RESEARCH ARTICLE Indian Journal of Animal Research, Volume 54 Issue 6: 716-722 (June 2020)

Adaptation of wild strain of duck plague virus in cell culture systems

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10.18805/ijar.B-3832

ABSTRACT

Duck virus enteritis (DVE) is an acute, contagious herpes viral disease of ducks, geese and swans and members of the family *Anatidae* under the order *Anseriformes*. As per the recent taxonomic classification by ICTV, DEV has been classified into the genus *Mardivirus*, subfamily *Alpha-herpesvirinae* of the family *Herpesviridae*. Vaccination is the only option for the prevention and control of Duck plague. In India vaccination is done with chicken embryo adapted live virus which has many shortcomings. So, in this present situation a safe and potent vaccine development is the need of the hour. The use of chicken embryo fibroblast and a certified cell line may, probably, be the best option to achieve this. Therefore, the present study was undertaken for adaptation and propagation of local strain of duck plague virus in various cell culture systems. During the study a wild strain of DPV (DP/As-Km/0019) which was isolated in Duck Embryo Fibroblast, available in the Department of Microbiology was selected. The selected wild strain was used for adaptation in various cell culture systems *viz*. Chicken embryo fibroblast cell culture (CEF), Vero cell line and QT-35 cell line. The virus was passaged up to 12th passage levels and the presence of viral antigen was demonstrated by appearance of cytopathic effect (CPE), by Polymerase Chain Reaction (PCR) and by Sandwich ELISA. PCR was able to detect virus from all the cell cultures from 5th passage onwards and virus titre was detected at 10th and 12th passage level by S-ELISA. It was observed that various cell culture systems can be a good candidate for propagation of DPV and further study is required to study its immunogenicity and its feasibility as a vaccine candidate.

Key words: Adaptation, Chicken Embryo Fibroblast, Polymerase Chain Reaction, QT-35 Cell line, S-ELISA, Vero Cell Line.

INTRODUCTION

Duck rearing is a profitable poultry industry because of its multiple out-put in the form of egg, meat and feather (Rajput *et al.,* 2014) India has a duck population of 23.539 million constituting 3% of total poultry population (GOI, 2012) and concentrated in the states of Assam, West Bengal, Andhra Pradesh, Tamil Nadu, Uttar Pradesh, Bihar and Orissa (Narhari, 2009).

Assam ranks as highest duck populous state of the country having 7.31 million ducks. However, population of duck as a whole has decreased at a rate of 14.85 per cent during the period 2007 to 2012. One of the main reasons of declining duck population may be attributed to frequent occurrence of infectious diseases like duck plague and duck cholera (Neher *et al.,*2018).

The duck virus enteritis (DVE) also known as duck plague is an acute contagious herpes virus infection of duck and other domestic and wild waterfowls (Leibovitz, 1991). As per the recent taxonomic classification by ICTV, duck plague virus (DPV) has been classified into the genus *Mardivirus*, subfamily *Alpha-herpesvirinae* of the family *Herpesviridae* (King *et al.,* 2012 Fadly *et al.,* 2008; Li *et al.,* 2009). The disease is known to have global distribution and due to high mortality, decreased egg production and hatchability, significant economic losses are associated with DPV across the world (Wang *et al.,* 2013).

The disease was first reported by Baudet in Netherlands (Baudet, 1923). After that the disease was subsequently reported in many part of the world. In India, DVE was first diagnosed from a severe outbreak in the state of West Bengal during early nineteen sixties (Mukherjee *et al.*, 1963a, 1963b). Thereafter, the disease has spread to different parts of the country and frequent outbreaks have been recorded in the duck-rearing areas including Kerala (Kulkarni *et al.*, 1995), Tamil Nadu (Chellapandian *et al.*, 2005), West Bengal (Bhowmik and Chakrabarty, 1985) and Assam (Chakrabarty *et al.*, 1980).

