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RESEARCH ARTICLE

ADAPTATION OF DUCK PLAGUE VIRUS (DPV) IN BABY HAMSTER KIDNEY CELL LINE (BHK-21) TOWARDS VACCINE DEVELOPMENT

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ABSTRACT

Duck plague (DP) is a worldwide acute, lethal, sometimes chronic, contagious virus infection of ducks caused by duck plague virus (DPV), also known as duck enteritis virus (DEV). The control of duck plague is considered as one of the biggest challenges in which vaccination is an efficient way to control DPV. Till now, vaccination is done mainly with chicken embryo adapted live virus that is known to be poorly immunogenic and elicits only partial protection. Further, the presence of other avian pathogens in the embryo propagated vaccine may pose a threat. To overcome these drawbacks, an attempt was undertaken for the first time regarding propagation and adaptation of isolates of DPV in baby hamster kidney (BHK-21) cell line. In this study, duck embryo fibroblast cell (DEFC) culture method was used to isolate DPV which were then adapted in the BHK-21 cell line. The characteristic cytopathic effects (CPE) of clumping and fusion of BHK-21 cells were observed starting from the 5th passage onwards. The presence of the virus and its multiplication in BHK-21 cell line was confirmed by detection of viral specific DNA by using polymerase chain reaction (PCR) targeting DNA polymerase gene (446 bp). The BHK-21 cell culture system is free from other infectious agents by comparing to chicken embryo adapted duck plague vaccine. Therefore, we can consider BHK-21 cell line as a suitable candidate for cultivation and propagation of DPV for vaccine development. The present study provides a scope to undertake further research on duck plague to explore the feasibility of utilizing this BHK-21 cell culture adapted DPV isolates for developing an attenuated vaccine against duck plague.

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INTRODUCTION

Bangladesh is considered as an Agriculture based country with a variety of agricultural resources of which scavenging duck rearing is considered to have potential effect on both for poverty alleviation and food production. Asia is considered to have most of the ducks of the world as east and south Asia has around 460 million of duck population. Bangladesh is the third largest country of duck population after China and Indonesia (FAO, 1991).

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With the huge amount of duck population, popularity of duck production is increasing in Bangladesh. Therefore, there are many constraints for the development of large scale duck farming in Bangladesh. Among these, the major hindrance for the development of duck farming in Bangladesh is the occurrence of diseases that devastate almost every year (Sarker *et al*., 1980). Among the prevailing infectious diseases of ducks in Bangladesh, duck plague (DP) is the most feared disease (Hanan *et al*., 2014) causing high mortality rates around 60%-70% (Sarker, 1980) which is caused by duck plague virus (DPV). DP is an acute, sometimes chronic, contagious virus infection that occurs naturally only in ducks, geese and swans of all ages, all members of the family Anatidae of the order Anseriformes (Breese and Dardiri,

1968). The infection has not been reported in other avian species, mammals, or people and does not pose a zoonotic risk. The etiological agent of DP is duck herpes virus 1 which is a member of the subfamily Alphaherpesvirinae of the family Herpesviridae (Davison *et al*., 1993; kaleta, 1990; Plummer *et al*., 1998; Shawky and Schat, 2002). DPV was first reported in Bangladesh by Starker *et al*. (1980, 1982). The specific pathologic response to DPV is dependent upon the species affected; age, sex and susceptibility of the affected host; stage of infection; and virulence and intensity of virus exposure. The germ of this disease spread through the affected ducks, polluted water and feed. DPV may undergo latency like other herpesviruses and the trigeminal ganglion seems to be a latency site for the virus. Recovered birds may carry the virus in its latent form, and viral reactivation may be the cause of outbreaks in susceptible wild and domestic ducks (Campagnolo *et al*., 2001). Adult ducks usually die in higher proportions than young ones, increasing the economic significance of the disease. In avian medicine, the control of DP is considered as one of the biggest challenges. Vaccination is an efficient way to control DPV. At present, two kinds of vaccines (Attenuated and inactive vaccines) are commercially available in market in which commonly used attenuated live vaccine provides a good protection against the disease (Lian *et al*., 2011; Huang *et al*., 2014). In order to get attenuated vaccine, different types of primary cell cultures particularly of avian origin and cell line of duck origin have been reported for propagation of DPV in homologous systems viz., duck embryo fibroblast (DEF-CCL-141), chicken embryo fibroblast (CEF) and duck embryo liver (DEL) (John *et al*., 1990; Mondol *et al*., 2010; Doley, 2013). However, these cultures have a limited life span (2-3 weeks) with time consuming cumbersome maintenance procedure. Alternatively, cell lines can be sub-cultured indefinitely or maintained as a seed-lot for storage in liquid nitrogen. At present, vaccination with a chicken embryo adapted live virus is followed to control DP which is poorly immunogenic and affords only partial protection against the disease. Further, the embryo propagated vaccine virus also pose risk of harboring other infectious agents. The cell lines of avian origin are also costly and not easily available or maintained. DPV has been propagated only in homologous primary cell culture systems of avian origin, and no cell culture adapted vaccine strain has been claimed to be used as vaccine strain for DP till now. The present study is conducted to overcome these drawbacks in which a commonly available continuous and heterologous cell line system known as BHK-21 is used for the propagation and adaptation of DPV which is isolated by using duck embryo fibroblast (DEF) cell culture method. It does have significant value as a potential vaccine candidate for developing first cell culture vaccine against this important avian pathogen which is confirmed by studying characteristic cytopathic effects (CPE) in BHK-21 cells during passages and checking viral multiplication by detection of viral specific DNA by using polymerase chain reaction (PCR).

