FACTORS AFFECTING BIOMASS PRODUCTION OF BABY HAMSTER KIDNEY CELL LINE IN ROLLER BOTTLE CULTURE SYSTEM FOR THE PRODUCTION OF FOOT AND MOUTH DISEASE VIRUS

QAISER AKRAM

2007-VA-432

A THESIS SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

UNIVERSITY OF VETERINARY AND ANIMAL SCIENCES, LAHORE

2012


بسم الله الرحمن الرحيم
٨٦ حامدا ١٣

To,

The Controller of Examinations,

University of Veterinary & Animal Sciences,

Lahore.

We, the Supervisory Committee, certify that the contents and form of the thesis, submitted by **Qaiser Akram, Regd. No. 2007-VA-432** have been found satisfactory and recommend that it to be processed for evaluation by the External Examiner (s) for award of the degree.

SUPERVISOR _____

Prof. Dr. Khushi Muhammad

MEMBER _____

Prof. Dr. Masood Rabbani

MEMBER _____

Prof. Dr. Kamran Ashraf

TABLE OF CONTENTS

DEDICATED

To

The HOLY PROPHET MUHAMMAD

(Peace Be Upon Him)

To

My Lovely Family

(Father, Mother, Sister: Dr. Madiha Akram and Brothers)

To

My Honorable Teachers

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am thankful to the most Gracious, Merciful and Almighty **ALLAH PAK** who gave me the health, thoughts and opportunity to complete this work, I bow before my compassionate endowments to **HOLY PROPHET (Peace Be Upon Him),** who I sever a torch of guidance and knowledge for humanity as a whole.

Words are inadequate in the available lexicon to avouch the excellent guidance given by my major advisor/ supervisor **Prof. Dr. Khushi Muhammad** (supervisor), Chairman, Department of Microbiology**, Prof. Dr. Masood Rabbani** (member)**,** Director, University Diagnostic Laboratory (UDL) and **Prof. Dr. Kamran Ashraf** (member)**,** Department of Parasitology**,** University of Veterinary and Animal Sciences (UVAS), Lahore, for their keen interest, guidance and valuable suggestions during my studies.

I am deeply thankful to **Dr, Jawad Nazir** (Assistant Professor UVAS, Lahore) and **Dr. Huaguang Lu**, Associate Director, The Animal Diagnostic Laboratory (ADL), The Pennsylvania State University, USA, for their valuable advice in my research work. Their constructive condemnation and their moral support persuade me to improve my work.

I am extremely thankful to **Higher Education Commission (HEC), Islamabad, Pakistan** for funding me for PhD studies and research. I am also grateful to HEC for providing me opportunity to visit and get training from **American Diagnostic Laboratories** under its **International Research Initiative Support Program (IRISP).** This training improved my practical skills in cell culture, molecular diagnosis and gene sequencing of animal viruses.

(QAISER AKRAM)

CHAPTER-1

INTRODUCTION

Cell culture is collection of cells from an animal or its embryo and *in vitro* propagation by providing their physical and chemical requirements. It is widely used in biological sciences to propagate viruses either for diagnostic or vaccine production and many other purposes (Butler, 2003). The cultured cells are categorized as primary cells, when taken directly from the animals, or established cell line with a potential to grow indefinitely under laboratory conditions. Cell lines derived from immortal cells are used for a variety of research purposes. Cells are grown as adherent culture like epithelial or fibroblastic cells or in suspension form like cells of lymphoblastoid lineage. Most cells derived from solid tissues are adherent in nature. Moreover, adherent cell lines are provided matrix coated micro-carriers for their attachment. These micro-carriers enable their survival and proliferation in suspension culture (Freshney, 1998). Based upon the tissue of their origin, the cells are categorized as fibroblasts, skeletal muscle, cardiac, and epithelial (liver, breast, kidney, etc) cells. A number of cell lines are developed from mammalian, primates, reptiles and insects. Most common mammalian cell lines include Baby Hamster Kidney (BHK-21), Vero, HeLa, Chinese Hamster Ovary (CHO), Median Durbine Chicken Kidney (MDCK), Median Durbine Bovine Kidney (MDBK) etc. BHK-21 is an adherent kidney fibroblastic cell line that was established in 1962 and is widely used for propagation of animal viruses (Wei-Wei et al. 1998) and production of biologics (Bundo et al. 1989).