The disease has caused much havoc in Assam. Several studies have been carried out to study the virus epidemiology, pathology, diagnostics and vaccine development. Occurrence of the disease is associated with heavy mortality in domestic and wild ducks (Dhama *et al.,* 2017). In a recent study it was reported that as many as 8 districts of Assam were found to be affected with DPV infection causing heavy mortality of 81.01% (Neher *et al.*, 2018).

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How to cite this article: Konwar, N., Sarmah, H., Gogoi, S.M., Vizo, K.E., Bharali, A., Barman, N.N., Bora, D.P. and DasIndian, S.K. (2020). Adaptation of wild strain of duck plague virus in cell culture systems. Journal of Animal Research. 54(6): 716-722.

Submitted: 23-04-2019 **Accepted**: 10-06-2019 **Published**: 14-08-2019

Vaccination is the only option for the prevention and control of Duck plague. In India, vaccination is done with chicken embryo adapted live virus vaccine. This vaccine is not free from egg borne infection, influence of maternal antibody and involves high production cost (Kulkarni *et al.*, 1998; Ferguson *et al.,* 2005; Mondal *et al.*, 2010). In this present situation, a safe, potent and cheap DPV vaccine is required for controlling the menace of Duck plague. The vaccine must be easy to produce in large scale and free from other adventitious infectious agents. The use of chicken embryo fibroblast and a certified cell line may, probably, be the best option to achieve this. Although, work is being done on adaptation of vaccine strain of DEV in various primary cell cultures, very few reports are available on the adaptation of the local strain of duck plague virus in CEF and certified cell lines. Furthermore, studies are required to understand the potential of a locally isolated DPV as a vaccine candidate. This paper describes about adaptation of wild strain of DPV in primary cell culture as well as in established cell line.

MATERIALS AND METHODS

Source of wild strain of duck plague virus

The virulent strain of Duck Plague Virus available in the virus repository of the department of Microbiology (DP/AS-Km/0019) was used to adapt in cell culture. The virus was revived in 1-2 months old maternal antibody free ducklings. Internal organs like liver, spleen, kidney, oesophagus, annular ring and heart were collected and samples were processed for isolation in duck embryo fibroblast (DEF) primary cell culture which was used for adaptation in chicken embryo fibroblast (CEF) as well as in cell lines.

Adaptation of DPV in CEF

The primary chicken embryo fibroblast cell culture was prepared from 9-11 day old embryonated chicken eggs as per standard protocol (Rai, 2010). The CEF monolayer was grown in tissue culture flask (25cm^2) at 37°C under 5% CO_2 in Eagle's Minimum Essential Medium (EMEM) containing 10% foetal bovine serum (FBS, Hyclone) supplemented with 2mM L-Glutamine and antibiotics at the rate of penicillin 100 units, streptomycin 100 µg and amphotericin B 25 µg per ml. A local strain isolated in DEF was used for propagation in chicken embryo fibroblast. The lyophilized virus (DP/AS-Km/0019) of DPV) was reconstituted in Hank's balanced salt solution (HBSS) and treated with cocktail antibiotic solution (Sigma 100X, USA) @10µl /ml of inoculums. The monolayer was thoroughly washed and inoculated with 1 ml virus inoculums per 25cm^2 cell culture flask (Nunc, Denmark) and kept at 37°C for 1 hour to facilitate proper adsorption of virus on to the cells. The un-adsorbed viruses were washed with Ca^{2+} Mg^{2+} free PBS. Then, maintenance medium containing 5 percent foetal bovine serum was added to the flask and incubated at 37°C for 3-4 days. Un-inoculated healthy monolayer was kept as control. The cell monolayer was observed daily under inverted microscope for any change of cell morphology. The virus was harvested after appearance of cytopathatic effect (CPE) and was stored at -30°C for future use.

