11,12]. Interspecies transmission also occurs of fowlpox virus[13]. Latest case report of Fowlpox virus infection is found in pigeon[14], Hungarian partridges (Perdixperdix) [15], white-tailed sea eagle (Haliaeetusalbicilla) [16], and Hungarian great tits (Parus major)[17].

MATERIALS AND METHODS

A local isolate of APV, maintained in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, N.D.University of Agriculture and Technology, Kumarganj, Faizabad was used.

The APV hyper immune serum raised in cockerels using egg adapted live virus vaccine (Vankies) by Yadav[18]was used.

The fertile white leghorn chicken eggs were procured from the poultry unit of college of Veterinary science and A.H. The eggs were cleaned with 70% alcohol and incubated at 37^0C in the egg incubator under strict hygienic conditions and candled daily after 5th day until used for virus inoculation. The FPV scab samples collected from infected birds and preserved in glycerin saline were washed in PBS and triturated using sterile sand and chilled sterile PBS in pestle and mortar, to make a 20 per cent (w/v) suspension and subjected to three cycles of freezing and thawing. The suspension was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected. Antibiotics, penicillin (@100 IU/ml) and streptomycin (@100 mg/ml) were added and the resultant suspension was used for inoculation into embryonated chicken eggs (ECE).

The virus suspensions thus prepared were tested for FPV antigen in Agar gel immune diffusion (AGID) test using FPV specific anti sera in. The AGID was used to presence of cross reaction antibodies to fowlpox virus in the sera[19] and were processed further for isolation and characterization. The supernatant was further diluted to give a 10% (V/V) suspension, filtered through 0.45 µm membrane filter and used as inoculum for virus isolation in embryonated chicken eggs and cell culture.

The field isolate of fowlpox virus was inoculated on chorioallantoic membrane of 9-11 day old ECE using the method of Lennette[20]. The eggs those died at first 48 hours were discarded and remainders were observed for 6 days. On 6th day Post inoculation(PI), the CAM was collected, washed in PBS and examined for presence of pock lesion. The CAM showing changes were collected and stored -20^0 C till further use.

Baby gravid monkey kidney cell line (BGM-70) available in the Department was used. The cells were grown in Dulbaccos Minimum Essential Medium(DMEM) supplemented with 10% fetal calf serum and sub cultured every 5 to 7 days. The maintenance media consisted to DMEM supplemented with 2-5% fetal calf serum.The confluent monolayer in culture bottles and Leighton tubes were infected with 1.0 ml and 0.1 ml of virus inoculum respectively. The infected monolayerwere incubated at 37^0C and examined at every 12 hours interval for virus specific cytopathic effects for a period of 5 days. The cells were then frozen and thawed thrice and then the suspension was centrifuged at 4000 rpm for 30 minutes. The supernatant was collected, treated with Penicillin @100 IU/ml and Streptomycin@ 100 mg/ml, filtered through 0.45µm membrane filter and stored until used as for subsequent passage.

The BGM-70 cells grown on coverslips in Leighton tubes were infected with 0.1 ml of fowl pox virus along with controls. After 45 min of adsorption at 37^0 C, 2 ml of maintenance medium was added in each tube. The coverslips were removed at 24 hrs intervals up to 168 hrs (PI), fixed and stained with May-Grunwald and Giemsa (MGG).The cells were fixed in absolute methyl alcohol for 5 min and stained as per the method described by Garg and Mayer[21].

RESULTS AND DISCUSSION

A local field isolate of fowl pox virus isolated from a natural outbreak was successfully cultivated on chorioallantoic membrane (CAM) of developing chicken embryo. Initially no clear cut evidence of virus growth was evident up to 2nd passage except slight congestion and moderate thickening of the CAM at the site of inoculation. At the 3rd passage the inoculated area was hemorrhagic and a coalesced mass of whitish grey material covering the entire inoculation site was noticeable. At 4th passage distinct whitish grey pock lesions with 0.5 to 2 mm in diameter were observed. The pocks were isolated, diffused, firm and necrotic and were mostly present along with blood vessels. Few whitish grey elevated patches were also observed at the secondary sites. At 5th passage onwards the pock lesion became more discrete with extensive congestion and foci of hemorrhages covering entire inoculated site. The secondary pock lesions were comparatively small and few in number. On subsequent passages the secondary pock lesions progressively regressed and no secondary lesions were observed at secondary sites at 8th passage.

To develop an alternate method of cultivation of FPV, the field isolate was grown in BGM-70 cells.The growth in BGM-70 cell monolayers was evidenced by appearance of characteristic cytopathic effects and production of intra cytoplasmic inclusion bodies. The growth was evident after 4th passage in the form of cellular clumping and increased granularity of the cells. At 4th passage, the CPE further intensified and involved almost entire cell monolayer. CPE included cellular granularity, cellular clumping, increased refractivity of the cells and formation of micro and macro plaques. In MGG stained preparations, the changes were appreciable at 4th passage in the form of rounding and clumping of cells, cellular vacuolation, and presence of intra cytoplasmic inclusions and formation of micro and macro plaques. Overall 5 passages were given in BGM-70 cells.

Keeping these effects in view present study was undertaken to cultivate and characterized a local field isolate of FPV recovered from fowl pox outbreak in a poultry flock previously vaccinated with live fowl pox vaccine. The field virus was inoculated onto CAM of developing

Table 1. Changes observed in CAM infected with FPV.