Foot and Mouth Disease (FMD) is a viral disease of cloven footed animals such as cattle, buffalo, sheep, goat, deer, camel etc (Klein, 2008; Pereira, 1981). The disease is characterized by pyrexia, lameness, and vesicular lesion on dorsum of tongue, feet and udder (Grubman et al., 2004). This disease causes massive economic losses in dairy animals that are measured in terms

of high mortality and morbidity, loss in milk production, working efficiency, quality of hide and weight gain, abortions, cost of treatment and consternation to the farmers (Murphy et al. 1999; Awan et al. 2009). Causative agent of the disease belongs to genus Apthovirus of Picornaviridae. There are seven dissimilar serotypes of FMD virus such as "O", "A", "C", "Asia-1", SAT-I, SAT-II, and SAT-III. None of the serotypes have cross immunity against each other. Serotype "O" is the most common worldwide. The disease is endemic in Pakistan and outbreaks are mainly caused by "O", "A" and "Asia-1" serotypes (Ajmal et al. 1989; Awan et al. 2009 and Khawaja et al. 2009). FMD virus was isolated from clinical specimen by growing on bovine thyroid primary cells followed by subsequent adaptation on the BHK-21 cell line (Khawaja et al. 2009) and was confirmed through virus neutralization test (VNT), complement fixation test (CFT), Enzyme Linked Immunosorbant Assay (ELISA) or Reverse Transcriptase Polymerase Chain reaction (RT-PCR) (Murphy et al, 1994: Adam and Marquardt, 2002; Reid et al. 2003).

Currently, various approaches are used to control FMD including implementation of strict bio security measures, movement restrictions on the animal and their products from the infected regions, proper disinfection of animal housing facilities, culling of infected animal and use of effective vaccination (Kretzmer et al. 2002; Amass et al. 2004; Singh 2003 and Velthuis and Mourits, 2007**)**. Keeping in view poor economic condition of the farmers and without any compensation, culling of infected animals is not possible in developing countries. Mass vaccination of the susceptible population is the only effective way to control and eradicate the disease. Chemically inactivated gel based multivalent vaccines are used for immuno-prophylaxis, but outbreaks are common even after their use (Meyer and Kundersen, 2001). In Pakistan, inactivated trivalent vaccine is used to vaccinate the animals (Muhammad 2012; Altaf et al. 2012). The maternally derived anti-FMD virus antibodies protect the newborn animals against

the disease immediately after birth and the antibodies may also interfere with development of the active immunity which follows post vaccination (Kitching and Salt 1995). Currently, used vaccines have a narrow antigenic spectrum, quite expensive and provide short-term protection to the inoculated animals (Rodrigue and Grubman, 2009).

FMD virus serotypes cab be propagated on Baby Hamster Kidney (BHK-21) cells in stationary culture (Roux flasks) but doesn't provide the required titer of the virus immunogen (Khuwaja et al. 2009). High cell density is required for mass production of virus serotypes. Various cell culture systems like roller culture bottles, multilayer dishes, micro carriers and suspension cultures are available for enhanced bio mass production of FMD virus serotypes (Ferrari et al. 1990; Freedman et al. 1996 and Slivac et al. 2006). The roller culture system may be an economical way to propagate high density cells and subsequently augment biological titer of the virus. The present study is therefore designed to attain the following objectives.

OBJECTIVES

Following factors affecting biomass production of baby hamster kidney cell line (BHK 21) through roller culture bottle system are optimized.

