



## Cell culture adaptation of Avipox viruses isolated from different species of birds

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### ABSTRACT

Avianpox (AP) is an infectious, slow spreading viral disease that has been reported to affect numerous species of birds including poultry. There is very limited information available regarding the molecular and biological characteristics of the avipox viruses (APVs) circulating in India. In the present study, APVs from fowl [FP/As-K(R)], pigeon [P2/As-K9(R)] and duck [D2/As-N] origin isolated from natural outbreaks of the disease in different areas of Assam were selected for adaptation in different cell culture. All the three isolates were propagated in chicken embryo fibroblast (CEF) primary cell culture and Vero cell line and their propagation was confirmed by observing virus specific cytopathic effects (CPE) and by performing PCR targeting the *4b* gene of APV. All three isolates were adapted in the CEF primary culture with production of virus specific CPE. However, a high degree of CPE was observed in the cultures infected with the fowl isolate from an earlier passage (P-11) in comparison to the other two isolates. Even the time required for completion of CPE was considerably less in case of the fowl isolate. The log TCID<sub>50</sub> of the fowl isolate was found to be  $4.18 \pm 0.11$ ,  $5.29 \pm 0.06$  and  $6.29 \pm 0.13$ , respectively in the 5<sup>th</sup>, 15<sup>th</sup> and 20<sup>th</sup> passage, which was higher in comparison to the other two isolates. Interestingly, none of the isolates showed any virus specific CPE in vero cell line on propagation till the 10<sup>th</sup> passage. Moreover, all the passages were found to be negative by PCR.

**Keywords:** Avianpox, Chicken embryo, Chicken embryo fibroblast (CEF), Cytopathic effects (CPE), PCR

The poultry industry in India has registered a phenomenal growth during the last few decades. It is one of the fastest growing segments of the agricultural sector in India today (Chatterjee and Rajkumar 2015). Assam being a state where majority of the population is non-vegetarian, there is a huge demand for eggs and poultry in the form of meat however, prevalence of many infectious diseases among the poultry population are posing a threat to this fast growing industry. Among the prevalent diseases Avianpox, the earliest described avian disease is very common (Pathak *et al.* 2017). The disease is caused by Avipoxviruses (APVs) and represent a large group of virus present in bird population (Gyuranecz *et al.* 2013), which belong to the Chordopoxvirinae subfamily of the Poxviridae family (Carulei *et al.* 2017).

Avipoxviruses (APV) are distributed worldwide causing diseases in domestic as well as wild birds of all ages and is responsible for causing significant economic impact

(Tripathy and Cunningham 1984). In India, fowl pox has been described in backyard poultry of different states including Trichy district in Tamil Nadu (Conroy *et al.* 2005, Durairajan *et al.* 2022), three districts in Assam (Gawande *et al.* 2007), Midnapur and North 24 Parganas districts of West Bengal (Dana *et al.* 2000, Biswas *et al.* 2011). A recent study conducted in Assam described incidences of Avipoxvirus infection in different species of domestic birds (Pathak *et al.* 2017)

Avipox virus can be isolated by different methods like bird inoculation, embryo inoculation and various cell culture systems (Gilhare *et al.* 2015, Baguory *et al.* 2016). Increasing detection of Avipox in various domestic as well as wild birds including recent spatial and host taxonomic range expansion suggests the disease be an emerging disease (Lawson *et al.* 2012) and hence, virus isolation is crucial for the prevention and control of the disease. In view of above facts, present study aimed at adaptation of Avipoxvirus isolated from different species of birds in primary cell culture and continuous cell line and the presence of virus was confirmed by appearance of CPE and PCR.

### MATERIALS AND METHODS

*Ethics committee approval:* The research procedures were approved by the Institutional Animal Ethics Committee, Assam Agricultural University.