Adaptation of DPV in Vero cell line and QT-35 Cell line

The Vero and QT-35 cell lines were maintained in the Department of Microbiology, College of Veterinary Science, AAU, Khanapara using Eagle's Minimum Essential Medium (EMEM) containing 10% foetal bovine serum (FBS) supplemented with 2 mM L-Glutamine. Antibiotics were added to the growth medium at the rate of penicillin 100 units, streptomycin 100 µg and amphotericin B 25 µg per ml. The cells were grown in 25 cm^2 cell culture flask (Nunc, Denmark) and the flasks containing confluent monolayer were used for adaptation of virus. For maintaining the cells, maintenance medium containing EMEM with 5% foetal bovine serum (FBS, Sigma) was used.

The wild strain of DPV adapted in CEF upto fifth passage level was further propagated in Vero cell line and QT-35 cell line. Cell free supernatant from CEF primary culture was filtered through 0.45 µm membrane filter (Nalgene) and treated with cocktail antibiotic (Sigma, 100X) @ 10 µl/ml. A 24 hour old 60-70% confluent cell monolayer was used for infection by duck plague field strain. Growth medium from the tissue culture flask was removed; 1 ml of antibiotic treated sample was then added and the flasks were incubated at 37°C in CO_2 incubator for virus adsorption. Hank's balanced salt solution (HBSS) was used to wash away the un-adsorbed viruses and approximately 5 ml of the maintenance medium containing 5% FBS was added. The flasks were then incubated at 37°C in a CO_2 incubator for a period of 4-5 days. The cells were monitored everyday till CPE appeared. After completion of 70-80% CPE the virus was harvested by three freeze thaw cycles. After clarification by centrifugation at 3000 rpm for 15 minutes, the virus containing fluid was aliquoted in vials and used for further passaging.

Confirmation of cell culture adapted DPV

Duck plague virus adapted in CEF, Vero as well as at QT cell line was subjected for confirmation at different passage level by PCR and s-ELISA.

Polymerase chain reaction (PCR)

Extraction of genomic DNA was done by Trizol method according to manufacturers guidelines. The 5th, 10th and 12th passaged virus in various cell culture systems was tested for presence of virus by PCR assay. For detection of DNA polymerase gene *UL30* specific primers Aravind *et al.,* (2015) were used. The details of the primers used in the present study are given below.

Name of the gene	Primer	Sequence (5′-3′)	Amplicon size
DNA polymerase gene(*UL30*)	Forward	5′CCGGATCCATGGCAG AGTCGGGTAGAAAC3′	1510 bp
	Reverse	5′CCGTCGACTCACCG TTTATCTTAACC TTAC3′	

For amplification of DNA polymerase gene a 50 µl reaction mixture containing 25 µl of master mix, forward primer (10 pmol/µl) 2 µl, reverse primer (10 pmol/µl) 2 µl, nuclease free water 13 µl and DNA template 8 µl was prepared. Thermal cycling conditions used were initial denaturation at 95°C for 3 mins followed by 30 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 DNA polymerase gene of DPV was confirmed by agarose gel electrophoresis in 1X Tris Acetate EDTA (TAE) buffer with 1.7% Agarose (Amresco) containing ethidium bromide (10 mg/ml). Electrophoresis was carried out at 80V for 1 hour and amplified product was visualized at 312 nm wavelength as a single compact band of expected size under UV light using a UV transilluminator (DNR, Bio-imaging system, Minilumi).

Sandwich-ELISA

A sandwich-ELISA (s-ELISA) was developed for the detection of virus adapted in various cell culture system (Hung *et al.,* 2004). Purified DPV was used to raise antibody in duck as well as in rabbit using standard protocol. A 96 well microtitre plate was coated with 50 µl per well of 1:800 diluted rabbit anti-duck plague virus antibody in carbonate-bicarbonate buffer (pH9.6) and was incubated overnight at 4°C. The coated plate was washed with PBST for 3-4 times and pre-blocking was done with blocking buffer containing 3% LAH + 2% goat serum + 5% horse serum in coating buffer @ 50 µl/well. The plate was incubated at 37°C for 1 hour. The plates were washed for three times and the test samples were diluted in diluting buffer (PBST+2% Horse serum) in the ratio of 1:2 by adding 50 µl to each well. Incubation was done for 1 hour at 37°C. After thorough washing duck anti-duck plague antibody (1:300) diluted in blocking buffer was added 50 µl to all the wells and incubated for 1 hour. The plate was washed thoroughly and goat anti-duck HRPO conjugate (1:300) diluted in blocking buffer @ 50 µl was added to all the wells and incubated for 1 hour. The plates were washed and 50 µl of freshly prepared substrate (H_2O_2) and chromogen (OPD, Sigma was added to all the wells and allowed to react for 15 minutes. The reaction was stopped with 1M H_2SO_4. Reading was done using 492 nm filter. The cut-off value of each sample was calculated by subtracting the mean optical density (O.D) value of the negative control from that of the test samples. Difference of O.D value $\geq$ 0.1 was considered positive.

