Journal of Animal and Veterinary Advances 2 (8): 425-429, 2003

Adaptation of Reovirus on Vero Cell Line

K. M. D. Islam, T. Ahmed, M. M. Ahasan, M. M. Billah, M. E. Islam and K. M. Hossain
Biotechnology Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh

Abstract: In the present experiment, adaptation of avian reovirus (Uchida, TS-17 AVREOV-1) on vero cell line and characterization of adapted virus by serological tests were carried out. For adaptation, the anchorage-dependent vero cell was first sub-cultured in Eagle's minimum essential medium (EMEM) to form semi-confluent monolayer. This monolayer was then infected by the wild strain of reovirus. Subsequent passages of reovirus on vero cell line were performed by serially infecting the cell line with the virus. The first infection was done by the wild virus. The passage 1 (P1) virus was harvested and used for the next passage In this way, the viruses were given three serial passages on vero cell line where characteristic cytopathic effects (CPEs) were observed. During the third passage, the virus produced characteristic CPE for reovirus as in the adapted cell line. Syncytia and giant cells were clearly observed after 30-36 h, which were followed by plaque formation after 72 h of infection. The tissue culture infective dose 50 ($TCID_{50}$) was performed in adapted vero cells to determine the infectivity titre of P3 adapted virus and it was found $10^{-5.5}$ $TCID_{50}$, which indicated $10^{-5.5}$ times diluted vero cells adapted P3 virus were able to produce CPE in 50% of the cell cultures inoculated. The P1 virus showed less infectivity but highly virulent whereas the P3 virus became progressively more cytopathogenic or infective to vero cells. The purity of P3 adapted reovirus was detected by serum neutralization test (SNT), using specific anti-reovirus serum from rabbit. The homologous SNT against reovirus was $0.8x10^4$ units per ml, whereas the non-homologous SNT was almost negative.

Key words: Reovirus, vero cell line

Introduction

The term "Reo" is an acronym for respiratory enteric orphan. Reovirus is an important enteric virus and is presumably widely distributed (Enriquez and Willcox, 1990). The initial avian reovirus was isolated in 1954 by Fahey and Crawley from the respiratory tract of chickens (Closas *et al.*, 1986). It has a broad host range spectrum including human, cattle, horse, cat, rabbit and monkey in mammals and trout and birds in avis. Three different serotypes of mammalian reovirus do not cause obvious symptoms in mammals (Della-Porta, 1985). Avain reovirus, however, are important pathogen in chicken, turkey and ducks and causes significant economic losses through variety of diseases and abnormalities. Viral arthritis, tenosynovitis, ruptured gastrocnemous tendons, pericarditis, pasting in young chicken and acute and chronic respiratory syndrome are observed in chickens and turkeys caused by the reoviral infection (Gordon and Jordon, 1982; Timoney *et al.*, 1988 and Jordon, 1990). Poor feed conversion, poor flock uniformity, reduced weight gains, mortality and severe lameness are some economic losses associated with reoviral infection. The occurrence of avian reovirus in Bangladesh has been detected on June 1997 in Animal Health Research Division, Bangladesh Livestock Research Institute (personal communication).

Reovirus can infect and grow on various primary cell cultures of avian origin and certain cell lines of mammalian origin. Commonly used cell lines for reovirus in different laboratories are chicken embryo fibroblast (CEF), duck embryo fibroblast (DEF), chicken embryo kidney (CEK), HeLa cells, WI-38, L 929, BHK 21 etc. Reovirus grows in primary chicken cell cultures of embryo, lung, kidney, liver and testicles. Primary chicken kidney cells of 2-6 weeks are satisfactory, but for plaque and isolation, primary embryo liver cells are preferred (Guneratne, 1982). Following infection of chicken kidney cells, syncytia form and float free, leaving holes in the monolayer and giant cells floating in the media. With fully adapted virus, CPE occurs, as early as 24-48 h, but on original isolation, 3-5 blind passages may be needed to demonstrate typical CPE in the cultures. Reovirus can be grown in mouse L-fibroblast cell line, BHK 21/23, ITT cells, chicken embryo fibroblast (CEF), duck embryo fibroblast (DEF), WI-38 and HeLa cell lines.