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*Source of the virus:* The *Avipoxvirus* isolates of fowl [FP/As- K(R)], pigeon [P2/As- K9(R)] and duck (D2/As- N) origin, available in the virus repository of the Department of Microbiology, C.V.Sc, A.A.U, Khanapara were selected for the study. The isolates were obtained from outbreaks in different parts of Assam and were confirmed by PCR analysis in a previous study (Pathak *et al.* 2017). The NCBI GenBank accession numbers of FP/As- K(R), P2/As- K9(R) and MT877437.1 are MT877439.1, MT877436.1 and MT877437.1, respectively. The isolates were revived in 10-12 day old embryonated chicken eggs (ECE) employing the chorioallantoic membrane (CAM) route of inoculation (Roy *et al.* 2013). The revival of the virus was demonstrated by appearance of lesions on the CAM post inoculation and further confirmed by polymerase chain reaction (PCR). The revived isolates of fowl, pigeon and duck origin were respectively designated as Fowl/PoxV, Pigeon/PoxV and Duck/PoxV and were used for adaptation in CEF and Vero cell lines.

*Polymerase chain reaction:* The presence of the viral nucleic acid was confirmed by performing PCR analysis as described by Lee and Lee (1997) based on *4b* gene of *Avipoxvirus*. DNA extraction was carried out using Trizol method following manufacturer's protocol. PCR was carried out in 25 µl reaction mixture containing 14.5 µl of Nuclease Free Water (NFW, Thermo Scientific), 2.5 µl of 10× buffer, 1.5 µl 25 mmol MgCl<sub>2</sub>, 1 µl of 10 mmol dNTP, *Taq* polymerase 0.5 µl, 1 µl of both forward primer (10 pmol) and reverse primer (10 pmol) and 3 µl DNA template. The forward primer 5'-AGCAGGTGCTAAACAACAA-3' and reverse primer 5'-CGGTAGCTTAACGCCGAATA-3' were used to amplify a product of 578 bp. Thermal cycling conditions used were initial denaturation at 94°C for 5 mins followed by 35 cycles each of 94°C for 30 sec, 57°C for 1 min, 72°C for 1 min with a final extension of 72°C for 8 min. The amplified PCR products of *4b* core gene was confirmed by agarose gel electrophoresis in 1× Tris Acetate EDTA (TAE) buffer with 1.2% agarose containing ethidium bromide.

*Adaptation of Avipox virus in various cell culture systems:* The selected isolates were propagated in Chicken Embryo Fibroblast and Vero cell line. The primary CEF cell culture was prepared following the method described by Rai (1985). The cell suspension was adjusted at 10<sup>6</sup> cells were cultured into 25 cm<sup>2</sup> cell culture flask and incubated at 37°C for 24-48 hrs. The flask showing 80% confluent monolayer was used for inoculation with selected APVs. The inoculums of virus (Fowl/PoxV, Pigeon/PoxV and Duck/PoxV) were treated with 1× cocktail of antibiotics (Antibiotic antimycotic solution, 100×, Sigma) per 1ml for half an hour. The virus inoculum @ 1 ml per 25 cm<sup>2</sup> cell culture flask (Nunc, Denmark) was allowed to absorb for 1 hr. After 1 hr, the inoculated flasks were washed with serum free media (Eagle's Minimum Essential Media, Hyclone) to remove the unabsorbed viruses. Maintenance media containing 5% FBS was added into the flask and incubated at 37°C for 4-5 days. Un-inoculated healthy monolayer

was kept as control. The cells were observed daily under microscope for any changes in the cell morphology. The infected cells showing complete CPE along with the control flask were harvested by giving three cycles of freezing-thawing in every passage.

*Adaptation in Vero cell line:* The fifth passage of Chicken Embryo Fibroblast primary cell culture adapted APVs were used for adaptation in Vero cell line. The cells were propagated using Eagle's Minimum Essential Medium (EMEM, Hyclone Inc.) containing 10% foetal bovine serum (FBS, Sigma) supplemented with 2mM L-Glutamine. The cells were grown in 25 cm<sup>2</sup> cell culture flask (Nunc, Denmark), incubated at 37°C with 5% CO<sub>2</sub>. The flask containing confluent monolayer were used for adaptation of virus. For maintaining the cells, maintenance medium containing EMEM with 5% FBS was used. Inoculation of the virus was done similarly to procedure followed by Konwar *et al.* (2019).