RESULTS AND DISCUSSION

Adaptation of wild strain of DPV

The primary chicken embryo fibroblast (CEF) monolayer was prepared from 9-11 days old embryonated eggs. Confluent monolayer of CEF was developed within 24 hour (Fig 1). The DPV virus was passaged in CEF up to 12 passage level**.** CPE observed in 4th passage comprised of vacuolation, rounding, syncytia formation and finally detachment of cells (Fig 2, 3, 4). It was seen that in 4th and 5th passage CPE

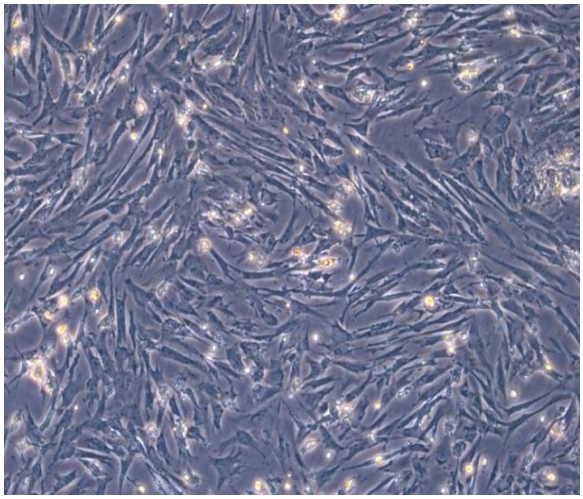

Fig 1: Un infected monolayer of CEF 100X.

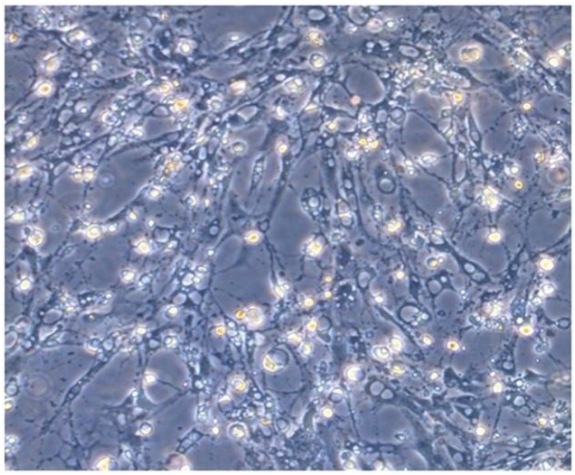

Fig 2: Infected cell monolayer showing Vacuolation & rounding 100X.

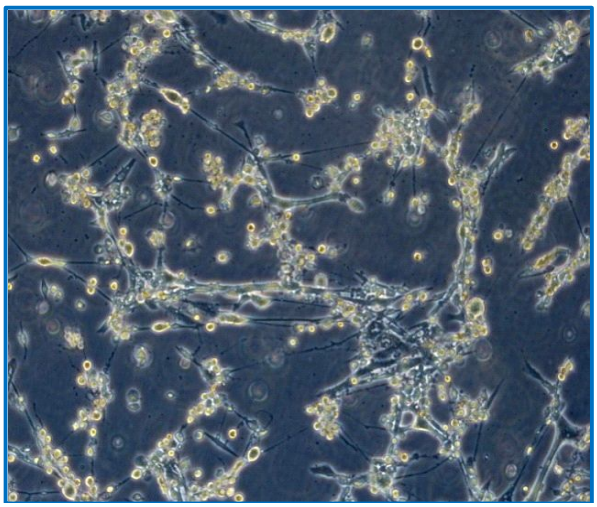

Fig 3: Infected cell monolayer showing Detachment of cells 100X.

