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AUGMENTATION OF BIOLOGICAL TITER OF FOOT AND MOUTH DISEASE VIRUS IN *IN VITRO* CULTURE

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ABSTRACT

Foot and Mouth Disease (FMD) virus grew well on BHK-21 cell line. The virus showed poor tissue culture infective dose 50 ($TCID_{50}$) that was log $10^{3.2\pm0.2}$ in BHK-21 cell line having Glasgow Minimal Essential Medium (GMEM) without Fetal Calf Serum (FCS). Addition of one per cent FCS in the medium @ one percent increased log $10^{7.1\pm0.2}$ units of virus $TCID_{50}$. Incubation temperature of 35^0 C and 37^0 C supported the multiplication and maintenance of BHK-21 cells and yielded log$10^{6.6\pm0.1}$ and log$10^{7.0\pm0.2}$units of virus $TCID_{50}$, respectively. Each serotype of FMD virus showed log$10^{6.29\pm0.07}$ units of virus $TCID_{50}$ in the stationary monolayer of BHK-21 cells in roux flask (75cm^2), log$10^{7.66\pm 0.02}$ units of virus $TCID_{50}$ in roller bottles (490cm^2) and log$10^{8.34 \pm 0.07}$ units of virus $TCID_{50}$ on 0.2 g of micro-carriers suspending in 200 ml of the growth medium in stirring bottle. The infectivity titer/$TCID_{50}$ of each of the virus serotypes was significantly higher in roller bottles than that achieved in roux flasks ($p<0.05$) and was significantly higher in stirring bottle containing micro-carriers suspending in the growth medium than that of harvested in roller bottle ($p<0.05$). It is concluded that the infectivity titer of the virus is directly proportional to number of BHK-21 cells in the culture system.

Key words: Anchorage dependant BHK-21 cells, roller bottles, infectivity titer, FMD virus

INTRODUCTION

FMD is a highly contagious viral disease of cattle, buffaloes, sheep, goats, etc. The disease is the main hurdle in the export of animal and their products. The disease spreads in a large population of susceptible animals (Awan et al., 2009) and is caused by an Aphthovirus of Picornaviridae (Grubman and Baxt, 2004). The virus has seven serotypes such as "A", "O", "C", "Asia-1", "SAT-1", "SAT-2" and "SAT-3" (Muhammad *et al.,* 2012:). Out of these, only "A", "O" and "Asia-1" types of FMD virus are prevailing in Pakistan (Khuwaja *et al*., 2009: Awan *et al.,* 2009). The virus grows in unweaned mice, foot pad of guinea pigs, bovine thyroid cells (BTY) and Baby Hamster Kidney cells (BHK-21) (Ferris *et al.,* 2002: Khuwaja *et al.,* 2009). The field FMD virus when grown first time on BTY cells show poor infectivity titer or biological titer. However, its infectivity titer is enhanced by repeated passage on BHK-21 cell line. BHK-21 cell lines are either anchorage dependent (adherent) or anchorage independent. Adherent BHK-21 cells grow on roux flask, roller bottle or surface of micro-carrier (Giard *et al.,* 1978). Each roux flask and roller bottle yields 1.7x10^7 and 4.4x10^7cells, respectively. However, cell yield in micro-carrier culture system depends upon number of micro-carrier / ml and total amount of growth medium in culture vessel yield (Akram et al., 2012). For vaccine production high biological titer of the virus is the basic requirement. In most of the biologics production units, FMD virus serotypes are grown in roux flasks (still culture system). This is a major impediment in production of vaccine to meet the requirements of the dairy industry. Present study is therefore designed to evaluate the factors (fetal calf serum, Temperature and culture system of adherent BHK-21 cells) augmenting biological titer of FMD virus serotypes in *in vitro* culture.