*Haematoxylin and eosin staining of cell culture for demonstration of CPE:* The infected CEF monolayer showing characteristic CPE was stained using haematoxylin and eosin staining method described by Rai (1985).

*Evaluation and quantitation of Avipoxvirus adaptation in various cell culture systems at different passage level:* The adaptation of the *Avipoxvirus* isolates in different cell culture system was assessed at different passage levels by observing the characteristic CPE produced in the infected cells. Further confirmation was done by PCR analysis of the cell culture fluid in all the passages from 4<sup>th</sup> passage onwards. The isolates exhibiting CPE and confirmed by PCR were further studied for determination of virus titre. The virus titre was estimated at 5<sup>th</sup>, 15<sup>th</sup> and 20<sup>th</sup> passage. TCID<sub>50</sub> was determined following guidelines of Virology Methods Manual (Brian and Hillar 1996) by Karber's method.

## RESULTS AND DISCUSSION

In this study, the *Avipox* virus isolates preserved in the virus repository in the Department of Microbiology, CVSc, AAU was revived by the chorioallantoic membrane route using 10 to 12 day-old embryonated chicken eggs. Some changes were evident in the infected CAM, i.e. the CAMs appeared opaque, leathery, edematous and thick. Congestion, swelling and occasionally minute haemorrhagic areas were seen in some of the infected CAMs (Fig. 1). The present observation was supported by Haligur *et al.* (2009) who observed CAM lesions of varied diameter and grayish-white in colour. Depending on the virulence of the virus, pock lesions in case of APV infection measuring 0.5-1.5 mm were observed on the CAM in 3-5 days after inoculation (Holt and Krogsrud 1973 and Cox 1980). The generalized thickening of infected CAM was also observed by (Prukner-Radovic *et al.* 2006). Similar way of isolation in CAM was carried out by various workers (Diallo *et al.* 1998, Weli *et al.* 2004, Yadav *et al.* 2007 and Fasaei *et al.* 2014, Roy *et al.* 2013).

The presence APV in the infected CAMs was confirmed

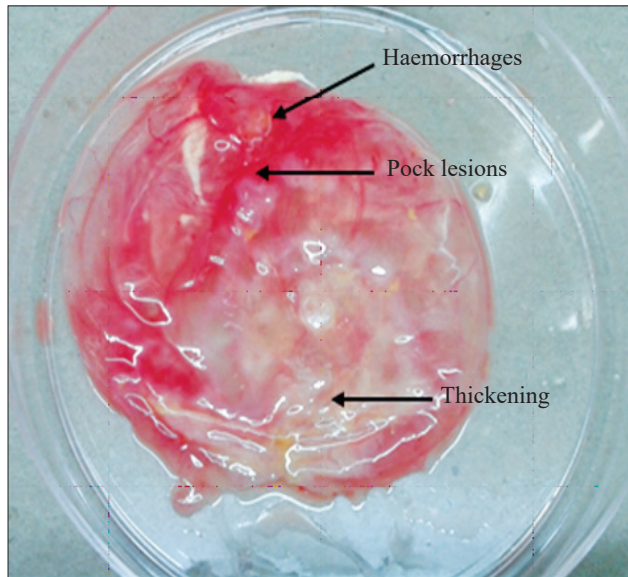


Fig. 1. Avipox virus infected chorioallantoic membrane showing haemorrhages, thickening and pock lesions at 96 hr post infection.

by using PCR. Standard vaccine virus strain was used as positive control. Amplified product of *4b* gene of DP virus was 578 bp against no amplification in negative control. Gel electrophoresis of the amplified product revealed presence of specific band at 578 bp (Fig. 2). PCR based on amplification of a 578 bp region of the highly conserved *4b* gene of avipox virus has been increasingly used for diagnosis in the last few years (Luschow *et al.* 2004). Several other workers have also reported use of polymerase chain reaction in amplification of *4b* gene sequence and used it as a rapid diagnostic tool for the detection of avian pox virus (Lee and Lee 1997, 2005, Rampin *et al.* 2006,

