INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2007/09–6–821–826
http://www.fspublishers.org

Studies on *In Vitro* Culture Characteristics of Adherent Baby Hamster Kidney-21 (BHK-21) Cell Line

SADEEQ-UR-RAHMAN[1], M. RABBANI, SAHIDULLAH, K. MUHAMMAD AND Z. IQBALL
University Diagnostic Lab and Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[1]Corresponding author's e-mail: sadeeqonline@gmail.com

ABSTRACT

A study was conducted to analyze the *in vitro* culture characteristics, growth pattern, growth requirements, growth effectors and cryopreservation of Baby Hamster Kidney-21 (BHK-21) cell line to optimize the *in vitro* culture requirements and conditions for maintenance and long time cryopreservation in liquid nitrogen. This would help persistence cultivation and maintenance of this cell line in cell culture laboratories. BHK-21 cells multiply fast during first 48 h and make a complete layer and got confluency with in 72 h post incubation, followed by a decline phase. Fetal calf serum has a growth stimulating effect and 5 - 10% serum level is satisfactory for the maintenance of cell line. While harvesting the cells from a flask, Trypsin (0.25%) with neutralization by fetal calf serum (5 - 10%) was found better. For cell storage 10% Dimethylsulfoxide (DMSO) through gradual cooling maintain maximum recovery of viable cells during cryopreservation.

Key Words: BHK- 21; Dimethylsulfoxide; Fetal calf serum; Cryopreservation

INTRODUCTION

Cell/tissue culture has been devised at the beginning of this century (Harrison, 1907) as a method for studying the behavior of animal cells, free of systemic variations that may arise in the animals either during normal homeostasis or under the stress of an experiment. As the name implies the technique is elaborated first with un-disaggregated fragments of tissue, with occasional mitosis in the outgrowth (Freshney, 1998).

Baby Hamster Kindney-21 fibroblast cell line has been established in March of 1961 (McPherson & Stoker, 1962). BHK-21 cells are susceptible to human adenovirus D, reovirus 3 and vesicular stomatitis virus (Indiana strain), but are resistant to poliovirus 2. In addition, the cells are negative for reverse transcriptase, indicating the lack of integral retrovirus genomes (McPherson & Stoker, 1962).

BHK-21 cell line is extensively used all over the world for propagation and maintainace of Foot and Mouth Disease Virus (FMDV) (Kalanidhi *et al*., 1993), for the extraction of Pseudorabies virus DNA (Wei-Wei *et al*., 1998) for production of ELISA antigen of Japanese encephalitis (JE) virus (Bundo *et al.,* 1989).

The dairy animals are an important component of our livestock, could not become a profitable industry in Pakistan due to infectious diseases, genetic discrepancies of animals, manage-mental, nutritional problems etc. Amongst bovine diseases, Foot and Mouth Disease (FMD) is a major constraint to the dairy industry and main cause of consternation to the farmers.

Although commercial virus vaccines are available for the immunoprophylaxis of FMD but these vaccines are either imported or prepared by conventional methods and therefore, not proved potent in controlling the outbreaks of FMD. To combat the disease, there is a dire need to develop a cell culture based virus vaccine containing local isolates of FMDV by adapting them on BHK-21 cells, which would need the maintenance of BHK-21 cell line. There are multiple steps such as growth, harvesting, storage and revival for persistent cultivation and maintenance of adherent BHK-21 cell line in Cell Culture Laboratories. The present study will help evaluate the behaviors of BHK-21 cells, maintenance of the cell line, preservation/storage of cells, growth and adaptation of FMDV to this cell line and thus produce cell culture based virus vaccine against FMD.

MATERIALS AND METHODS

Study on growth of BHK-21 cell line (cell division). The BHK-21 cell line was cultivated on 12 Carrel Flasks to develop monolayer using the technique as described by Hussain *et al.* (2003 - 04) explained below.

Production of BHK-21 cells monolayer. Three Roux flasks containing BHK-21 cell monolayers were dispersed by adding 0.25% v/v Trypsin solution. The growth medium (M 199, Biomedical; USA) containing 5% fetal calf serum, 300 mL for 12 carrel flasks at the rate of 25 mL medium per carrel flask was added in the Roux flask subsequent to detachment. After proper mixing 25 mL media was transferred to each carrel flask and was labeled and placed in incubator at 37^{o}C. After 24 h incubation three carrel flasks were taken and cells were harvested as previously discussed. After proper mixing an aliquot was taken for cell counting to determine live to dead ratio of cells by staining with Trypan Blue (0.4% w/v), as modified earlier (Davis, 2002).