MATERIALS AND METHODS

Source of Baby Hamster Kidney (BHK-21) cells and its propagation: Baby Hamster Kidney (BHK-21) cells were procured from Department of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. The cryopreserved cells were revived using standard procedure (Altaf *et al.,* 2012). BHK-21 cells were grown in filter sterilized Glasgow minimum essential medium (GMEM) with Earl's salts (Biomedical; USA) supplemented with 5 % fetal calf serum (FCS). The liquefied cells (10^7 cells) were transferred to roller bottle (480 cm^2) containing 100 ml of the growth medium. The bottle was incubated at 37 °C with 5 % CO_2 for 60 hours and the cells were harvested and transferred to roux flask (175 cm^2) with 35 ml of the medium (Khuwaja et al., 2009), the roller bottle (Duran, GmbH, Germany) containing 100 ml of the medium and spinner flask containing 0.2g microcarriers/ 200 ml of the growth medium (Giard *et al.,* 1978) in order to observe the effect of culture system on the biomass production of FMD virus.

Source of Foot and Mouth Disease (FMD) virus and its cultivation: Each of the serotype of FMD virus ("O",

“A” and Asia-1”) was obtained from the Department of Microbiology and cultivated on monolayer of BHK-21 cells in the above mentioned different culture systems.

Fetal calf serum: Eighteen roller bottles having complete monolayer of BHK-21 cells with maintenance medium were divided into six groups (A, B, C, D, E and F), each containing three bottles. Sterilized FCS was added @ 0, 1, 2, 3, 4, and 5 percent aseptically in each bottle of group A, B, C, D, E and F, respectively. Monolayer in each of the bottle was infected with 3ml of freshly grown the FMD “O” virus and was incubated at 37^0C for 48 hours.

Incubation temperature: Twelve roller bottles having complete monolayer of BHK-21 cells with 100 ml of maintenance medium (one percent FCS) were divided into four groups (A, B, C and D), each containing three bottles. Monolayer in each of the bottles of each groups was infected with 3 ml of freshly grown the FMD “O” virus. Each of the bottles was incubated at 33^0C, 35^0C, 37^0C and 39^0C for 48 hours.

Culture system: Nine culture vessels in each of the culture systems (roux flask, roller bottle and spinner flask with micro-carrier) were divided into three groups (A, B and C), each containing three flasks of each of the culture system. Each of the culture vessels of each group was infected with 3, 5 and 7 ml of each serotype of FMD virus, respectively. Each of the vessels was incubated at 37° C for 48 hours

Biological Titration of the Virus ($TCID_{50}$): After incubation periods, cytopathic effects (CPE) of the virus was observed under inverted microscope and the harvested culture fluid was processed for biological titration ($TCID_{50}$) (Alhaji et al., 2011).

Statistical Analysis: Log value of each $TCID_{50}$ value of the virus was calculated and was processed for calculation of mean and SD values. The data thus recorded as a result of each of the above mentioned parameters was compared using one way analysis of variance (ANOVA) at 5% level of probability (SPSS 13.0).

RESULTS AND DISCUSSION

Cell culture adapted FMD virus grew well on BHK-21 cell line (Figure 1-3). There are many cell lines including BHK-21 cells, lamb kidney cells and bovine thyroid (BTY) cells used for growth of the virus. The virus has ligand molecules and host cells have specific receptors. The virus serotypes from field do not grow directly on monolayer of BHK-21 cells. This could be possibly due to coating of secretary antibodies on receptors of the cells (Jackson *et al.,* 1996). However, the virus can directly grow on primary monolayer of BTY cell then after 2nd, 3rd passages can adapt on BHK-21 cells (Ferris *et al.,* 2002: Khuwaja *et al*., 2009). First time growth of the virus on BHK-21 cells after passages on BTY gives 10^3titer. Repeated passages on BTY cells give increase $TCID_{50}$ of FMD up to 10^6 (Khuwaja *et al.*, 2009). The virus showed poor growth in BHK-21 cell line having cell culture medium without FCS (Figure 1). This could be due to rapid death of cells in the maintenance medium that don’t support replication of the virus. Addition of FCS in GMEM supported the maintenance of BHK-21 cells and also supported the replication of FMD virus. Addition of FCS in GMEM @ 1 and 2 % concentration proportionally increased the infectivity titer of FMDV, i.e., 10^3, 10^7, 10^7, respectively (Figure 4). However, further increase in FCS in GMEM didn’t improve the replication of FMDV and hence didn’t show significant increase in the infectivity titer of the virus. FCS neutralizes the residual amount of trypsin in inoculums and supports the attachment of cells with substrate and also support the maintenance of BHK-21 cells. This could be possible reason of having higher infectivity titer of virus at 1% FCS in the cell culture medium. Further increase of the serum in the GMEM improved the multiplication of BHK-21 cells but may not support the replication of the virus. Incubation temperature of 33^0C and 39^0C did not support the multiplication of BHK-21 cell line in the presence of GMEM supplemented with FCS and hence didn’t support the replication of FMD virus. This could be possible reason of having poor infectivity titer of the virus at above mentioned temperature. However, incubation temp of 35^0C and 37^0C supported the multiplication and maintenance of BHK-21 cells that might had supported the replication of FMD virus (Figure 5). This might be possible reason of high infectivity titer of $10^{6.6}$ and $10^{7.2}$ at 35^0C and 37^0C respectively. The replication/infectivity titer of virus is directly proportional to number of cells (Razdan *et al.,* 1996).