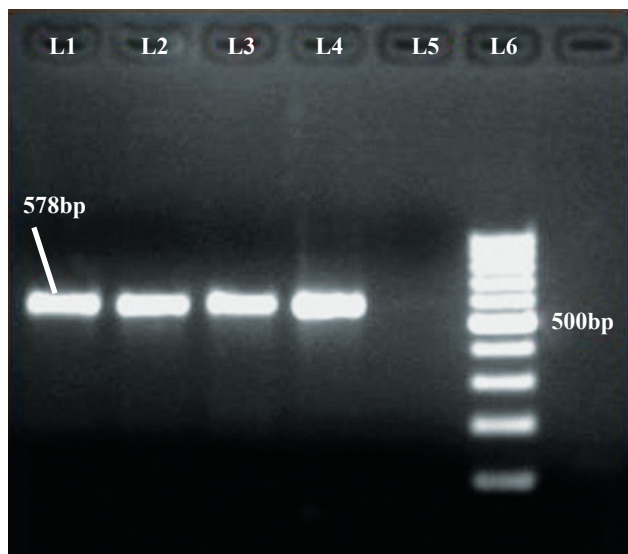


Fig. 2. Gel confirmation of PCR amplicons of avipoxvirus isolates revived in embryoted chicken eggs. L1: FP/As- K(R), Fowl isolate, L2: D2/ As- N, Duck isolate, L3: P2/ As- K9(R), Pigeon isolate, L4: Commercial vaccine, L5: Negative control, L6: 100 bp ladder.

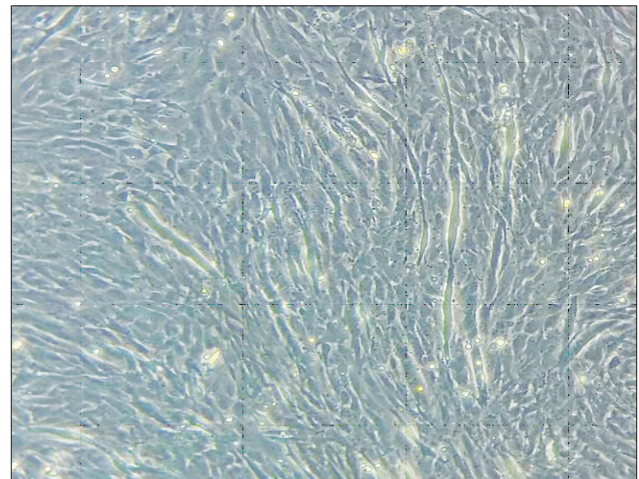


Fig. 3. Uninfected monolayer of CEF (100 $\times$ ).

Hanan *et al.* 2009, Manarolla *et al.* 2010, Roy *et al.* 2013, Fasaei *et al.* 2014 and Zhao *et al.* 2014).

**Adaptation in Chicken Embryo Fibroblast (CEF) cells:**  
In this present investigation, the selected Avipox virus of Fowl, Duck and Pigeon origin was propagated in the chicken embryo fibroblast primary cell culture up to 20<sup>th</sup> passage. In the infected CEF, CPE was evident from 4<sup>th</sup> passage. CPE was mild to moderate in the first few passages. Uninfected monolayer did not show any CPE (Fig. 3). Slight variation as regard to the period required for initiation of CPE was observed among the three isolates. The CPE consisted of cell degeneration appearing as rounding vacuolation, syncytia formation, granulation and finally detachment. The CEF inoculated with the fowl isolate showed no characteristic CPE up to 3<sup>rd</sup> passage level. The infected CEF cells showed granulation, vacuolation and rounding (Fig. 4) at 4<sup>th</sup> passage level 72 hrs P.I. As the passage level increased more prominent CPE could be seen which comprised of thinning, syncytia formation and detachment of cells (Supplementary Fig. 1). From the 9<sup>th</sup> passage CPE appeared within 48 hrs P.I and from 13<sup>th</sup> passage onwards changes could be seen within 24 hrs P.I. In the cultures infected with the fowl isolate a high degree of CPE was observed from an earlier passage in comparison to the other two isolates. Even the time required for completion of CPE was considerably less. Staining of the infected cells by H & E staining method revealed prominent CPE like syncytia formation (Supplementary Fig. 2) but no inclusion bodies could be detected. Comparable CPE was observed in CEF cells infected with the pigeon isolate, in which rounding and vacuolation of cells was observed from the 4<sup>th</sup> passage onwards. However, syncytia formation and detachment of cells were observed only from the 13<sup>th</sup> passage after 48 h P.I. In case of the duck isolate, CPE comprising rounding and vacuolation could be observed only after 72 h P.I up to the 10<sup>th</sup> passage level. Appearance of high degree of CPE within 24 hours P.I. was observed later in the 15<sup>th</sup> and 17<sup>th</sup> passage in case of the pigeon and duck isolate respectively. Irrespective of the isolate concerned, it was observed that with the increase in the number of passage,