1. Cell density
2. Rolling speed of the cell culture bottles
3. Volume of cell culture medium per bottle
4. Concentration of Fetal Calf Serum in growth medium
5. Incubation time and temperature

Moreover, following factors affecting biomass production of foot and mouth disease virus (FMDV) through roller culture bottle system are also optimized.

1. Incubation temperature for interaction of the virus with the cells
2. Biological titer of the virus
3. Concentration of Fetal Calf Serum in cell culture medium

CHAPTER-2

REVIEW OF LITERATURE

Agriculture and Livestock are economically imperative sectors of Pakistan. Livestock is the major supporting sector of about 30-35 million of people living in rural areas. Small scale livestock farmers keeps 4-5 cattle/buffalo, 6-7 sheep/goat and 10-12 chickens and ducks in the rural areas. The share of agriculture sector in Pakistan's (GDP) is over 20.90 percent, out of which the livestock sector has over 11.5 % share in national GDP. Overall the livestock has more than 55.1% share in the agriculture sector. Pakistan has nearly 68.3 million cattle and Buffaloes, 65.5 million goats, 29 million sheep and 1.3 million camels. Total 2.327 million tons of meat and 46.44 million tons of milk are being provided by these dairy animals every year (Economic Survey of Pakistan, 2011-2012). Pakistan's livestock faces many nutritional, managemental and infectious issues and are directly or indirectly affects the dairy farming. Out of many infectious diseases, Foot and Mouth Disease (FMD) is common in Pakistan and causing severe economic loss to dairy farmers (Nazir, 2006).

2.1. Foot and Mouth Disease (FMD)

Foot and Mouth Disease (FMD) is a highly contagious viral infection of cloven-hoofed animals (Klein, 2008), which has worldwide occurrence. The disease is endemic in Pakistan. FMD outbreaks have been documented in Argentina, Brazil, Botswana, Bolivia, China, Ecuador, Egypt, Guinea, Israel, Jordan, Kazakhstan, Korea, Lebanon, Palestine, Russia, South Africa and Turkey in 2006-2007.

2.1.1. Taxonomy

FMD virus was documented in 1963 by the International Committee on Taxonomy of Viruses (ICTV) as member of Aphthovirus in the Picornaviridae. The name, Picornaviridae is derived from a Latin word 'Pico' (meaning small) and 'rna' for RNA, which refers to the size and genome type of the virus. The genus name 'Aphthovirus' refers to the vesicular lesions developed in cloven-footed animals (OIE, 2009).

2.1.2. Historical background

Molecular structure of FMD virus was investigated using x-ray crystallography technique (Acharya et al. 1989). An Italian Monk Girolamo Fracastoro in 1546 reported first incidence of FMD outbreak (Sutmoller et al. 2003) while Friedrich Loeffler in 1897 proved that it is a viral ailment. Blood of the infected animal was passed through porcelain filter (0.2 μm) and the filtrate caused the disease in susceptible animals. This proved that the causative agent is not a bacterium because only viruses can pass through porcelain filters. This necessitated many scientists to work on the disease and the laboratories incepted conducting research on various aspects of the virus infected animals. On the other hand, gigantic economic loses demanded the establishment of separate FMD section in research centers. The first research center for FMD was established in Germany in 1909 followed by another research center in England at Pirbright. Similar measures were taken by USA in 1954 at Plum Island (Brown, 2003).

2.1.3. Etiology

FMD is caused by Apthovirus of Picornaviridae (Domingo *et al*. 2002; Ishimaru et al. 2004). The FMD virion is approximately 25 nm in diameter. Under the electron microscope, the

virion appears as round particle with a smooth surface. FMD virus is distinguished from other Picornaviruses by its being lack of a surface canyon, receptor- binding site for entero and cardio-viruses. Antigenically, seven different serotypes of FMD virus are contributing in the prevalence of the disease in animals (Murphy et al. 1999; Domingo et al. 2002). The genetic variation in FMD viral genome exists due to absence of proof-reading in the 3D-encoded RNA dependent RNA polymerase and competitive selection (Curry et al, 1995). Mutants with a selective advantage in the prevailing environment are better represented than other viruses. Mutation rate in FMDV ranges from 10^{-3} to 10^{-5} per nucleotide site per genome replication. This high error rate leads to differences in replicated genomes from the original parental genome of the virus (Grubman et al, 2004).