The vero cell line was derived from the kidney of a normal, adult, African green monkey (*Cercopithecus*) on March 27, 1962, by Y. Yasumura and Y. Kawakita at the Chiba University in Japan. The spindle shaped cells are anchorage-dependent that grow on the inner-lower surface of the flask (Hives, 1983). Initial characterization of vero cells as a substrate for vaccine production was performed by the Institut Mèrieux, now known as Aventis Pasteur. In addition to its use as a vaccine cell substrate, this cell line has been used extensively for virus replication studies and plaque assays. Vero cells are sensitive to infection with SV-40, SV-5, measles, arbovirus, rubella, simian

adenovirus, poliovirus, influenza virus, vaccinia and others. The rationale behind the use of vero cells rather than primary monkey kidney cells is that these cells can be banked and well characterized. It also eliminates the ethical and economical issues of animal use. In addition, vero cells are aneuploid, which does not form tumors at the passage level (Rebecca Sheets. 2002). History and Characterization of vero cell line. Meeting on the vaccine and related biological products advisory committee May 12, 2000).

Most virological research is performed with strains of virus adapted to produce characteristic CPE in cultured cells. Adaptation usually results from the selection of those viral mutants best equipped with the cellular synthetic machinery. During the isolation of viruses, there may emerge variants capable of multiplying more efficiently in the host cells used for this purpose than the original wild type virus. Often such variants damage the original host less severely than the wild type virus and are, therefore, said to be less virulent or attenuated. Viruses are often purposely adapted to alter the growth and virulence characteristics. An example is provided by the attenuated vaccine virus strain, which are obtained by repeated passaging of virus virulent for one host in some other hosts, until virus strain with decreased virulence for the original host has been selected. Following adaptation, the infectivity of the virus to the adapted cells is increased but the virulence of the virus to the original host is decreased.

Literature survey indicates that till to date no research work has been undertaken to adapt reovirus on vero cell line. Therefore, the research work was carried out to adapt the reovirus on vero cell line and to characterize the adapted virus by serological tests.

Materials and Methods

The experiment was conducted at Virology Laboratory, Animal Health Research Division (AHRD), Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka-1341, Bangladesh during January 1999 to October 1999.

Vero Cell line: Vero cell line was collected from Gonoshastha Vaccine Research Laboratory, Dhaka, Bangladesh and maintained on EMEM (Sigma) maintenance media. The maintenance medium was supplemented with 1% heat-inactivated (56^0 C) fetal calf serum (Sigma), 2% L-glutamine, 2% sodium bicarbonate and 1% penicillin, neomycin and streptomycin solution (PNS). The culture media, EMEM, supplemented with 5% heat-inactivated fetal calf serum, 2% L-glutamine, 2% sodium bicarbonate and 1% PNS, was used for culturing the virus in the cell line. The cell line was maintained by subculturing in every 5 days interval. For subculturing, 1 ml of 0.5% trypsin (Sigma) solution was used to disperse the confluent monolayer evenly up to a suspension of vero cells.

Reovirus: The reovirus was a generous gift from Prof. M. Yoshimura, Consultant, Japan International Co-operation Agency (JICA), who collected the wild type reovirus strain Uchida, TS-17 AVREOV-1 from infected chickens in Dhaka, Bangladesh. The virus was stored in –80^0 C. Before starting any work with this stored virus, it was thawed to 25 cm^2 flasks by incubating in the incubator.

