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Adaptation of Newcastle Disease Virus (NDV) on Vero Cell Line

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Abstract: Newcastle disease virus (NDV) is the infectious agent of Newcastle disease in poultry. This virus can grow within different animal cells including primary cell culture and established cell line. In order to adapt NDV on African green monkey kidney (Vero) cell line, five consecutive passages were done. Eagle's minimum essential medium (EMEM) with supplements was used for both culturing Vero cells and maintaining NDV on Vero cells. During first and second passage, wild NDV didn't produce any clear evidence of cytopathic effect (CPE), but in third passage changes in the characteristics of cell monolayer were observed. During fourth and fifth passages, clear and consistent CPE were observed within 50 to 60 hours of infection. CPE was characterized by formation of syncytium, giant cell, dendritic-shaped cell and finally plaque. The titer of passage 5 (P5) virus was $10^{-3.9}TCID_{50}$, whose purity was tested by serum neutralization test (SNT) and the result was 1.6×10^4 units/ml.

Key words: Adaptation, cytopathic effect (CPE), newcastle disease virus (NDV), vero cells

Introduction

The essential prerequisite for the experimental investigation of a virus or the disease it causes is the production in some laboratory host because virus can't replicate or multiply without any host system. Animal cells in culture have been using for cultivation of virus since 1950s, because it is an excellent host for virus growth. Newcastle disease virus (NDV) is the infectious agent of Newcastle disease in poultry (Doyle, 1927). Different types of primary cells particularly of avian origin and certain cell lines of mammalian origin are readily infected by NDV. Commonly used cell lines to replicate NDV are rabbit, pig, calf, chicken, monkey kidney cells, chicken embryo fibroblast (CEF), chicken embryo kidney (CEK), baby hamster kidney (BHK-21), HeLa cells, KB cells (Fenner *et al.*, 1974) etc. Vero cells, fibroblast like cells from African green monkey kidney, are anchorage-dependent in culture.

Adaptation refers, during isolation of viruses, there may emerge variants capable of multiplying more efficiently in the host cells used than the original wild type virus. Following adaptation, the infectivity of the virus to the adapted cells is increased but the virulence is decreased. Often such adapted variants damage the original host less severely than the wild type virus and are therefore said to be less virulent. NDV is purposely adapted on Vero cell line to alter growth and virulence characteristics so that Vero cells become a suitable laboratory host for cultivation, mass propagation attenuation and genetic modification of NDV.

Materials and Methods

The experiment was conducted at Virology Laboratory, Animal Health Research Division (AHRD), Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka-1341, Bangladesh.

Table 1: Data from tissue culture infective dose 50 ($TCID_{50}$) assay plate

Column No. in 96-well plate	log_{10} titer of virus dilution	Observed CPE percentage
A	0	100%
B	-1	100%
C	-2	100%
D	-3	90%
E	-4	40%
F	-5	10%
G	-6	0%
H	No virus (control)	0%

Culture media: Eagle's minimum essential medium (Eagle, 1955) with supplements were used for culturing Vero cells and maintaining NDV on Vero cells. Culture media was prepared by adding 5% heat-inactivated fetal calf serum (FCS), 2% L-Glutamine, 2% sodium bicarbonate and 1% antibiotic (penicillin, neomycin and streptomycin) solution. Similarly, maintenance media contained 1% FCS in place of 5%.

Adaptation of NDV on vero cell line: An established Vero cell line was collected from Gonoshastha vaccine research laboratory, Savar, Dhaka, Bangladesh. This collected cell line was sub-cultured at plateau state of it's growth phase. Cells were cultured in 25 cm^2 flask and incubated 37 ^oC. Cultured cells were observed carefully under inverted microscope until the formation of semi-confluent monolayer. Usually 24 hours of sub-culturing, semi-confluent monolayer of Vero cells developed within 25 cm^2 flask. At this stage, cells were infected by wild NDV. Each 25 cm^2 flask was infected with 0.25 ml NDV inoculum of 10^5 pfu/ml (plaque forming unit/ml). The culture medium was replaced by