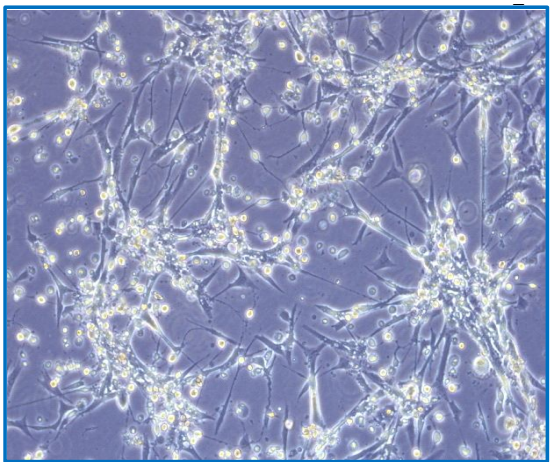

Fig 4: Infected cell monolayer showing Syncytia formation 100X.

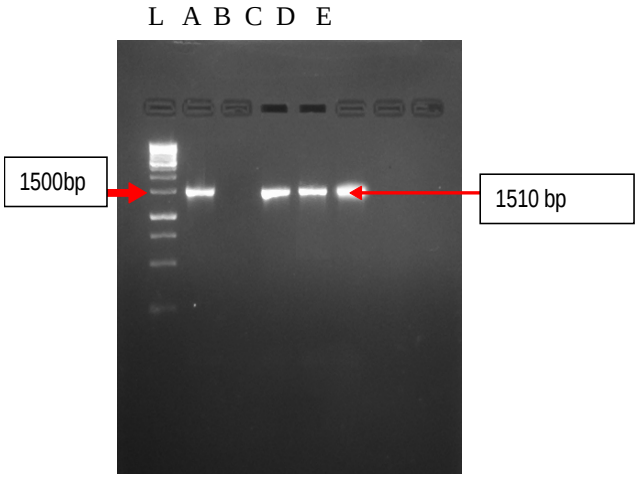

Fig 5: L = 1 kb Ladder A = Positive Control B = Negative Control C = P5 of CEF D = P10 of CEF E = P12 of CEF.

developed within 48 hours of infection and CPE observed were vacuolation and rounding. In 6th passage level the time of appearance of CPE was within 48 hours and CPE consisted of vacuolation, rounding, syncytia and detachment of cells. From eight passage level all characteristic CPE could be observed within 24 hours post inoculation. It was observed that the time taken for appearance of CPE decreased with passage levels. The uninfected cell monolayer retained normal cellular details throughout the passage levels. PCR was performed on 5th, 10th and 12th passage level and viral nucleic acid could be detected (Fig 5). On the other hand Sandwich ELISA could detect viral antigen from 10th passage onwards (1:16). The virus titre increased gradually with increase in passage level and in 12th passage the titre was found to be 1:64. Comparable results were observed in a study conducted by Kumar and Ponnoose (1997) where the virus was adapted in CEF cultures by serial passaging upto 10th passage level and reported demonstration of rounding and clumping of cells, syncytium formation, vacuolation of cytoplasm and eosinophilic intra-nuclear inclusion bodies. Similarly, Dinh *et al.,* (2004) conducted an experiment on adaptation of DPV in CEF and observed that in 4th to 7th passage level CPE was observed after 72 hours while from 8th passage the time taken for demonstration of CFE reduced to 48 hours. Mondal *et al.,* (2010) opined that first signs of CPE were evident at 12 hour post infection and by 48 hour more than 80% cells became rounded with vacuolation, cytoplasmic extension and detachment. Adaptation of DPV virus in CEF culture were also conducted by Doley *et al.,* (2013) and Neher (2016) who reported that characteristic CPE appeared within 24 hours post infection and along with increase in the number of passages, the CPE appeared earlier. Complete CPE could be detected by 48 hours. Adaptation of DPV field strain in chicken embryo fibroblast cell culture at different passages was confirmed using PCR. PCR could detect the viral nucleic acid from the fourth passage itself. Similar findings were also reported by Dinh *et al.,* (2004) where they could detect virus from CEF cell culture from 1st passage onwards by PCR.