Adaptation of Reovirus: Semi-confluent monolayer of vero cells developed within the 25 cm^2 flasks after 24 hours of subculturing. For the infection of reovirus, the growth medium was removed from the cell culture and the monolayer was washed twice with phosphate buffer saline (PBS). The cells in 25 cm^2 flasks were infected with 250 μl reovirus inoculum of 10^5 plaque forming unit per ml. The inoculum was spread uniformly over the monolayer and incubated at 37^0 C for 45 min with intermittent rotation to allow absorption. Five ml of sterile culture medium was added to each 25 cm^2 flasks. The flasks were incubated at 37^0 C and monolayers were examined daily under microscope for CPEs. The virus samples were ready to harvest when 80% CPEs were observed. For harvesting, the flasks were transferred to -20^0 C for overnight. After thawing, the suspension from the flasks were taken into vials and centrifuged at 600Xg for 15 min to pellet the cell debris. The supernatant fluid containing the virus was collected and stored in –80^0 C. This was the passage 1 (P1) virus and used for the subsequent infection of the vero cells in the similar fashion. The P3 virus showed characteristic CPE and was used for further serological testing.

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 37^0 C for 72-96 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 1–6 for homologous SNT and rows 7–12 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 reovirus-specific serum (anti-reovirus antibody previously collected form Professor M. Yoshima, (Consultant, Japan International Co-operation Agency {JICA}, BLRI, Savar, Dhaka) 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° C for 45 min. Then 200 µl suspension of vero cell ($5X10^5$ cells ml^{-1}) was added to all wells of the plate. The plate was incubated at 37° 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 1-5 were for SNT against reovirus, column 7–11 were against Newcastle disease virus (NDV) and wells of row 6 and 12 were control.

Results

Cytopathic Effects (CPEs): Cytopathic effects involved rounding, granulation, vacuolization, clumping, syncytia formation of vero cell monolayer due to infection by reovirus. Following 24 h of infection no CPEs was found, the cells were looked as confluent monolayer (Fig. 1). After 24 h of infection, the cells were gradually started to changes in shape to produce CPEs. CPE was characterized by granularity in cytoplasm, rounding of infected cells, development of microplaque, clustering of infected cells, intracytoplasmic bridge connecting those clusters, vacuolization in the cell system and formation of syncytia. After formation of syncytia, the nuclei of the few Vero cells were aggregated in several places on the monolayer. These multinucleated aggregated cells were defined as giant cells (Fig. 2). 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. 3). Plaques were formed during 36 to 42 h following infection. The plaques of Reovirus on vero cells have special characteristics that are clear, red as well as intermediate turbid form and several size classes ranging from 0.5 to 4.0 mm in diameter.

Day-by-day observed CPES were

* About 0 to 24 h following infection: Vero cell monolayer
* About 24 to 28 h following infection: Rounding, granulation and vacuolization of cells
* About 30 h following infection: Syncytia formation
* About 36 h following infection: Giant cells formation
* About 36 to 72 h following infection: Plaque formation

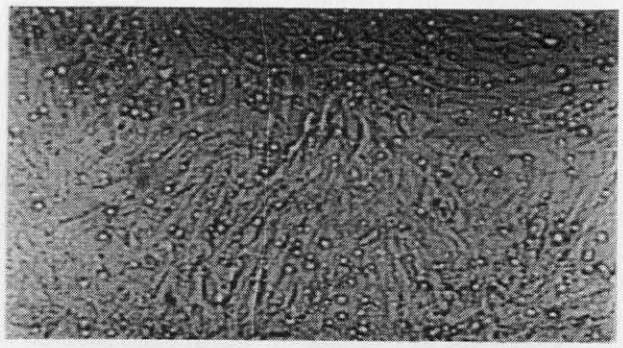

Fig. 1: Vero cells infected by reovirus following 24 h of infection

Outcome of $TCID_{50}$: About 36 h after infection reovirus produced suitable CPEs on vero cells. Stained plate was observed under microscope for percentage of CPE or cell damage obtained at each well (Table 1). Column H and row 6 did not produce any cell damages, because they were virus control. Wells of column A, B, C and D showed 100% CPE and column G did not produce any CPE. Wells of column E and F produced fractionated CPE, that is, column E showed 90% CPE,

Table 1: Data obtained from $TCID_{50}$ assay

Column	Log_{10} titer of virus dilution	Observed CPE percentage
A	0	100
B	-1	100
C	-2	100
D	-3	100
E	-4	90
F	-5	30
G	-6	0
H	No virus	0