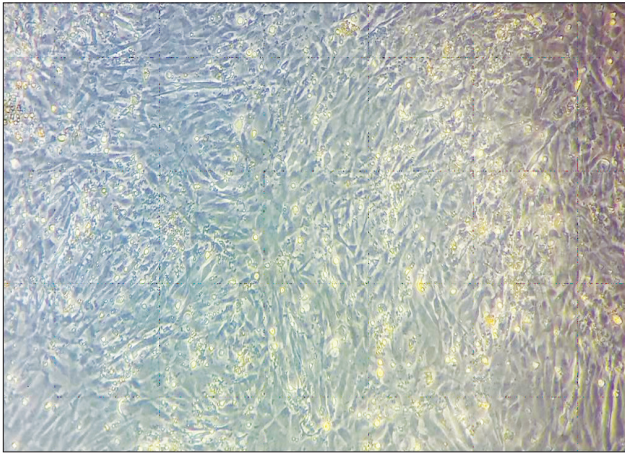


Fig. 4. CEF cells infected with Fowl pox virus showing vacuolation and rounding at 24 hours p.i (100 $\times$ ).

the time taken for appearance of CPE decreased. From 17<sup>th</sup> passages it was observed that CPE appeared with 24 hrs in all the 3 isolates. There was no CPE observed in the uninfected cell monolayer and the cell remained unchanged morphologically. CPE was consistent throughout all the passages. The virus propagated in different passage level was confirmed by PCR analysis (Supplementary Fig. 3).

Different workers have reported the adaptation of Avipox virus in chicken embryo fibroblast cell culture (Tripathy 1991, Tadese *et al.* 2002, Yadav *et al.* 2007, Balachandran *et al.* 2012, Gilhare *et al.* 2015). Presence of CPE consisting of rounding, vacuolation, degeneration of cells appearing as bunches of grapes, syncytia, detachment of cells, intracytoplasmic inclusions were common findings. The present findings are comparable to the observations made by Gilhare *et al.* (2015), who reported that the CEF inoculated with FPV showed no characteristic CPE up to the second passage level. At third passage level, CEF cell culture showed aggregation of cells which progressed rapidly and appeared as floating cells at 72 h PI. Massive detachments of cells were observed at 120 h PI. They also cited that on subsequent passage, the CPE was observed earlier. Similarly, Yadav *et al.* 2007 also reported that the CPE was observed earlier in case of FPV in comparison to Turkey Pox Virus, Quail Pox Virus, PPV. More or less similar results were also reported by (Olfat *et al.* 2005 and Jarmin *et al.* 2006). The better adaptability of the fowl isolate, in the present study may be attributed to the fact that fibroblast cells of chicken origin were used and the other two isolates were of different host origin namely pigeon and duck.

**Adaptation in vero cell line:** In the present study, the Vero cell line was used for adaptation of CEF cell culture adapted Avipox virus. The cell line, being versatile in nature, could be easily maintained in the laboratory. There are few reports on adaptation of viruses of avian origin in cell lines. Baguory *et al.* (2016) propagated fowlpox virus (Baudette strain) on BGM (Baby Grivet Monkey Kidney cell line) and African green monkey vero cell lines. Their study indicated high susceptibility of BGM but the vero

cells were found to be abortive to the virus. The highest titre recorded in Vero cells was  $10^{2.1}$  which indicate that the Vero cell line wasn't suitable for the growth of fowlpox virus. In the present investigation, all the three isolates were propagated up to the 10<sup>th</sup> passage. There were no morphological differences the in infected monolayer. Due to the absence of characteristic CPE and negative results in PCR further passages were not performed.