Cell counting procedure. An aliquot 0.5 mL was collected

and mixed with 0.1 mL Trypan Blue 0.4% w/v solution in a dilution tube. Haemocytometer was loaded so that fluid entirely covered the polished surface of each chamber. The cells in the center and four corner primary squares of each grid (ten primary squares) were counted. When the Haemocytometer is properly loaded, the volume of cell suspension that will occupy one primary square was 0.1 mm cube (1 mm. Sq. X. 1 mm) or 10^{-4} mL. The cells with in ten primary squares (five primary square per chamber), were counted to give number of cells with in 1 mm^3 (10 x 0.1 mm^3) or 1 x 10^{-3} mL.

Total cell concentration in the original suspension in cells mL^{-1} was then:

Total count x 1000 x dilution factor.

A dilution factor was calculated as (volume of sample + volume of diluent/volume of sample Appendix-1).

Effect of chemical detachment agents on adherent BHK-21 cell line. The experiment was designed to see the effect of different chemicals; Trypsin (0.25%), Versene and Trypsin versene solution, which are used usually in Cell Culture Laboratories for cell harvesting (David *et al*., 1998) The cells (9.9 x 10^4 cells mL^{-1}), were equally distributed after proper mixing and transferred into three replicates of carrel flasks (25 cm square) containing 25 mL of medium 199 (modified). Each replicate containing three carrels was harvested after 72 h post incubation using Trypsin (0.25%), versene (0.012%) and Trypsin Versene solutions, respectively. Assessments of viable cells was done subsequent to harvesting, two hours and four hours post harvesting. Experiment was performed at 25 degree centigrade.

Effect of fetal calf serum on the growth of BHK-21 cells. The BHK-21 cells from Roux flask were harvested and transferred to 96 well gamma radiated cell culture plates such that each well receive 100 uL serum free medium 199 containing cells (1 x 10^5 cells mL^{-1}). The plate was divided into six replicates such that each replicate containing 16 wells column wise. Each well was added with 0, 2.5, 5, 7.5, 10 and 12% fetal calf serum and incubated at 37 degree centigrade. MTT Assay was performed 72 h post incubation (Muhammad, 1993).

Effect of different sources of serum on the growth of BHK-21 cell line. The BHK-21 cells from Roux flask were transferred to 96 well gamma radiated cell culture plates as previously, such that each well receive 100 uL serum free medium 199, containing cells (1 x 10^5 cells mL^{-1}). The plate was divided into six replicates such that each replicate containing 16 wells column wise. Each replicate was added with 10% cattle calf serum, Allanto-amniotic fluid, Sheep serum, Poultry serum, Goat serum and Fetal calf serum (all sera samples were gamma radiated & filterd by 0.2 um pore sized, syringe filter), respectively. The plate was incubated at 37°C for 72 h in which a complete monolayer is formed under normal circumstances that is when we use fetal calf serum as a growth promoting agent. After 72 h post cultivation MTT Assay was performed as discussed above.

Effect of cryoprotective agents (Dimethylsulfoxide (DMSO) and glycerol on cryopreservation of BHK-21 cell line. The cells for cryopreservation were selected at log phase log phase (Freshney, 1998). The harvested cells were resuspended (centrifuged @ 1000 rpm per three min) at the rate of 9.1 x 10^5 cells mL^{-1} (with 92% viability) in cell culture medium 199 (modified) containing 20% fetal calf serum and distributed in 30 (15 for each cryoprotective agent) aliquots. Each aliquot was containing 0, 2.5, 5.0, 7.5 and 10% DMSO and glycerol. The aliquots were transferred to -20°C for thirty minutes then -70°C for 3 h followed by storage in liquid nitrogen. On 10 days post storage, each of the aliquot was removed, liquefied in water bath at 37 C, centrifuged at 1000 rpm for 3 min. The pellet of each vial was suspended in the growth medium containing 10% fetal calf serum and transferred to carrel flasks containing 25 mL of the growth medium. An aliquot was taken to determine the cells viable ratio through counting. On 24 h post cultivation, the attached and those cells that had attained the normal shape were counted in the inverted microscopic fields.

Effect of different temperatures on preservation of BHK-21 cell line. BHK-21 cell line monolayers were harvested, resuspended in storage medium containing 20% fetal calf serum and 10% DMSO and trasfered to 18 aliquots, as previously discussed. Each aliquot stored at temperature mentioned below. On 7 days post storage, each of the aliquot was removed and revived as previously discussed. On 24 h post cultivation, the attached and those cells that had attained their normal shape were counted in the inverted microscopic fields. (Table I).

Cultivation and adaptation of viruses on BHK-21 cell line. BHK-21 cell line is widely used all over the world for propagation and isolation of FMDV. In this study we cultivate and adopt the local isolates of FMDV (Serotype "O" & "A"). It also contains an effort to cultivate.