The details of the CPE produced, PCR confirmation and S-ELISA detection is shown in the Table 1.

A confluent monolayer of Vero cell was developed within 24 hours (Fig 6). The cell monolayer attaining 60-70% confluence was infected with CEF adapted DPV. The field strain was propagated up to 12th passage and presence of virus was confirmed from 5th passage onwards by PCR

Table 1: Virus propagation in CEF showing different degree of cpe along with confirmation by s-ELISA and PCR.

Passage levels	Degree of CPE	Time of CPE appeared (hrs)	Detection by	
			ELISA	PCR
P4-P5	++	48	-ve	+ve
P6-P7	+++	48	-ve	+ve
P8-P9	+++	24	-ve	+ve
P10	+++	24	1:16	+ve
P11	+++	24	1:32	+ve
P12	+++	24	1:64	+ve

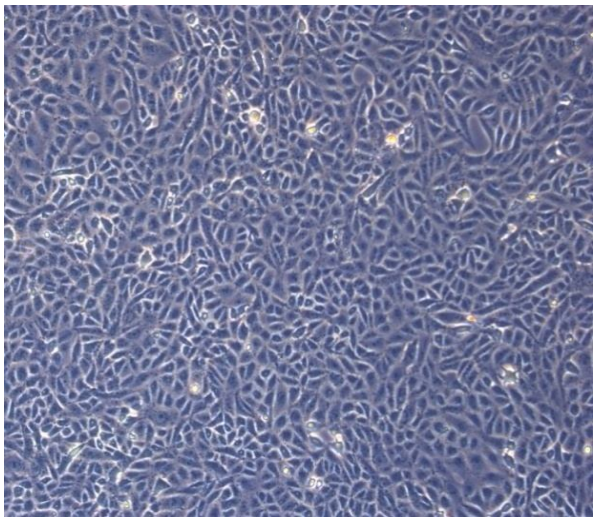

Fig 6: 24 hour confluent monolayer of Vero cell line (100X).

(Fig 5). The detection of virus in Vero cell line by S-ELISA was possible only from 10th passage. The titre of the virus at 10th and 12th passage level was found to be 1:16 which was less than the titre of DPV adapted in CEF. Though the virus was detected by PCR, no prominent CPE was observed up to 12 passage level (Fig 7). The adaptation of field strain of DPV in Vero cell line at different passage level and their confirmation by PCR and ELISA is depicted in the Table 2.

The Vero cell line, being versatile in nature, could be easily maintained in the laboratory. There are many reports on adaptation of viruses of avian origin in Vero cell line like Marek's disease (Jaikumar *et al.*, 2001), Newcastle disease virus (Ahamed *et al.*, 2004), Avian reo virus (Nwajei *et al.*, 1988) and Chicken Infectious Anaemia virus (Samah *et al.*, 2013) but there is only one report on adaptation of DPV in Vero cell line. In the study conducted by Aravind *et al.,* (2015), it was observed that gradual CPE could be seen from the 7th passage onwards and from 15th passage there was development of prominent CPE.

The QT-35 cells were maintained by continuous sub culturing in growth media. A confluent cell monolayer was developed within 48 hour (Fig 8). The cell monolayer attaining 60-70% was infected with CEF adapted DPV. The virus was propagated up to 12th passage level. The characteristic CPE observed in CEF cell were not evident in QT-35 cell line, however intermittent thinning of cells followed by detachment was found from 8th passage (Fig 9). PCR was positive from 5th passage level and S-ELISA was done for determination of virus titre at different passages. The titre of the adapted virus at 10th and 12th passage was found to be 1:16. The details of the PCR confirmation, appearance of CPE and ELISA titre is shown in the Table 2.