DP is directly or indirectly causing huge economic loses to the farmers. Therefore, it is essential to take necessary steps to control this disease for the development of the poultry industry. The present study was undertaken with the following objectives:

- Isolation of duck plague (DPV) virus using primary duck embryo fibroblast cell and subsequent observation of cell cytopathic effects.
- Propagation and adaptation of DPV in BHK-21 cell line towards the development of potential vaccine candidate.

MATERIALS AND METHODS

Cell cultures

The primary duck embryo fibroblast (DEF) cell culture was used for isolation and initial propagation of duck plague virus (DPV) which was prepared from 10 day-old embryonated duck eggs followed by standard protocol. The monolayer of primary DEF cell was grown in tissue culture flasks (25 cm2) at 37.5 ºC under 5% CO2 in growth media containing Minimum essential medium (MEM) supplemented with fetal calf serum (FCS), L-glutamine, sodium bi carbonate, HEPES and antibiotics (Penicillin and Streptomycin). The baby hamster kidney (BHK-21) cell culture was used for adaptation of DPV which was processed from cryovials preserved BHK-21 cells (in liquid nitrogen) and grown the monolayer as followed for primary DEF cell culture.

Samples Used

The samples were taken from virology laboratory, Animal Health Research Division, BLRI, Savar, Dhaka. A total of 40 PCR positive samples (previously identified under the project on "Prevalence and molecular characterization of duck plague virus in selected areas of Bangladesh" carried out during the financial year 2015-2016) were used for adaptation in BHK-21 cell line followed by isolation using duck embryo fibroblast cells (DEFC) towards the development of vaccine candidate.

Sterility test of inoculums

Bacteriological media was used for sterility test. For this purpose, little amount of inoculums were inoculated into fresh blood agar (BA) media at 37°C for 24 h to identify the presence of any type of bacteria in the collected samples.

Isolation of virus using primary DEF cell culture

The DEF cells were ready for infecting with virus inoculum after removing growth media from the tissue culture flask and washing for 2 times with phosphate buffer saline (PBS) respectively. Then the DEF cells were inoculated with 500 µl virus inoculums per 25 cm2 flask. The flask was then incubated at 37.5 ºC in the presence of 5% CO2. The flask was tilted every after 5-7 minute. After 45 minutes, the flask was removed from the incubator and added 7 ml maintenance media. After 48 hours, cell-cytopathic effects (CPE) were observed using an inverted microscope. After CPE observation the flask was kept at -20 ºC for preservation.

Passage and adaptation of DPV in BHK-21 cell line

Culture flask which contains confluent monolayer of BHK-21 cell line decontaminated with 70% alcohol. Growth media was discarded from the culture flask. Culture flask washed for two times with 6ml PBS. BHK-21 cells were Inoculated with 200 µl of DPV inoculums which were isolated using DEF cell culture method. Then the culture flask was incubated for 1h at 37.5 ºC and shook for 8 minutes interval so that cells layer could not dry. Maintenance media (5ml-6ml) was added to the culture flask. Finally the culture flask was incubated at 37.5 ºC and examined daily for the appearance of CPE. Infectious

fluids (IF) containing DPV were harvested after 48 to 72 hrs of post infection and kept at -20°C until further use.

Detection of DPV DNA in infected cell cultures by polymerase chain reaction (PCR)

QIAGEN Protease One-step Mini Kit was used for extraction of genomic DNA from the virus infected DEF and BHK-21 cell cultures at different passage levels. The extracted DNAs were subjected to PCR amplification by targeting DNA polymerase gene (446 bp) of DPV from the infected DEF cell cultures and BHK-21 cells. The details of the primer set are presented in Table 1.