Source of viruses. The Foot and Mouth Disease Virus (FMDV, serotype "O" & "A") was taken from the FMD project running in the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. Canine Parvo Virus (CPV) and Canine Distemper Virus (CDV) were obtained from University Diagnostic Laboratory (UDL). Newcastle Diseases Virus Vaccine (NDVV, LaSota) was obtained from commercial markete. HPSV infected liver was obtained from the Department of Microbiology, UVAS, Lahore. The inoculums of each virus was prepared properly and filtered through a syringe filter of 0.2 µm pore size.

RESULTS

Growth curve of BHK-21 cell line. Initial concentration of cells was 9.9 x 10^4 cells mL^{-1}. After 24 h incubation an increase in the live cells population i.e., 1.800 x 10^5, 1.300 x 10^5 and 2.100 x 10^5 with a mean value of 1.733 $\pm$ 0.621 x 10^5. During next 24 (48 h post incubation), hours the live

cell number was increased however a slight increase in the live cell population was observed in the next 24 (72 h post incubation). A complete monolayer was observed on the glass surface. There was a decline in the live cell number in the last 24 h (96 h post incubation) 2.000 x 10^5, 2.200 x 10^5 and 2.100 x 10^5 with mean value 2.100 $\pm$ 0.144 x 10^5 (Fig. 1).

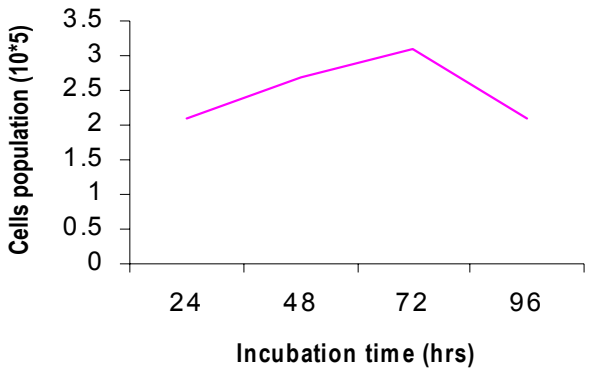
Effect of chemical detachment agents on adherent BHK-21 cell line. Subsequent to harvesting with Trypsin the cells viability was 95%, after two hours was 89.1% and after four hours 89.9%. Similarly subsequent to harvesting with versene the viable cells were 90%, after two hours were 84% and after four hours were 82.2%. The viable cells subsequent to harvesting with Trypsin versene solution were 93.3%, after two hours 84% and after four hours were 85% (Fig. 2).

Effect of fetal calf serum (FCS) on the growth of BHK-21 cells. The OD values with 0% FCS was with a mean value of 0.07 $\pm$ 0.008, with 2.5% FCS was 0.13 $\pm$ 0.02, with 5% FCS was 0.35 $\pm$ 0.05, with 7.5% FCS was 0.35 $\pm$ 0.05, with 10% FCS was 5 $\pm$ 0.12 and with 12% FCS 0.48 $\pm$ 0.10 were recorded (Fig. 1).

Effect of different sources of serum on the growth of BHK-21 cell line. OD values of Cattle calf serum, 0.406, 0.423, 0.467, 0.446, 0.428 and 0.326 with a mean value of 0.41 $\pm$ 0.05, of Allanto-amniotic fluid were 0.132, 0.128, 0.133, 0.112, 0.117 and 0.086 with a mean value of 0.14 $\pm$ 0.06, of Sheep serum, 0.457, 0.519, 0.464, 0.503, 0.468 and 0.503 with a mean value 0.49 $\pm$ 0.06, of Fetal calf serum, 0.590, 0.5820, 0.536, 0.553, 0.502 and 0.554 with a mean value 0.55 $\pm$ 0.03 of Goat serum, 0.556, 0.472, 0.520, 0.511, 0.523 and 0.598 with a mean value 0.55 $\pm$ 0.05 and of Poultry serum, 0.141, 0.119, 0.130, 0.137, 0.126 and 0.115 with a mean value 0.13 $\pm$ 0.009 (Fig. 3).