Table 2: Data from serum neutralization test (SNT) plate

Test type	No. of row in 96-well plate	No. of column in 96-well plate	Dilution of serum used	Percentage of CPE observed
Homologous SNT	1-5	A	1 : 10	0
		B	1 : 20	0
		C	1 : 40	0
		D	1 : 80	0
		E	1 : 160	0
		F	1 : 320	40
		G	1 : 640	80
		H	No serum	100
	6	All column	No serum	100
Non-homologous SNT	1-5	A	1 : 10	90
		B	1 : 20	90
		C	1 : 40	100
		D	1 : 80	100
		E	1 : 160	100
		F	1 : 320	100
		G	1 : 640	100
		H	No serum	100
	6	All column	No serum	100

maintenance medium. Infected flasks were incubated at 37 ^{o}C, because at this temperature infectivity and reproducibility of NDV to any host is optimal (Foster and Thompson, 1957). Cell monolayer of infected flasks were examined twice per day under inverted microscope for CPE. This passage 1 (P1) virus was harvested by centrifugation at 600 × g for 15 minutes and stored at -70 ^{o}C. Harvested P1 virus was infected again to Vero cells using same media and techniques. Virus harvested through this second passage was designated as passage 2 (P2) virus. Similarly 5 subsequent passages were done and CPE was observed carefully during all passages. P5 virus was assayed and tested for its purity.

Tissue culture infective dose 50 ($TCID_{50}$): Vero cell suspension containing $5x10^5$ cells per ml was prepared from confluent monolayer by trypsin (0.5%) digestion and then by adding maintenance media. In a 96 well microtitre plate, 180 µl pre-warmed Hank's balanced salt solution (HBSS) was added to each well. Twenty µl virus suspension was added to each well of column A except A6. Through row A to G, 10 fold dilution of virus was performed. Wells of column H and row 6 did not get any virus and thus they were control. Eighty µl suspension was poured off from all wells. Then 200 µl of previously prepared cell suspension was added to each well and the plate was incubated at 37 ^{o}C for 72 to 96 hours. The gradual development of CPE was observed twice daily. The 96 well plate was stained with 1% crystal violet when complete CPE was observed. The cell monolayer was washed with deionized water until excess violet was removed. After drying, the plate was observed under inverted microscope and data was calculated to determine $TCID_{50}$. The final $TCID_{50}$ was the mean of four counts.

Serum neutralization test (SNT): The cell suspension was prepared as the same way of $TCID_{50}$ assay. A 96 well plate was recognized as rows 1 to 6 for homologous SNT and rows 7 to 12 for non-homologous SNT. Then 180 µl of pre-warmed HBBS was added to all wells of column A and 100 µl of pre-warmed HBBS was added to all the remainder wells of plate. Twenty µl NDV-specific monoclonal antibody containing serum was added to all wells of column A except A6 and A12 because they were serum control. Wells of row 6 and 12 and column H did not contain any serum because they were control. Then 100 µl serum suspension from column A was transferred to column B. Two fold dilution of serum through column A to G was performed by transferring 100 µl of suspension from column B to C, C to D and so on. Hundred µl virus suspension (10^2TCID_{50}) was added to all wells of the plate and 100 µl suspension was poured off from all wells. The plate was incubated at 37 ^{o}C for 45 min. Then 200 µl suspension of vero cell ($5O10^5$ cells/ml) was added to all wells of the plate. The plate was incubated at 37 ^{o}C for 3 days and observed twice per day for CPEs. Finally, the plate was stained as the same way of $TCID_{50}$ assay and observed under microscope to obtained data. In this test, wells of column 1 to 5 were for SNT against NDV, column 7 to 11 were against Reovirus and wells of row 6 and 12 were control.