Table 1. Primers used in the detection of DPV

Specificity	Primer name	Sequence
DPV	Forward	5'-GAA-GGC-GGG-TAT-GTA-ATG-TA-3'
	Reverse	5'-CAA-GGC-TCT-ATT-CGG- TAA-TG-3'

PCR products were then analyzed by 1.5% agarose gel, respectively stained with ethidium bromide and examined against UV light using an image documentation system.

RESULTS

Sterility test of inoculums

No bacterial growth was found in blood agar (BA) media which proved the samples were sterile.

Isolation of DPV using primary DEF cell culture

Five serial passages of each DPV containing samples were done on primary duck embryo fibroblast cells (DEF) to observe the cytopathic effects (CPE) and in every case the normal spindle shaped of the duck embryo fibroblast cells were degenerated and transformed into round shaped cells (Fig. 1). Extensive clumping of DEF cells was found after 48h post-infection with DPV. Then these viruses were confirmed by PCR (Fig. 3) using specific primer sets of DPV. After observing CPE, isolation of the virus was done. Out of 40 samples, 27 duck plague virus were isolated.

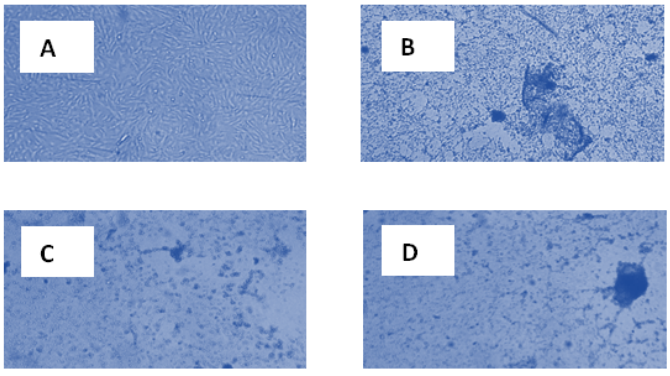

Fig. 1.Normal DEF cells (A). DPV infected DEF cell (1st passage: Initiation of infection and cell rounding started, photo taken after 12 hours, 40x), (B). DPV infected DEF cell (4th passage: Almost 100% cell infected, photo taken after 48 hours of infection, 40x), (C). DPV infected DEF cell (5th passage), (D).

Adaptation of duck plague virus and their effects on BHK-21 cell line

Six representative isolated samples of duck plague virus (DPV) out of 27 isolated samples were inoculated into BHK-21 cell line for adaptation in BHK-21 cell line. During initial four passages of DPV, infected BHK-21 cells showed no characteristic CPE followed by gradual development of CPE in the form of rounding of cells from 5th passage onwards. No detachment of cells was noticed during the first five passages. At the 7th passage CPE comprised of clumping of BHK-21 cells. Up to 8th passages, CPE was characterized by aggregation and clumping of rounded cells, finally death and detachment of BHK-21 cells(Fig. 2). Confirmation was done by PCR. The result of adaptation of DPV in BHK-21 cell line is showed in Table 2.

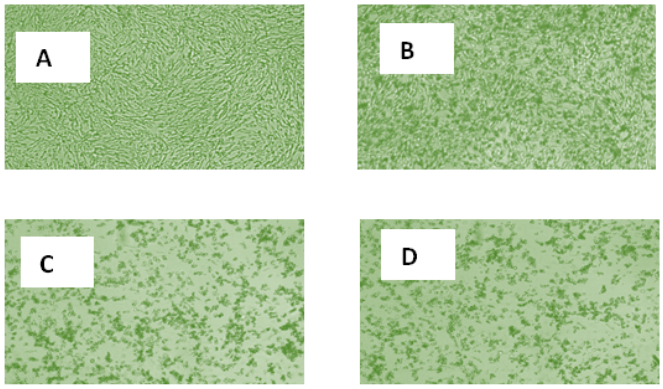

Fig. 2. Normal BHK-21 cells (A). DPV infected BHK-21 cell (5th passage: Initiation of infection and cell rounding started, photo taken after 12 hours, 40x), (B). DPV infected BHK-21 cell (7th passage: Almost 100% cell infected, photo taken after 48 hours of infection, 40x), (C). DPV infected BHK-21 cell (8th passage), (D)

Detection of DPV by Polymerase Chain Reaction (PCR)

The DNA of the virus infected DEF and BHK-21 cell cultures at different passage levels were processed for screening of target polymerase gene (446 bp) by PCR method. The PCR products were analyzed using 1.5% agarose in TAE buffer gel which contained 0.5 µl /ml ethidium bromide.