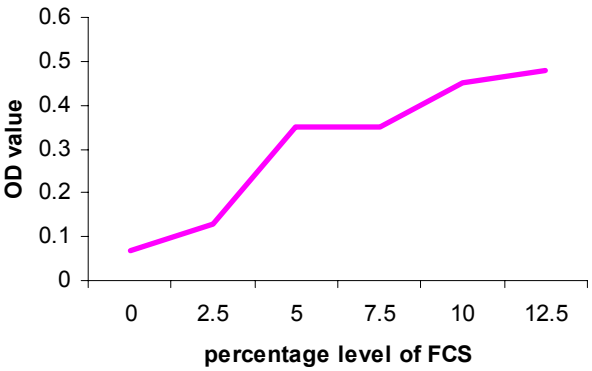
Effect of cryoprotective agents (DMSO & glycerol) on cryopreservation of adherent BHK-21 cell line. With trypane blue stain live to dead ratio of cells were counted, which showed 62% viability with 10% DMSO and 34% viability with glycerol. Number of live, attached and normal shape cells with 0.0% DMSO were 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, with average value 0, with 2.5% DMSO were 40, 41, 50, 35, 45, 30, 50, 33, 29 and 39 with average value 39.2 with 5.0% DMSO the cells were 55, 70, 63, 75, 75, 60, 79, 70, 58 and 58 with average value 66.3. similarly number of live, attached and normal shape cells with 7.5% DMSO were 96, 80, 100, 82, 110, 91, 83, 60, 105 and 92 with average value 89.9 and with 10% DMSO the cells were 145, 151, 110, 189, 170, 186, 215, 210, 190 and 220 with average value 178.6. Similarly cells with 0.0% glycerol were 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, with average value 0, with 2.5% glycerol were 36, 40, 33, 46, 40, 38, 50, 52, 45 and 32 with average value 41.20, with 5.0% glycerol the cells were 32, 38, 50, 37, 28, 36, 35, n 40, 58 and 60 with average value 41.4. Similarly numbers of live, attached and normal shape cells with 7.5% glycerol were 47, 50, 63, 44, 46, 52, 55, 42, 42 and 47 with average value 48.8 and with 10% glycerol the cells were 29, 32, 34, 20, 34, 40, 54, 73, 74, 57 and 65 with average value 51.2 (Fig. 4).

Fig. 1. Growth curve of BHK-21 cell line



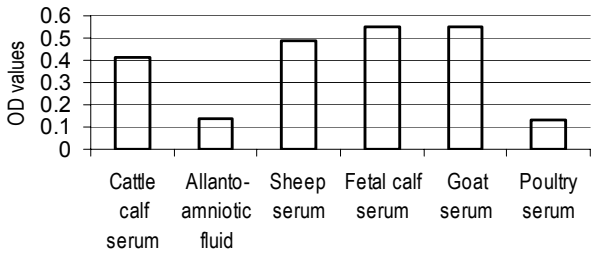
Above figure shows that the cell line population increases till 48 h after that stationary phase is achieved which is followed by decline phase.

Fig. 2. Effect of fetal calf serum on the growth of BHK-21 cells



The above figure shows the effect of FCS on the growth of BHK_21 cell line. It is clear that the FCS has growth promoting effect and 7-10 % serum has the desired stimulating effect to keep the cells for persistant cultivation and maintenance.

Fig. 3. Effect of different sources of serum on the growth of BHK-21 cells



The above figure shows that FCS has the maximum growth stimulating effect for BHK_21 cell line. These results should be shifted to the text and remove from here

Effect of different temperatures on preservation of BHK-21 cell line. On 7 days post storage, each of the aliquot was reviewed and incubated at 37°C. 24 h post incubations the cells were counted and recorded in (Table II). The number of attached and live cells of the aliquots at 25°C were "0"(zero), at -20°C were 39, 19, 33, 13, 18, 25,

Table I. Effect of chemical detachment of adherent BHK-21 cell line

Name of chemical	Time post detachment (hours)	Number of cells per mL of growth medium (cells x 10^5)			Percentage of cells	
		Live	Dead	Total	live	Dead
Trypsin (0.25%)	0	3.22	0.15	3.35	95	5.00
	2	3.15	0.41	3.55	89.1	10.90
	4	2.08	0.42	3.50	89.9	10.10
Versene (0.2%)	0	3.11	0.31	3.42	90	10.00
	2	2.57	0.48	3.05	84	16.00
	4	2.55	0.52	3.07	82.2	17.80
Trypsin (0.25%) + EDTA (0.0.012%)	0	3.01	0.21	3.21	93.3	6.70
	2	2.70	0.50	3.20	84	16.00
	4	2.80	0.40	3.20	85	15.00