In this context, Weli and Tryland (2011) opined that in non-permissive African green monkey cells (CV-1, Vero), there is a blockade of the APV morphogenesis cycle following the formation of immature virus and that this blockade may not be associated with cell receptors.

**Quantitation of cell culture adapted virus:** To estimate the virus titre, TCID<sub>50</sub> of the adapted isolates was calculated at 5<sup>th</sup>, 15<sup>th</sup> passage and 20<sup>th</sup> passage. The mean log TCID<sub>50</sub> value of the 5<sup>th</sup> passage of the CEF adapted Fowl/RV/CEF P-5, Pigeon/RV/CEF P-5 and Duck/RV/CEF P-5 isolates was found to be  $4.18 \pm 0.11$ ,  $3.89 \pm 0.14$  and  $3.6 \pm 0.05$  respectively. At the 15<sup>th</sup> passage, the fowl isolate (Fowl/RV/CEF P-15) exhibited a titre of  $5.29 \pm 0.06$ , the pigeon isolate (Pigeon/RV/CEF P-15) a titre of  $5.13 \pm 0.12$  and the duck isolate (Duck/RV/CEF P-15) a titre of  $4.47 \pm 0.13$ . Finally in the 20<sup>th</sup> passage, a titre of  $6.29 \pm 0.13$ ,  $5.72 \pm 0.20$  and  $4.81 \pm 0.09$  was recorded in case of the fowl (Fowl/RV/CEF P-20), pigeon (Pigeon/RV/CEF P-20) and duck (Duck/RV/CEF P-20) isolate in the same order. A gradual increase in the titre in the 15<sup>th</sup> and 20<sup>th</sup> passage was evident in case of all the three isolates. The mean log TCID<sub>50</sub> of the CEF adapted fowl isolate showed a higher titre in comparison to the other two isolates in all the three passages studied. These findings are in agreement that of Baguory *et al.* (2016), who reported an increase in the logTCID50 value from 5.0 at the 4<sup>th</sup> passage to 6.5 after the 9<sup>th</sup> passage in CEF. In a similar study, Soud *et al.* (2018), propagated PPV in CEF and observed that the virus CPE appeared clearly after 6-days post inoculation at the 3<sup>rd</sup> passage and on the 5<sup>th</sup> day post inoculation by the 4<sup>th</sup> passage with a titre of 4.0 log<sub>10</sub> TCID<sub>50</sub>/ml. At the 5<sup>th</sup> passage, they found that the titre increased to 4.3 log<sub>10</sub> TCID<sub>50</sub>/ml. Other workers have attempted to determine the virus titres in embryonated eggs. Pradhan *et al.* (1996) observed that the EID<sub>50</sub> titre of FPV (V) strain was  $10^{6.6}$  EID<sub>50</sub>/ml at the seventh passage level. Siddique *et al.* (2011) reported that the EID<sub>50</sub> titre per ml of suspension was  $10^{6.2}$  and  $10^{6.5}$  for PPV-1 and FPV-2, respectively.

In the present study, it was observed that all the three isolates of fowl, pigeon and duck origin could be successfully adapted to grow in the CEF cell culture with characteristic CPE production. CPE observed in different passages included rounding, vacuolation, clumping/fusion, syncytia formation and detachment. It was seen that the degree of CPE observed in CEF cells was variable in case of the three isolates. In the fowl isolate, high degree of CPE was observed within 24 hrs P.I from the 13<sup>th</sup> passage. However, in case of the pigeon and duck isolate similar CPE was seen within 24 hrs only after the 15<sup>th</sup> and 17<sup>th</sup>

passage, indicating better adaptability of the fowl isolate.

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