Table 2: Data obtained from SNT

Type of SNT	No. of row	No. of column	Dilution of serum	Observed CPE percentage
Homologous SNT		A	1:10	0
		B	1:20	0
		C	1:40	0
		D	1:80	0
	1-5	E	1:160	20
		F	1:320	40
		G	1:640	80
		H	No serum	100
	6	A, B, C, D, E, F, G, H	No serum	100
Non-homologous SNT		A	1:10	90
		B	1:20	100
		C	1:40	100
		D	1:80	100
	7-11	E	1:160	100
		F	1:320	100
		G	1:640	100
		H	No serum	100
	12	A, B, C, D, E, F, G, H	No serum	100

F showed 30%. These observed data were sufficient to calculate $TCID_{50}$ titer of P3 adapted reovirus sample by using Karber method (Karber, 1931) and it was found that the infectivity titer of P3 adapted reovirus sample was $10^{-5.5}TCID_{50}$.

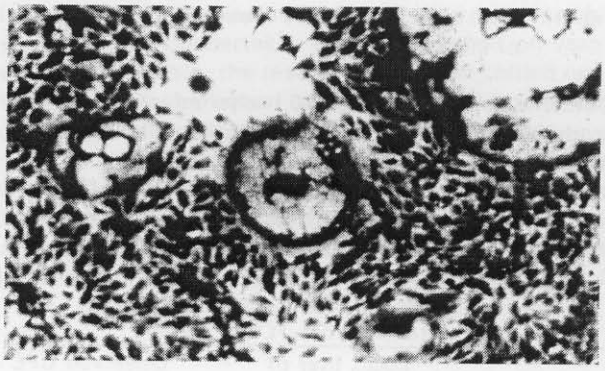

Fig. 2: Giant cell formation in vero cell monolayer following 30-36 hr of infection by reovirus

Outcome of SNT: It is found that all control wells (wells of row 6, 12 and column H) showed 100% CPE, because they did not contain any serum and all the viruses were active. In case of homologous SNT (row 1-5), column A, B, C and D did not show any CPE, because high dilution of serum present at that dilution which inactivated the virus. Column E, F and G showed 20%, 40% and 80% CPE respectively. In case of Non-homologous SNT (row 7-11), about 100% CPE was found all over the wells, because reovirus-specific serum can not neutralize any other virus. From the data obtained from SNT (Table 2), it is found in case of homologous SNT, CPE was obtained at 1:80 dilution of serum and the titer of virus used was 10^2TCID_{50}. The

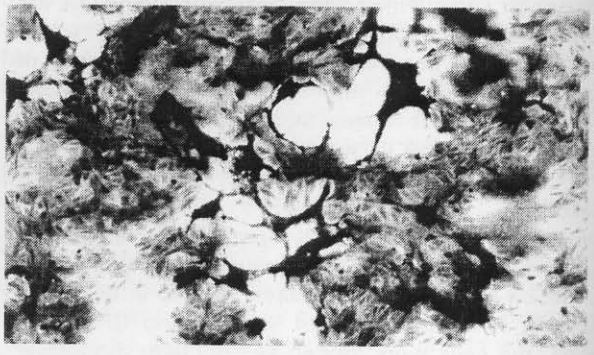

Fig. 3: Plaque formation in vero cell monolayer following 72 hr of infection by reovirus

homologous SNT against reovirus was $0.8x10^4$ units per ml. In case of non-homologous SNT, CPE was observed only in 1:10 dilution and the titer of virus used was 10^2TCID_{50}. The non-homologous SNT against NDV was $0.1 x10^4$ units per ml and it was negative.

Discussion

To adapt reovirus on vero cell line, the collected reovirus was given three serial passages on vero cells.