DISCUSSION

A proper host system is prerequisite for an experimental investigation involving a virus or the disease it causes. As virus cannot replicate without the host system, selection of appropriate host system is essential for its reproduction. Since 1950s, animal cells which are an excellent host for virus growth have been used for virus cultivation. The research work was undertaken for isolation of DPV in DEFC culture and adaptation of DPV in BHK-21 cell line in order to vaccine development against duck plague. Primary cell cultures were traditionally used by researchers to support the growth of duck plague virus. Limited life span is the major drawback of primary cultures. Therefore, they cannot be readily stored in liquid nitrogen for future passages. Moreover, various pathogens may be present during the preparation of CEF and DEF cell cultures on a regular basis from 10 to 11 days-old embryos derived from a flock. To keep the cell cultures free from avian pathogens, various tests has to be performed continuously which adds to the cost of vaccine production. A continuous cell line that would support DPV replication could have significant advantages for the rapid large-scale preparation of DPV vaccines. Till date, DPV has been reported to be adapted only in primary cell cultures of avian origin and only recently one report is available regarding its adaptation in duck cell line DEF-CCL-141 (Mondal *et al*., 2010).

Table 2. Adaptation of duck plague virus in BHK-21 cell line

No. of PCR positive sample	Virus isolation (DEFC)	No. of isolated DPV inoculated into BHK-21 cell	Adaptation of isolated DPV into BHK-21 cell	Confirmed by PCR
40	27	06	06	06

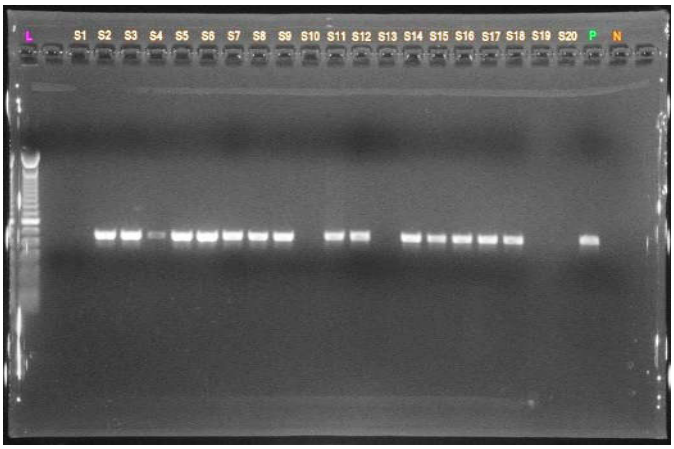

Figure 3. Electrophoresis of PCR products on 1.5% agarose gel stained with ethidium bromide

However, very few information are available for DPV adaptation in heterologous system of continuous cell line. In this context, an attempt was made to adapt DPV in BHK-21 cell line followed by studying parameters including observation of characteristic CPE and detection of viral specific DNA by PCR. In this study, primary DEFC culture was used for initial propagation and isolation of DPV. With characteristic CPE, 27 of DPV were successfully isolated from 40 PCR positive samples. Among the 27 of isolated DPV, six representative samples were then inoculated into BHK-21 cell line for adaptation. After propagating isolated DPV, infected BHK-21 cells showed characteristic CPE only after 5th passage. CPE comprised of clumping of host cell at the 7th passage and finally death and detachment of cells occurred at 8th passage. Similar CPE were observed showing rounded cells occurring throughout the monolayer followed by extensive clumping during DPV infecting the DEFC. These findings are strongly supported by findings of Guo *et al*. (2008) and Aravind *et al*. (2015). The DPV replication in BHK-21 cells was also confirmed by PCR amplification at 8th passage level. However, this BHK-21 cell line can be used to obtain an attenuation level for the adapted viral isolates of duck plague virus to develop a pathogen free and cost effective vaccine against duck plague.

Conclusion

Through this research work, 27 samples of duck plague virus out of 40 samples were isolated using duck embryo fibroblast cell culture method that was confirmed by PCR. Six representative samples among 27 isolated samples were used to adapt in BHK-21 cell line. These representative six samples were observed to be adapted in BHK-21 cell line by showing characteristic cytopathic effects on BHK-21 cell line. The presence of viral DNA was confirmed by PCR. The main focus of this study was to adapt duck plague virus in BHK-21 cell line in order to develop vaccine against duck plague virus. The result of the present study hopefully will be helpful in controlling duck plague disease through the development of vaccine in Bangladesh. The presently adapted DPV isolates in BHK-21 cells could have potential to be used as a vaccine candidate after following more passage and necessary attenuation, which might provide first cell culture vaccine for this important pathogen (DPV). However, explorative studies are suggested on vaccine development to control duck plague which is considered as a devastating disease of duck population in Bangladesh.

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