There are no previous reports on adaptation of DPV in QT cell lines. However, QT cell line has been extensively used for isolation and propagation of Fowl pox virus and Adeno virus (Yadav *et al.*, 2007; Kamal *et al.,* 2015). The characteristic CPE observed in CEF cell were not evident in QT-35 cell line, however intermittent thinning of cells followed by detachment was found from 8th passage onwards. The adaptation of DPV could be confirmed by performing PCR at various passage levels. PCR was positive from 5th passage level.

The adaptation of duck plague virus in different cell culture systems was demonstrated by PCR. Several workers

Table 2: Adaptation of DPV in different cell line and confirmation by demonstration of CPE, PCR and s-ELISA.

Passage level	Cell line					
	VERO			QT-35		
	CPE	PCR	ELISA TITRE	CPE	PCR	ELISA TITRE
P5	-ve	+ve	-ve	-ve	+ve	-ve
P10	-ve	+ve	1:16	+ve	+ve	1:16
P12	-ve	+ve	1:16	+ve	+ve	1:16

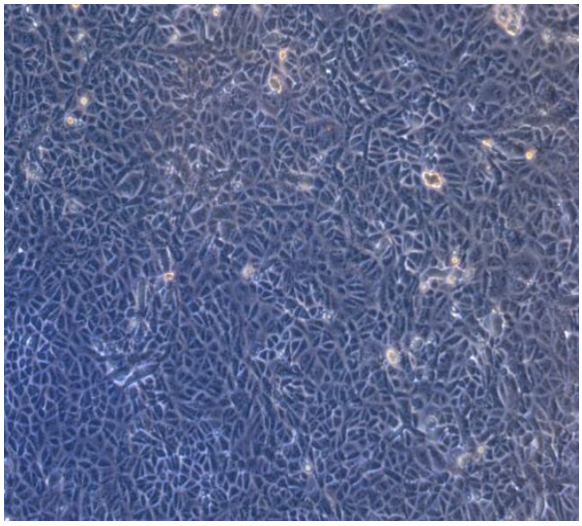

Fig 7: Infected monolayer of Vero cell line (100X).

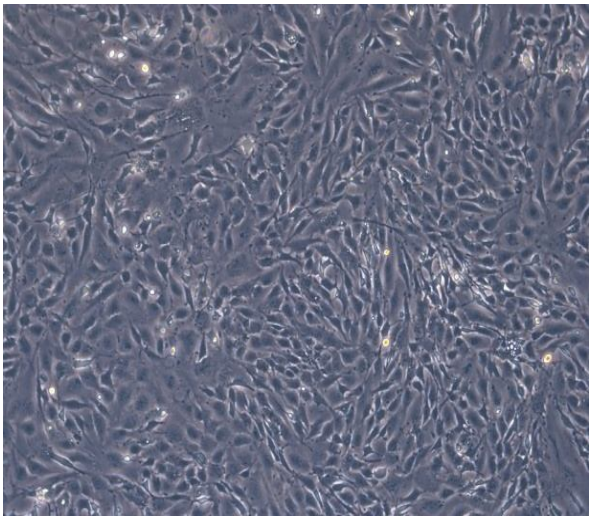

Fig 8: 24 hour confluent monolayer of QT-35 cell line (100X).