2.1.4 Geographical Distribution of Serotypes

Geographically, Europe has type A, O and C, Asia has type “A”, “O” and “Asia 1” and Africa has “A”, “O”, “SAT1, 2 and 3” (Fenner et al. 1993). Pakistan has serotype O, A and Asia-1. The disease is more prevalent in cattle. Primarily, Type “A” and “O” were identified (Vallee and Carre, 1922) then type “C” was identified (Trautwein, 1927). Later on new emerging strains from South African Territories (SAT) were demonstrated. From Pakistan, isolation of serotypes A, O, C and Asia-1 has been reported (Ahmad and Khan, 1988). Similar serotypes have been reported to infect the cattle population in India, Afghanistan, Iran and China (Kesy, 2002; Tosh et al. 2002; Perez et al. 2005; Zahur et al. 2006) whereas serotypes SAT-1, SAT-II and SAT-III are common in South African Territories. In Pakistan, the most commonly prevalent FMD virus serotype isolated from the infected animals is type “O” (Zulfiqar, 2003).These all seven serotypes are considered diverse and do not configure immunity against each other. There are lots of subtypes in each serotype (Buxton and Fraser, 1977). The disease is endemic in Middle

East. Pakistan, one of the Asian countries is also in the list where FMD is still persistent (Iqbal et al, 2011). Europe is FMD free, Australia, Korea and Japan have also been eradicated the FMD (Merck Manual, 2002). Phylogeny of FMD serotype "A" virus circulating in Pakistan and Afghanistan during 2002-2009 revealed the presence of A-Iran05 and 3 other unnamed lineages. The A-Iran05 lineage is still evolving as revealed by the presence of 7 distinct variants, the dominant being the A-Iran05AFG-07 and A-Iran05BAR-08 sub-lineages (Jamal et al. 2011). Characterization of serotype O FMD viruses circulating in Pakistan and Afghanistan between 1997 and 2009 revealed the presence of Pak98, Iran2001 and Pan-Asia lineages within the ME-SA (Middle East South Asia) prototypes. The Pan-Asia lineage is currently dominant in the area and is evolving with time as revealed by the appearance of distinct variants e.g. Pan-Asia-II and a new variant designated here as Pan-Asia-III (Jamal et al. 2011).

2.1.5. Economic Significance

FMD is worldwide considered to be economically important disease. According to OIE manual the disease, FMD is found on List "A" of infectious diseases and has been reported that it is the big hurdle in the intercontinental deal of animals and their products (Trautwein, 1927). Reduced yield of milk and meat, abortion in later stages of pregnancy, (Rauf et al. 1981), death of young animals are the main economic losses of the disease (Singh, 2003; Yadav, 2003). The disease is therefore described as transboundry animal (TADs) and highly contagious (Ferris et al. 2002; Zahur et al. 2006). Many countries restrict the import of dairy animals or their products on account of potential losses caused by the disease (Cottam et al, 2006).

2.1.6. Sign and symptoms

FMD causes high mortality in youngs and low in adults. In young ages, FMD progresses multi focal myocarditis which leads to heart failure (Sharma and Adhlakha, 1995). The disease is diagnosed by few cardinal symptoms such as pyrexia, increased salivation and formation of vesicular lesions on gum, epithelium of muzzle, on teets, interdigital space of claws of infected animals (Grubman et al. 2004). During disease period, vesicles rupture and form the ulcerative lesions (Rauf et al. 1981). The acute phase of the disease lasts for about 5 to 7 days. In few cases, the disease may enter into incurable condition such as panting which is due to disturbance in the production thyroxin. In lactating animals there is anorexia, depression and marked drop in milk production. However, clinical signs are mild in sheep and goat (Callen et al. 1998). Moreover, virulence of the various serotypes of the virus depends upon the host species (Donadson, 1998).