Results and Discussion

Cytopathic effect (CPE): Infected cell monolayer gradually developed histological evidence of cell

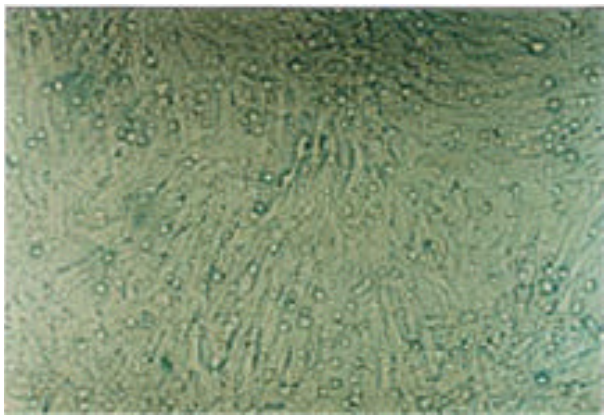

Fig. 1: Vero cells infected by NDV following 24 hours of infection

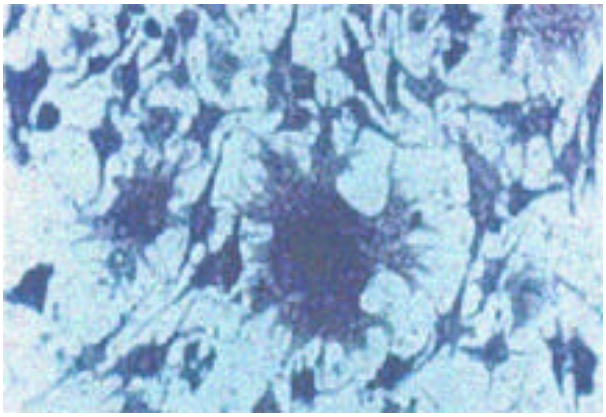

Fig. 3: Formation of dendritic shaped cells in Vero cell monolayer

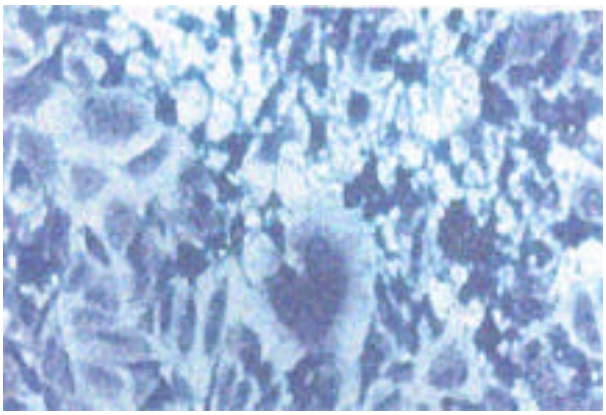

Fig. 2: Giant cell formation in Vero cells monolayer following 30 to 40 hours of infection by NDV

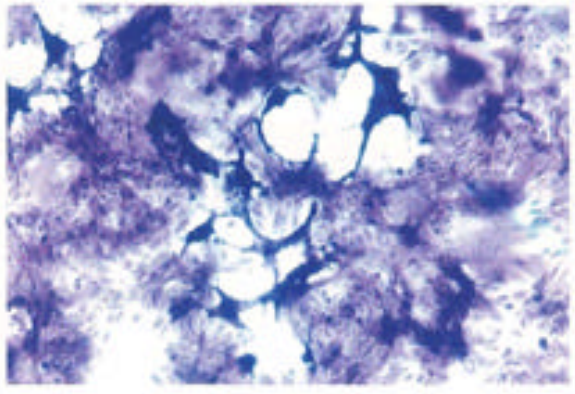

Fig. 4: Plaque formation in Vero cell monolayer following 50 to 60 hours of infection by NDV

damage, as newly formed virion spread to involve more and more cells in culture. During the first and second passages, wild NDV did not produce clear evidence of CPE. These viruses were just started to adapt on Vero cells and their infectivity to Vero cells were low. During the third passage, some changes in Vero cell monolayer began to develop after 24 hours of incubation following infection. Nevertheless, complete CPE of NDV on Vero cells were not found at this passage.