Table II. Effect of temperature on preservation of BHK-21 cell line

S. No.	Storage time (hours)				Live cells per microscopic field 24 hrs post-cultivation	Average (%)
	0.05	0.50	3.00	168		
1	+25^{0}C	25^{0}C	25^{0}C	25^{0}C	0, 0, 0, 0, 0, 0, 0, 0, 0, 0,	0
2	+25^{0}C	-20^{0}C	-20^{0}C	-20^{0}C	39, 19, 33, 13, 18, 25, 19, 26, 22, 29	24.3
3	+25^{0}C	-79^{0}C	-79^{0}C	-79^{0}C	174, 163, 190, 182, 169, 201, 190, 180, 179, 203	183.1
4	+25^{0}C	-20^{0}C	-20^{0}C	-196^{0}C	47, 66, 39, 47, 49, 40, 28, 42, 38, 44	44.0
5	+25^{0}C	-20^{0}C	-79^{0}C	-196^{0}C	156, 162 ,206, 254, 157, 191, 159, 152, 194, 198	183.3
6	+25^{0}C	-196^{0}C	-196^{0}C	-196^{0}C	27, 18, 26, 24, 41, 30, 33, 28, 33, 25	28.5

19, 26, 22 and 29 with average value of 24.3, at -79^{0}C, were 174, 163, 190, 182, 169, 201, 190, 180, 179 and 203 with average value of 183.1, at -196^{0}C were 27, 18, 26, 24, 41, 30, 33, 28, 33 and 25 with average value of 28.5. The aliquots, which were stored at -20^{0}C for three hours and directly transferred to liquid nitrogen (-196^{0}C) were 47, 66, 39, 47, 49, 40, 28, 42, 38 and 44 with average value 44.0. Similarly the aliquots, which were first stored at -20^{0}C for half hour, then at -79^{0}C for three hours and at the end at -196^{0}C (in liquid nitrogen) for seven days were 156, 152, 206, 254, 157, 191, 159, 152, 194 and 198 with an average value 181.3. After comparing the values tabulated in Table II.

Cultivation and Adaptation of Local Isolates of Different Animal Viruses on BHK-21 Cell Line

Inoculation of FMDV (Serotype "O" & "A"). Forty eight hours post incubation about 20% CP was observed, while the control flask was ok. The flask containing virus was passed through two regular freeze and thaw cycles 72 h post incubation. Second inoculation results into 100% CP. FMDV cytopathic effect is characterized by rounding of cells, detachment of cells, clumping of cells and death of cells.

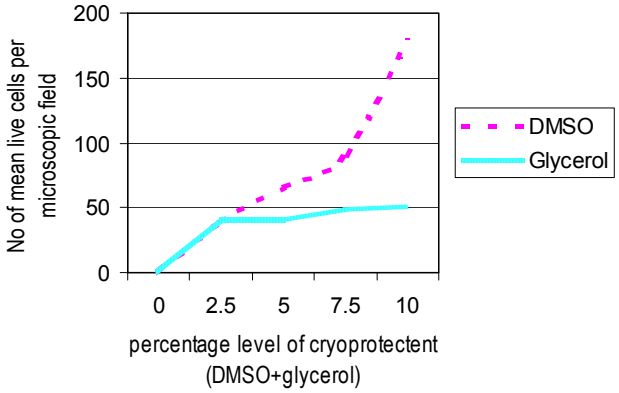
Cultivation of other animal viruses. The other animal viruses CPV, CDV, NDV and HPSV were given five blind passages but do not show any CPE, which shows that the cell line does not support the growth of these viruses.

Statistical analysis. All results were subjected to statistical analysis. Mean and standard deviation was calculated and compared using one way analysis of variance according to Minitab software Programme.

DISCUSSION

Cell culture has been developed into a sophisticated technique as a tool for diagnosis, isolation, identification and propagation of viruses and for the detection of viruses neutralizing antibodies.

Fig. 4. Effect of varying level of DMSO and glycerol on survival of BHK-21 cells during cryopresevration



Baby Hamster Kindney-21 fibroblast cell line has been established in march, 1962 and has been extensively used all over the world for propagation and maintenance of Foot and Mouth Disease Virus (FMDV) (Kalanidhi *et al.*, 1993), for the extraction of Pseudorabies virus DNA (Wei-Wei *et al.*, 1998) for production of ELISA antigen of Japanese encephalitis (JE) virus (Bundo *et al.,* 1989).

Existence of FMD has detrimental effects on export of dairy animals and their products (Chenard *et al.,* 2003). To control this most drastic viral infectious disease, there is a dire need to develop cell culture based viral vaccine, for which the cultivation and maintenance of cell line is necessary. Standardization of the protocols, requirements and conditions is necessary to maintain the cell line in cell culture laboratories. This project contains efforts to study and optimize the *In vitro* culture requirements, effect of harvesting chemicals, influences of different cryoprotective

agents and different temperatures during cryopreservation.

There are multiple steps such as growth, harvesting, propagation, storage and revival for the persistent cultivation and maintenance of BHK-21 cell line. In this study the effects of chemical detachment agents is determined. Several chemicals such as Trypsin, versene and trypsin-versene in different dilutions are used for the harvesting of cells. The process for sub-culturing and harvesting of cells with respect to concentration of harvesting agent have been optimized and the cells viability have been found satisfied by using trypsin (0.25% v/v) as a harvesting agent. These findings are in full agreement with ouyang *et al.* (2002), who recommended using 0.25% v/v trypsin for sub-culturing of keratenocytes.