Anchorage dependant BHK-21 cells are grown in three ways. The cells are grown in roux flasks, dishes, and multiwell plates in stationary cultures, in inner surface of roller bottles which are rotated (3 rpm) on motorized racks and on micro-carriers suspended in growth medium in mechanically stirred vessels. These rolling culture vessels (Roller bottles) are widely used for producing large quantities of cells (Kretzmer *et al.,* 2002). Although BHK-21 cells are adapted to grow on micro-carriers to achieve high cell density but the roller bottles are better choice for growing monolayer of adherent cells (Kunitake *et al*., 1997: Kretzmer *et al*., 2002). In the present study, FMD virus type “O” serotypes showed $10^{6.4 \pm 0.15}$units of infectivity titer (Tissue culture infective dose 50-$TCID_{50}$) in the stationary monolayer of BHK-21 cells in roux flask ($75cm^2$), $10^{7.2 \pm 0.2}$units of $TCID_{50}$ in roller bottles ($490cm^2$) and $10^{8.13\pm0.1}$units of $TCID_{50}$ on 0.2 g of micro-

carriers suspending in 200 ml of GMEM growth medium in stirring bottle (Figure 3). FMD virus type "A" serotype showed $10^{6.0 \pm 0.2}$units of infectivity titer (Tissue culture infective dose 50-$TCID_{50}$) in the stationary monolayer of BHK-21 cells in roux flask ($75cm^2$),$10^{7.2\pm 0.1}$units of $TCID_{50}$ in roller bottles ($490cm^2$) and $10^{8.2 \pm 0.05}$units of $TCID_{50}$ on 0.2 g of micro-carriers suspending in 200 ml of GMEM growth medium in stirring bottle (Figure 4). FMD virus type "Asia-1" serotypes showed $10^{6.1 \pm 0.2}$units of infectivity titer (Tissue culture infective dose 50-$TCID_{50}$) in the stationary monolayer of BHK-21 cells in roux flask ($75cm^2$), $10^{7.0 \pm 0.2}$units of $TCID_{50}$ in roller bottles ($490cm^2$) and $10^{8.0\pm0.3}$units of $TCID_{50}$ on 0.2 g of micro-carriers suspending in 200 ml of GMEM growth medium in stirring bottle (Figure 5). The infectivity titer of the virus serotype was significantly higher in roller bottles than that achieved in roux flasks ($p<0.05$) and was significantly higher in stirring bottle containing micro-carriers suspending in growth medium than that of harvested in roller bottle ($p<0.05$). The infectivity titer of the virus is directly proportional to number of BHK-21 cells in the culture system (Altaf *et al.,* 2012: Khuwaja *et al.,* 2009: Salivac *et al.,* 2006).