Cytopathic effect	P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8	P-9	P-10
Congestion of CAM	-	+	+	+	+	+	+	+	+	+
Thickening of CAM		+	+	+	+	+	+	+	+	+
Hemorrhages	-	-	+	+	+	+	+	+	+	+
Primary pock lesions	-	-	+	+	+	+	+	+	+	+
Secondary Pock lesions	-	-	-	+	+	-	-	-	-	-

Table 2. Cytopathic effects in FPV infected and MGG stained BGM-70 cells

Cytopathic effect	P-1	P-2	P-3	P-4	P-5
Cellular Rounding	-	+	+++	++++	++
Cellular Clumping	-	-	++	+++	++++
Cellular vacuolation	-	-	+	++	++
Intra cytoplasmic inclusions	-	-	-	++	+++
Plaques	-	-	-	+	+++
Extent of CPE	-	+	++	+++	++++

chicken embryos. There was little evidence of virus growth up to 2nd passage other than slight congestion in the inoculated area (fig-1). However, at 3rd passage the inoculated area appeared hemorrhagic with coalesce mass of white grey material. At 4th Passage, the CAM was greatly hemorrhagic and whitish grey pock lesions with a diameter of 0.5 to 2 mm were observed throughout the inoculated site. The pock lesions were isolated, diffused, firm and necrotic and were mostly present along with the blood vessels.The four strains of avipoxviruses (APVs) viz. FPV, TPV, QPV and PPV were passaged in susceptible embryonated chicken eggs, showed edematous thickening and diffused pock lesions in CAM at higher dilutions (10-4/10-5) of second passage level, while clear and distinct pock lesions were observed at the lower dilutions (10-1/10-2) of the same passage level[22].The FPV are reported to multiply on CAM of developing chicken embryo resulting in the formation of compact proliferative pock lesions that are sometime focal or diffuse[5]. CAM is commonly used for initial isolation of FPV[23]. Since then, it became the medium of choice for cultivation of FPV because the virus was earlier regarded as highly host specific and was believed to replicate only in avian cells[24,25,26]. The FPV was reported to produce characteristic pock lesions on the CAM. A field isolates to produce pock lesions that were distinct and fully developed within 5-6 days of incubation after inoculation of embryos by CAM method[27]. SimilarlyCAM was used to grow the Avipox virus isolated from wild birds and it produced characteristic pock lesions[5, 28].

To develop an alternate method of cultivation of FPV, the field isolate was grown in BGM-70 cells. The field isolate did not show apparent changes up to 3rd passage in unstained

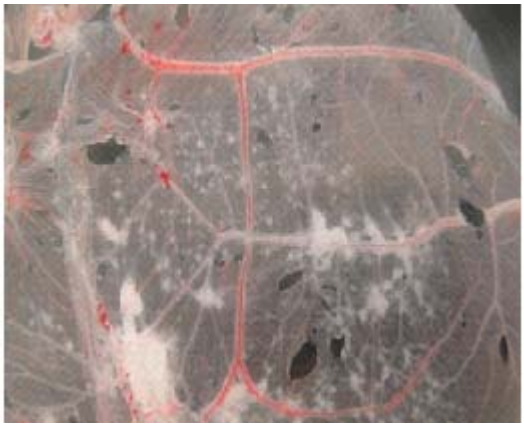

Fig. 1. FPV infected CAM showing discrete pock lesions at primary and secondary sites.

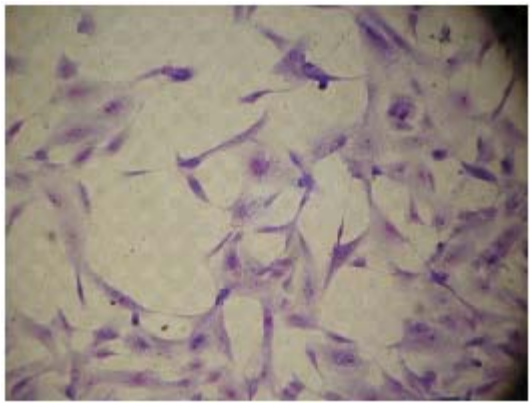

Fig. 2. FPV infected BGM- 70 Cells showing intracytoplasmic inclusions, vacuolation and plaques. (MGG staining)

monolayers however, there was little evidence of CPE in the form of rounding of cells at isolated places in MGG stained cells (fig- 2). At 4th passage the cellular clumping and marked increased in granularity of the cells was evident. The CPE became more pronounced and involved almost entire cell monolayer. The changes included cellular granularity, cellular clumping and increased refractivity of the cells and formation of micro and macro plaques. Similar changes were also reported earlier using cell lines of mammalian origins[18, 29, and 30]**.** It is also reported that the appearance of cytopathic effects only after 3 blind passages in BGM-70 cells[10.] A permanent cell line of avian origin QT-35 wasalso used for the propagation of avipoxviruses. In QT-35, the characteristics CPE consisting of rounding,vacuolation of cells, presence of intracytoplasmicinclusions and finally plaque formation was observedin all the four strains of APVs within 3-4 days postinoculation[22]. However it is also found that found no CPE in BHK-21 as well as Vero cells infected with FPV except rounding of the cells during 10 initial passages[30]. In present study, the changes in MGG

stained Monolayers included rounding and clumping of cells, cellular vacoulation, formation of micro and macro plaques and presence of intracytoplasmic inclusion bodies in the infected cells. Sometimes single cell contained many inclusion bodies. Similar changes were also reported by using BGM-70 cells[18].

Conflict of interest

Authors hereby declare that there is no conflict of interest in manuscript.

ACKNOWLEDGMENTS

The authors are thankful to Dean, College of Veterinary Science and Animal Husbandry, NDUAT, Kumarganj, Faizabad for providing the funding and facilities to carry out the work.

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