The resultant CPEs on each passage was observed carefully. Virus from each passage were harvested and clarified by centrifugation. The infectivity titer of reovirus present in each passage suspension was measured by $TCID_{50}$ assay. Homologous SNT as well as non-homologous SNT against reovirus recognized the purity of reovirus on passaged suspension, which was prevalent at that laboratory. During the first passage of reovirus on vero cells, P1 virus did not produce clear evidence of CPEs. The virus started to adapt on vero cells and their infectivity to vero cells were low. During the second passage, some changes in vero cell monolayer began to develop after 30 h of incubation following infection. Monolayer showed rounding, granulation, clumping of infected cells and vacuolization in the cell cytoplasm. During the third passage, CPE was rapid and consistent. In addition to above changes in cell monolayer, a large number of clear syncytia were observed after about 30-36 h of infection which were followed by formation of multinucleated giant cells. A large number of clear, red as well as intermediate turbid form plaques were also observed after 72 h of infection. The size classes of plaque ranged from 0.8 to 4.4 mm in diameter. These P3 viruses were well-adapted on vero cell lines.

Sil *et al.* (1996) studied the adaptation of Pesti des Petits Ruminants (PPR) virus on vero cell line after 5 serial passages. Virus produced typical CPEs in cells, such as formation of dendritic shaped cells, syncytium and gaint cells. Highest titre of virus was found on 96 hours post-infection, which decreased with increasd time of incubation. According to the findings of Peilin *et al.* (1997), complete CPEs of IBDV on vero cell line was stably produced in 65 to 72 h of inoculation during 4th passage. The present experiment showed that complete CPEs of reovirus were observed on vero cell line following 72 h of incubation during 3rd passage. This observation was found consistent with the findings of Sil *et al.* (1996) and Peilin *et al.* (1997).

Following three serial passage of reovirus on vero cell line, P3 viruses became progressively more cytopathogenic or infective but less virulent to vero cells. The infectivity titer of P3 viruses was $10^{-5.5}$ $TCID_{50}$. It indicates that $10^{-5.5}$ times diluted vero cell adapted P3 reoviruses were able to produce CPE in the 50% of the cell cultures inoculated. The purity of these P3 viruses was tested by SNT by using specific serum against reovirus. In case of homologous SNT test against reovirus, the result found was $0.8x10^4$ units per ml, which was very positive. In case of non-homologous SNT against reovirus, the result was $0.1x10^4$ units per ml almost negative. Compared to heterologous serum (anti-reovirus antibody), which clearly indicated that the resultant virus following adaptation in ver cell was reovirus.

Further study may help to determine the attenuation of virus for the production of live vaccine and mass production of test antigen.

References

Closas, A. S. G., G. Meulemenas and J. E. Lancaster, 1986. Update to avian disease. 1st edition, Office International des Epizootics.

Della-Porta, A. J., 1985. Veterinary Viral Disease: Their Significance in Southeast Asia and The Western Pacific. 1st edition, Academic Press International.

Enriquez, C. and G. E. Willcox, 1990. General characteristics of mammalian and avian Reoviruses. Poult. Abstract, 17: 621.

Gordon, R. F. and F. T. W. Jordon, 1982. Poultry Disease. 2nd edition. English Language Book Society.

Guneratne, E. M., 1982. Culture of Reovirus in primary chicken cell culture of embryo, lung, kidney, liver and testicles. Dev. Biol. Stand., 50: 355-359.

Hives, W. J., 1983. Biotechnology: Application of Animal Cell Culture. 2nd edition, Lea and Febiger.

Jordon, F. T. W., 1990. Poultry Disease, 3rd edition, Bailliere Tindall.

Karber, G., 1931. 50% end-point calculation. Arch. Exp. Pathol. Pharmak., 162: 480-483.

Peilin, W., C. Baoxiang and L. Jinsong, 1997. Adaptation and propagation of infectious bursal disease virus in Vero cells. Chinese J. Vet. Sci. and Technol., 27: 23-24.

Sil, B. K., M. M. Rahman, N. C. Debnath, M. J. F. A. Taimur, M. F. Rahman and A. J. Sarker, 1996. Adaptation of Pesti des Petits Ruminants (PPR) virus in Vero cell line. 14th Annual Conference of Bangladesh Society of Microbiology.

Timoney, J. F., J. H. Gillespie, F. W. Scott and J. E. Barlough, 1988. Human and Bruner`s Microbiology and Infectious Disease of Domestic Animals. 8th edition, Comstock Publishing Association.