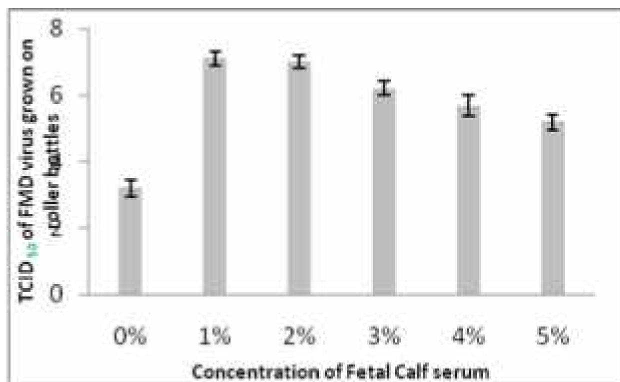

Figure 1. Effect of different concentrations of Fetal Calf Serum on the biomass production of FMD "O" virus ($TCID_{50}$)

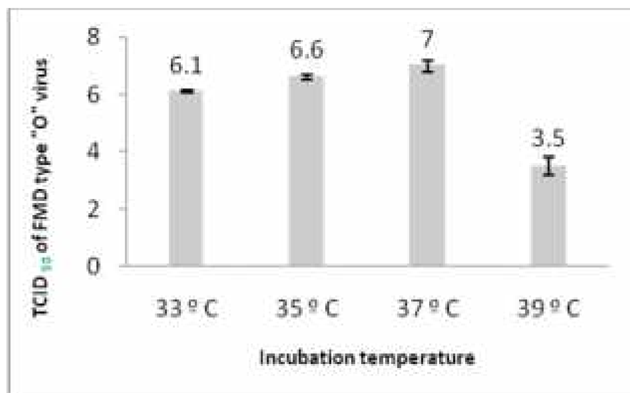

Figure 2. Effect of Incubation temperature on the biomass production of FMD "O" virus ($TCID_{50}$)

It is concluded that 1 – 2% of FCS, $35\text{-}37^0$ C incubation temperature and surface area of roller bottles for attachment of BHK-21 cells are critical factors for supporting maximum infectivity titer of FMD virus serotypes. However, infectivity titer of the FMD virus depends on number of micro-carriers suspended in growth medium in stirring vessels.

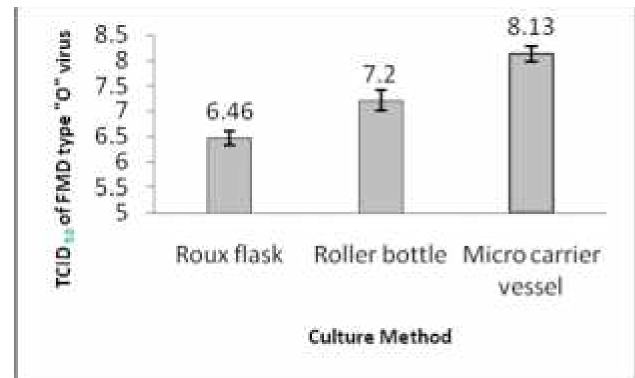

Figure 3. Biological titer of FMD "O" virus cultivated in Roux flask, Roller Bottle and Micro-carrier culture system

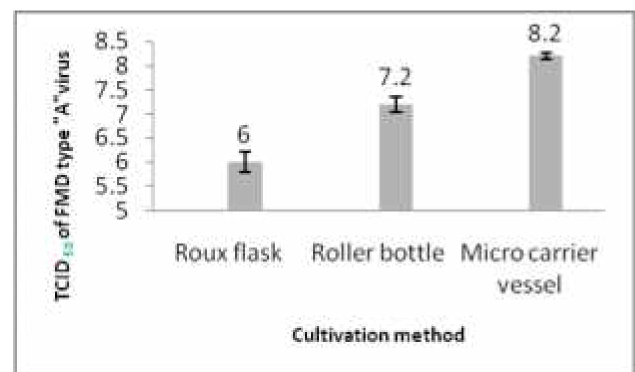

Figure 4. Biological titer of FMD "A" virus cultivated in Roux flask, Roller Bottle and Micro-carrier culture system

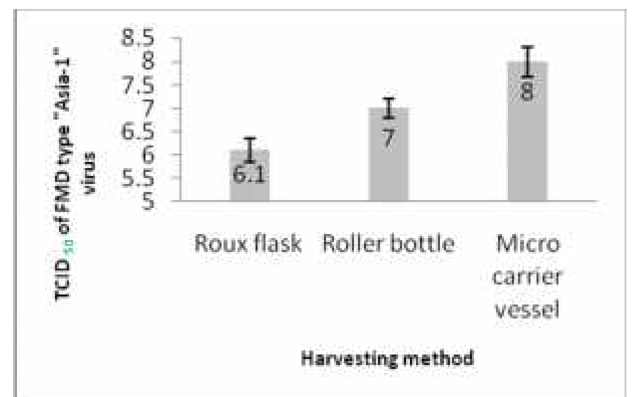

Figure 5. Biological titer of FMD "Asia-1" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

REFERENCES

Awan, F. N., M. A. Khan, K. Muhammad, M. Rabbani, M. Younus and R. M. Khawaja. (2009). Epidemiological investigation of foot and mouth disease in districts of punjab, Pakistan J. Zool., Suppl. Ser. No., 9: 179-185.