During fourth and fifth passages, CPE was rapid and consistent. Within 24 hours of infection, no CPE was found and the cells were looked as like as confluent monolayer (Fig. 1). After 24 hours of infection, cells were gradually started to change in shape to produce CPE. CPE was characterized by granularity in cytoplasm, rounding of infected cells, development of micro plaque, clustering of infected cells, intracytoplasmic bridge connecting those clusters, vacuolization in the cell system and the formation of syncytia. Syncytium is defined as irregularly shaped eosinophilic intracytoplasmic inoculation. A large number of clear syncytia were observed after about 30 to 40 hours of infection which were followed by the formation of multinucleated giant cells (Fig. 2). The nucleus of the few Vero cells was aggregated in several places on the monolayer. These multinucleated aggregated cells are called giant cells. After the formation of giant cells, further changes on cell monolayer occurred to form dendritic shaped cells (Fig. 3). Dendritic shaped cells were polygonal shaped to a stellate or dendritic appearance. During the terminal stage of CPE, the whole monolayer showed maximum degeneration of cells and large gaps throughout the monolayer, which are called plaque (Fig. 4). Plaques were formed during 50 to 60 hours following infection. The plaques of NDV on Vero cells have special characteristics, that is clear, red as well as intermediate turbid form and several size classes ranging from 0.5 to 4.0 mm in diameter.

Result of $TCID_{50}$: Table 1 represents data of $TCID_{50}$

assay plate. From these data, the infectivity titer of P5 adapted virus was calculated by using Karber method (Karber, 1931). The result found that P5 NDV suspension was $10^{-3.9}$ $TCID_{50}$. It indicates that, $10^{-3.9}$ times diluted Vero cell adapted P5 NDV are able to produce CPE in the 50% of the cell cultures inoculated.

Result of SNT: Table 2 represents data from SNT plate. From these data, the purity of P5 adapted virus was calculated. In case of homologous test against NDV, the result of serum neutralization test was 1.6×10^4 units/ml. Therefore, the result was very positive and it indicates that high concentration of NDV was present within P5 suspension. In case of non-homologous test, the result was almost negative, because almost 100% CPE was observed in all wells of the plate. This non-homologous SNT, against Reovirus, clearly indicated that the resultant virus following adaptation in Vero cells were NDV.

Different types of primary cells particularly of avian origin and certain cell lines of mammalian origin are used to cultivate and propagate NDV. Sinha (1958) reported that infection of NDV on chicken embryo is more rapid when inoculated embryos are young quality. Bankowski (1964) showed that NDV could be rapidly propagated in primary cells of avian origin. He used Chicken Embryo Fibroblasts (CEF) and Chicken Embryo Kidney (CEK) cells grown on monolayer on glass or plastic for infection by NDV.

Barahona and Hanson (1968) showed that lentogenic strain of NDV are cytopathic but fail to produce plaques in Chicken Embryo Fibroblasts (CEF) cells within 96 hours in absence of magnesium and dimethylaminoethyl (DEAE). They also found that velogenic strain of NDV, when grown on CEF monolayer usually exhibit large, clear or red plaques (2 to 4mm) or both and many also show small plaques (0.5 to 1.5mm) of either type. Granoff (1964); Schloer and Hanson (1968) showed that NDV induces formation of plaques of two basic types-clear and red as well as intermediate turbid form and several size classes ranging from 0.5mm to 4.0mm in diameter.

Kournikakis and Fildes (1988) cultured avirulent strain of NDV in LLC-MK_2 cells, plaque were visible after 2 days and maximum virus titer were reached in 3 days. Virus titer was not affected by continued incubation for 6 days, although plaque size increased.

Chicken embryo fibroblast (CEF) cells is the most popular throughout the world to grow NDV. But CEF cells are the source of many vertically transmitted diseases like Salmonella, Reovirus, avian leukemia, Marek's disease etc.

On the other hand, Vero cell line is used for cultivation, adaptation and attenuation of wide range viruses. In this case, 5 serial passages of NDV on Vero cell line confirm it's successful adaptation. During fourth and fifth passages CPE was regular and consistent with the formation of syncytium, giant cell, dendritic-shaped cell and finally plaque. The infectivity titer of P5 virus was $10^{-3.9}TCID_{50}$. Also, purity of P5 virus was tested by both homologous and non-homologous SNT and the resultant virus was completely pure.

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