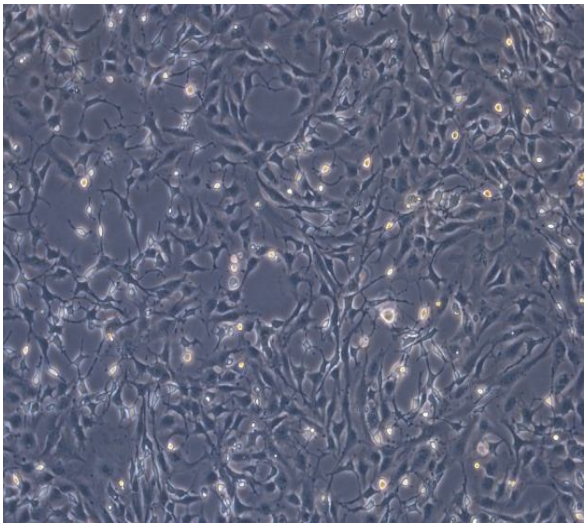

Fig 9: Infected confluent monolayer of QT-35 cell line (100X).

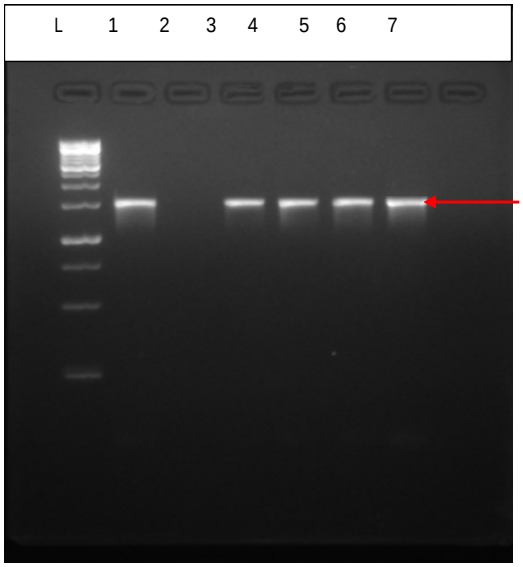

Fig 10: Confirmation of DPV adaptation in Vero and QT-35 cell line by PCR.

L-1Kb Ladder
1=Positive control
2=Negative control
3=P5 of DPV adapted in vero cell lines
4=P12 of DPV adapted in vero cell lines
5=P5 of DPV adapted in QT-35 cell lines
6=P12 of DPV adapted in QT-35 cell line

have opined that the speed, sensitivity and specificity of PCR greatly improved the diagnostic and research tool for studying of duck plague (Hansen *et al.*, 1999; Pritchard *et al.*, 1999. The DNA polymerase gene (*UL30*) was targeted because of the highly conserved nature of the gene (Xuefeng *et al.*, 2008a; Mondal *et al.*, 2010). The viral DNA was extracted from the cell culture fluid by trizol method and used for amplification. The amplicon size was 1510 bp (Aravind *et al.*, 2015) and there was no nonspecific amplification or smearing.

In the present study a Sandwich ELISA test was standardized for the detection of virus titre in cell culture. ELISA is reported to be simple alternative to standard duck inoculation method and neutralization test for diagnosis of DPV (Chandrika *et al.*, 1999) S-ELISA was able to detect virus at 10th passage level in CEF, Vero and QT-35 cell line. Neher *et al.,* (2018) have used S-ELISA for detection of DPV antigen from field samples and termed it as a rapid diagnostic test for detection of DPV antigen. Similarly S-ELISA has been used for detection of DPV antigen in field sample as well as from the CAM and allantoic fluids of inoculated birds (Tu *et al.*, 2004, Hung *et al.*, 2004).

Through this research work an attempt was made for adaptation of wild strain of DPV in both primary and continuous cell lines. The local strain was successfully adapted in various systems and the findings were found to be comparable with the observations made by other workers. It was seen that the titre of the virus adapted increased with passage level and further study is required for studying their immunogenicity and ability to be used as a vaccine candidate in future.

ACKNOWLEDGEMENT

The authors are thankful to the Dean and Director of Research (Veterinary), Faculty of Veterinary Science, AAU, Khanapara for providing necessary facilities to carry out the research work. This study was funded by Department of Biotechnology, Government of India under DBT Twinning project on "Development of novel molecular diagnostics and improved vaccine for Duck Plague virus"(Grant No. BT/372/ NE/TBP/ 2012).

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