While harvesting the cells, 2 - 5 mL trypsin (0.25% v/v, Gibco) was added to the flask containing cells after washing by PBS (A) solution. Cells detachment was monitored through inverted microscope. After complete separation of monolayers medium 199 conataining 5% FCS was added and incubated at 37^{o}C (Hussain *et al.,* 2003). It has been found that BHK-21 cells multiply fast during first 48 h, reaching highest concentration and making a complete monolayer till next 72 h post incubation. It is followed by a decline phase during, which the cells viability begins to decrease. Similar studies and results founded by (Ferri *et al.,* 1990; Hussain *et al.,* 2003).

This study reveals that FCS has growth stimulating effects on this cell line (kruman, 1981). MTT Assay has been employed to evaluate the growth stimulating effects of FCS and it has been shown that the color optical density is directly proportional to the density of viable cells. The activity of MTT Assay depends on the mitochondrial dehydrogenase activity, which transforms the MTT salt, (3 {4, 5-Dimethyzol-2-yl} 2, 5-Diphenyl Tetrazolium Bromide), into water insoluble Formazone in living cells. The quantity of Formazone is directly proportional to the number of living cells (Muhammad, 1993). These findings couple with those of Ciapti *et al.,* (1993), Gomez *et al*. (1997) and Ahne *et al*. (2005). Other animal sera have been tested for its growth supporting ability to BHK-21 cell line; it has been shown that FCS is the best growth promoter than bovine, sheep and goat serum. These findings are going parallel with (Kruman *et al*., 1985; Padamraj *et al*., 1991). Any how padamaraj *et al*. (1991) has also shown that PEG treated bovine serum has similar effect to FCS and better than goat serum. It has been thought that the enhancement in growth stimulating effect of bovine sera may be due to PEG treatment.

In this study project, cryopreservation of BHK-21 cells has been carried out to have a cell bank in the cell culture laboratory. Cell line at late log phase having shined and healthy sheath of monolayer and more than 90% viability was subjected for storage. It has been observed that long term preservation of cells can be carried out in liquid nitrogen at -196^{o}C using 10% DMSO as a cryoprotective agent and 20% FCS. These findings are in full agreements with findings of Birkland (1976), who stored cells with 8 - 10% DMSO. It has been founded that gradual cooling can minimize the shock (Freshney, 1998; Davis *et al*., 2002). Further more it has been studied that 10% DMSO with gradual cooling gives better cells viability than same concentration of glycerol, after long term storage. Similar observations were recorded by Armitage and jusa (2003). However Birkland (1976) found better results with 10% glycerol, which may be due to the equilibrium time of 15 min after the addition of storage medium to cell suspension. Cells cryopreservation has been performed by using 10% DMSO and 20% fetal calf serum, cells viability before storage in liquid nitrogen is 92% and after revival is 62%, which is better than at -79^{o}C. These findings go parallel with findings of (Hibno *et al.,* 1996; Davis *et al.,* 2002).

This study indicates that BHK-21 cells are susceptible to FMDV infection and these viruses can be adapted to this cell line by blind passages. FMDV inoculum has been introduced and passaged on a confluent monolayer (Esterhusy *et al*., 1988). The serum free media is replaced with 1% FCS after allowing the virus to adsorb. Forty two hours post incubation CPE of the virus has been observed. After two simultaneous freezing and thawing cycles, it has been inoculated again. CPE of the virus this time enhanced and it causes the rounding, detachment and death of cells. These findings are going parallal with the finding.

Total and percent viable cell count. Aliquot 0.7 mL collected from 25 mL cell suspension and mixed with 0.1 mL tryphan blue solution.

GRID: A. 21/0, 26/0, 24/0, 25/0, 18/1 = 115/1 (Numerator viable cells and the denominator are nonviable cells).

GRID: B. 20/1, 28/0, 26/1, 29/0, 26/0 = 129/2

Total cells = (viable + nonviable) = 247

% viable cells = 98%

Dilution factor = volume of sample + volume of diluent/volume of sample 0.7 + 0.1/0.0.7 = 1.14.

Cells per mL = total cells x 1000 x dilution factor

247 x 1000 x 1.14 = 2.8 x 10^5

Total number of cells = cells per mL x 25, 2.2 x 5 x 30 = 6.8^7.

Viable cells mL^{-1}, 2.7 x 10^5.

Non-viable cells mL^{-1}, 3.4 x 10^3.