Alhaji, S., (2011). A modified arithmetical method of Reed and Muench for determination of a relatively ideal median lethal dose (LD50. African Journal of pharmacy and pharmacology, 5(12): 1543-1546.

Altaf, I., M. Saddique, M. Irshad, M.Z. Khan, A.A. Anjum and M. Kamran (2012). Antibody response of Rabbits to combined Hemorrhagic Septicemia and Foot and mouth disease virus vaccine. The J. Anim. Plant Sci. 22(2): 501-504.

Buxton, A. and G Fraser (1977). Animal Microbiology. Vol. 1 and 11. Blackwell Scientific Publication, Edinburgh. pp 339-341 & 611-618.

Berinstein, A., M. Roivainen, T. Hovi, P.W. Mason and B. Baxt (1995). Antibodies to the vitronectin receptor (integrin v 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J. Virol. 69:2664-2666.

Ferris, N.P., G.H. Hutchings, H.J. Moulsadle, J. Golding and J.B. Clarke (2002). Sensitivity of primary cells immortalized by oncogene transfection for the detection and isolation of foot-and-mouth disease viruses. *Vet. Microbiol.,* 84: 307-316.

Giard, D.J., W.G. Thilly, D.I. wang and D.W. Levine (1978). Virus production with newly developed microcarrier system. Applie and envr. Micro. 34(6): 668-72.

Grubman, M.J., B. Baxt and S. Curry (2004). Foot-and-mouth disease. Clinical Microbiology Reviews. 17(2): 465-493.

Jackson, T., F. Ellard, R. Abu-Ghazaleh, S.M. Brooks, W. E. Blackmore, A.H. Corteyen, D. Stuart, J. Newman, and A. King (1996). Efficient infection of cells in culture by type O foot-and-mouth disease virus require binding to cell surface heparin sulphate. J. Virol. 70: 5282-5287.

Kunitake, R., A. Suzu, H. Ichihashi, S. Matsuda, O.H. Osamu and K. Morimoto (1997). Fully-automated roller bottle handling system for large scale culture of mammalian cells, *J. Biotechnology.* 52: 289–294.

Kretzmer G (2002) Industrial processes with animal cells. Applied Microbiol. Biotechnol, 59: 135–142.

Khawaja, R. M., K. Muhammad, W. Shahzad, I Hussain, Z. I. Choudhry, F. N. Awan, A. Bukhari, I. Altaf and A. Hanif (2009). Isolation, adaptation and sero-typing of local strains of foot and mouth disease virus Pakistan J. Zool., Suppl. S# 9: (Part II): 173-177. Sutmoller, P. (2003). Control and eradication of FMDV, Virus Res., 91: 101-144.

Muhammad, K. (2012). Augmentation of combined oil based Foot and Mouth Virus (‘O’, “A” and “Asia-1”) and Hemorrhagic Septicemia vaccine production potential and immunoprophylaxis of buffalo and cattle against the diseases. Final technical and financial report, Department of Microbiology, University of Veterinary and Animal Sciences, Lahore,

Razdan R, AK Sen, BV Rao and VV Suryanarayana, 1996. Stability of foot-and-mouth disease virus, its genome and proteins at 37°C. Acta Virol, 40: 9-14.

Slivac I, Sr ek V G, Radoševi K, Kmeti I and Kniewald Z 2006 Aujeszky’s disease virus production in disposable bioreactor; *J. Biosci.* 31: 363–368.

Villegas, P and H.G. Purchase (1998). Titration of biological suspensions, In: *A laboratory manual of isolation and identification of avian pathogens*. Edited by The American Association of Avian Pathologists, USA, pp. 248-253.