Similarly cells were counted 48, 72 and 96 h post incubation and the record was tabulated in Table I.

REFERENCES

Ahn, J., C.H. Joo, I. Seo, D. Kim, Y.K. Kim and H. Lee, 2005. All CVB serotypes and clinical isolates induce irreversible cytopathic effects in primary cardiomyocytes. *J. Med. Virol.,* 75: 290–304

Armitage, W.J. and B.K. Juss, 2003. Freezing monolayers of cells without gap junctions. *Cryobiol*., 46: 194–6

Barnard, B.J. and H.J. Geyer, 1981. Attenuation of turkey meningo-encephalitis virus in BHK-21 cells Onderstepoort. *J. Vet. Res*., 48: 105–8

Ben, T.A., N. Kitabatake and E. Doi, 1990. Toxicity of dimethyl sulfoxide as a solvent in bioassay system with HeLa cells evaluated colorimetrically with 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl-tetrazolium bromide. *Agric. Biol. Chem*., 54: 2961–6

Birkeland, S.A., 1976. The influence of different freezing procedures and different cryoprotective agents on the immunological capacity of frozen-stored lymphocytes. *Cryobiol*., 13: 442–7

Bostandzhieva. R., Y. Ivanov and K.H. Kharalambiev, 1989. Micro-complement fixation test for antibodies to bovine respiratory syncytial virus. *Vet. Sbirka*., 87: 13–6

Bundo, M.K. and K.B. Morita, 1989. Detection of Japanese encephalitis virus antigens by the sandwich ELISA in infected cell culture fluid and cell homogenates. *Trop. Med. Nagasaki*., 31: 49–65

Ciapetti, G., E. Cenni, L. Pratelli and A. Pizzoferrato, 1993. In vitro evaluation of cell/biomaterial interaction by MTT assay. *Biomaterials,* 14: 359–64

Clavijo, A., P. Wright and P. Kitching, 2004. Developments in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. *Vet. J*., 167: 9–22

Cruz, H.J., A.S. Ferreira, C.M. Freitas, J.L. Moreira and M.J. Carrondo, 1999. Metabolic responses to different glucose and glutamine levels in baby hamster kidney cell culture. *Appl. Microbiol. Biotechnol*., 51: 579–85

Cruz, H.J., E.M. Dias, J.L. Moreira and M.J. Carrondo, 1997. Cell-dislodging methods under serum-free conditions. *Appl. Micro. Biotech.*, 47: 482–508

Davis, J.M., 2002. *In*: *Basic Cell Culture,* 2nd Ed, Pp: 167–70. Oxford University, Press U.K

Dujin, T., D. Panjevic, M. Popovic, M. Valcic and D. Cirkovic, 1990. Immunoprophylaxis of piglets against Foot and Mouth Disease. *Vet. Glasnik*., 44: 1061–6

Escarmis, C., E.C. Carrillo, M. Ferrer, J.F. Arriaza, N. Lopez, C. Tami, N. Verdaguer, E. Domingo and F.M.T. Franze, 1998. Rapid selection in modified BHK-21 cells of a Foot-and-Mouth Disease virus variant showing alterations in cell tropism. *J. Virol*., 72: 10171–9

Esterhuysen, J.J., G.R. Thomson, W.A. Ashford, D.W. Lentz, M.D. Gainaru, A.J. Sayer, C.D. Meredith, D.R.V. Janse and A. Pini, 1988. The suitability of a rolled BHK21 monolayer system for the production of vaccines against the SAT types of foot-and-mouth disease virus. I. Adaptation of virus isolates to the system, immunogen yields achieved and assessment of subtype cross reactivity Onderstepoort. *J. Vet. Res*., 5: 77–84

Ferrari, M., M.N. Losio, G.L. Gualandi and M.C. Bugnetti, 1990. Cultivation of Foot-and-Douth Disease virus in BHK21 cells grown in microcarrier culture system. *Micro*., 13: 161–3

Freshney, R.I., 1998. *In*: *Culture of Animal Cells: A Manual of Basic Technique,* 2nd Ed, Pp: 1–3. Alan R. LISS, Inc., N.Y

Gomez, L.A., A.E. Alekseev, L.A. Aleksandrova, P.A. Brady and A. Terzic, 1997. Use of the MTT assay in adult ventricular cardiomyocytes to assess viability: effects of adenosine and potassium on cellular survival. *J. Mol. Cell. Cardiol*., 29: 1255–66

Harison, R.J., 1907. *In*: Observation on Living Developing Nerve Fibers. *Proc. Soc. Exp. Biol. Med*., 4: 140–3

Hibino, Y., K. Hata, K. Horie, S. Torii and M. Ueda, 1996. Structural changes and cell viability of cultured epithelium after freezing storage. *Craniomaxillofac Surg*., 24: 346–51

Hosseini, M., R.A. Hawkes, P.D. Kirkland and R.J. Phelps, 1998. Rapid screening of embryonated chicken eggs for bluetongue virus infection with an antigen capture enzyme linked immunosorbent assay. J. Virol. Meth., 75: 39–46

Hussain, S., K. Muhammad, M. Arshad, M. Rabbani and Z. Iqbal, 2004. Passive immunization of Foot-and-Mouth Disease infected buffaloes. *Pakistan J. Sci. Res*., 56: 76–80

Jusa, E.R. and Y. Inaba, 1995. Salt- and pH-dependent hemagglutination with Kasba virus, a member of the Palyam serogroup of the genus Orbivirus. *J. Basic. Microbiol*., 35: 315–8

Kruman, I.I., N.F. Ostapenko, B.K. Gavriliuk and L.L. Marchenko, 1982. Presence of supposed growth factors in a serum fraction with a molecular weight of the components from 100,000 to 200,000 D. *Tsitologiia*, 24: 345–8

Kruman, I.I., N.F. Ostapenko, B.K. Gavriliuk, R. Ia Gordon and V.N. Karnaukhov, 1981. Arrest of BHK-21 cell culture growth and its stabilization in a resting state as affected by serum fractions. *Tsitologiia,* 23: 937–43

Kruman, I.I., S.N. Miakisheva and B.K. Gavriliuk, 1984. Age-related changes in the growth-stimulating effect of serum on BHK-21 cells. *Tsitologiia*, 26: 1043–7

Kurano, N.C., F. Messi, S. Kurano and A. Fiechter, 1990. Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor 2. Effects of medium components and waste products. *J. Biotechnol*., 15: 113–28

Macpherson, I. and M. Stoker, 1962. Baby Hamster Kidney Fibroblast Cells (BHK-21). *Virol*., 16: 147–51

Mehrotra, M.L. and D.C. Shukla, 1990. Seroprevalence, diagnosis and differential diagnosis of bluetongue virus disease in India. *Indian J. Virol*., 6: 98–103

Muhammad, K., 1993. "*In vitro* studies of chicken macrophages and their role in Salmonella related immunization failure". *Ph. D Thesis*, School of Biological Sciences, University of Surrey, Surrey

Ouyang, A.L., Y. Zhou, P. Hua and W.S. Tan, 2002. Effect of trypsin on the rat keratinocyte separation and subculture. *Sheng. Wu. Gong. Cheng Xue. Bao*., 18: 59–62

Padmaraj, A., K. Kumanan, A. Shyambabu and V.D. Padmanaban, 1992. Suitability of poly ethylene glycol (PEG) treated serum for growth of BHK-21 cell culture. *Indian Vet. J*., 69: 776–7

Ratajczak, J., W. Marlicz, T. Rozmyslowicz, B. Machalinski and M.Z. Ratajczak, 1996. Comparison of the different strategies for cryopreserving and storage of the bone marrow CD 34 + cells. Possibility of un-programmed rate freezing and storage at -80 degrees C mechanical freezer. *Annal. Transplant.*, 1: 35–8

Ratajczak, M.Z., W.I. Kuczynski and J. Ratajczak, 1994. Influence of different temperature of cryopreservation on the proliferative potential of human bone marrow progenitor cells. Transplantological implications. *Arch. Immunol. Ther. Exp*., 42: 217–21

Reddy, G.S.A. and V.A. Srinivasan, 1991. Immunogenicity of tissue culture Ranikhet disease (Newcastle disease) vaccine. *Indian Vet. J*., 68: 708–12

Shome, B.R., R. Shome, N. Srivastava and A.K. Bandyopadhyay, 1997. Infectious bursal disease in the Andamans: isolation and identification of the virus. *Indian Vet. J*., 74: 281–3

Simon, K.O., P.A.D. Whitaker, J.S. Youngner, C.C. Wendell and P.A.W. Dowling, 1990. Sequential disassembly of the cytoskeleton in BHK-21 cells infected with vesicular stomatitis virus. *Virol*., 177: 289–97

Swayne, D.E., R.G.M. Jhon, E.J.W. Jackwood and R.M.W. Pearson, 1998. *In*: *A Laboratory Manual for Isolation and Identification of Avian Pathogens,* 4th Ed, Pp: 223–34. American Association Avian Pathologists, Washington State University Press, Washington D.C

Wei, Wei., D. Zwang, D. Ran, J. Huang and Y. Meng, 1998. Cloning and probe preparation of pseudorabies glycoprotein gp 50. *Chinese J. Vet. Sci*., 18: 457–9

(Received 23 June 2006; Accepted 18 August 2006)