2.1.7. Pathogenesis

Incubation period of the disease is 2 to 14 days, depending upon initial infectivity titer of the virus at time of exposure, route of infection, host susceptibility and viral strain (Rubino 1946). Initial site for viral replication is lung epithelium or pharyngeal areas (Domanski at el. 1959) followed by quick diffusion of the virus to oral or pedal epithelium (Donaldson et al. 1970). Abrasion in skin and mucosal membranes can also infect the animals but needs high infectivity titer of the virus (Mkercher and Gailuinas, 1969). The virus is excreted from infected animals through saliva, feces, milk, urine, semen, exhaled air and nasal discharge (Donaldson 1983). After attachment of the virus onto the cell surface, the virus enters into the cell cytoplasm that is primary target site for its replication (Fenner at el. 1993). RNA of the virus acts as mRNA and is directly translated into large virus polypeptides. RNA of the virus enters into the particles

which are then released through cytolysis. The virus multiplies at the site of infection where primary vesicles are formed in 1-4 days. The virus localizes in the distant epithelia and secondary vesicles appeared (Meyer and Knudsen, 2001). Arial transfer of droplets is a common way of the disease transmission in close premises (Alexanderson et al. 2003). Vesicle formations on the tongue, lips, feet etc, in animals develops viremia results into pyrexia. Contagious period lasts for 24 hours followed by clinical onset of the disease. However, FMD infected cattle is the main source of the virus among all the species. The carriers animals recovered from the disease are potential source of the virus and cause of new outbreaks (Meyer and Knudsen, 2001: Cox et al., 2005). The long term survival of the virus in the refrigerated infected animal meat is source of its national and international spread through the food chain. The virus is resistant to dry condition. It can persist on hay and straw about 15 weeks and on hides for much longer time, in slurry up to 6 months, in urine for 39 days, on soil up to 3 to 25 days (Ryan et al. 2008). It is stable at $4^{o}C$ or at freezing temperature but is unstable in muscles due to acid production during setting of meat. Contagious nature of the virus, immunosupression and environmental stress are common factors for spread of the disease (Ryan et al. 2008).

2.1.8. Diagnosis

It is necessary for efficient control of the disease to differentiate the carrier and healthy animals before appearance of clinical signs (Callens and Clereq, 1997). Control of the disease and its laboratory diagnosis is a difficult job on account of its various serotypes and sub-serotypes (Doll, 2001). In morbid animals, the tissue samples are processed for virus diagnosis through serological tests such as complement fixation test (CFT) and serum neutralization test (SNT). In most of the laboratories these tests have been replaced by ELISA for its rapid diagnosis (Ferris and Dawson, 1988). On the other hand, the virus is isolated and characterized in

the laboratories using the latest and highly sensitive Polymerase Chain Reaction (PCR) technique (Doel et al, 1993,).

2.1.9. Complement Fixation Test (CFT)

Compliment fixation test (CFT) is used to differentiate main serotypes of FMD virus and also helps in identification of the vaccinal serotypes of the virus. In past, complement fixation test had unique worth in many FMD laboratories. It needs a complete practical knowledge of anti-complementary reactions. It is the technique that differentiates seven serotypes of the virus (Lobo et al. 1973). For the production of the vaccine and characterization of its serotype is matter of vital concern (Rauf et al. 1981). Sub typing and serological typing enhanced by means of panel of monoclonal antibodies that give precise antigenic outline of a fresh strain. Contrast in isolates and vaccine viruses with the application of conservative method of serotyping, virus neutralization tests and CFT, in addition to profiling the isolates by monoclonal antibodies was presented by author. Both etiquettes of examination mutually provided knowledge on the interactions between the viruses (Jung et al, 1995). The antigenic identification of antigen and antibody through CFT is founded on fixation of complement by antigen-antibody combination (Cowan and Trautman, 1967). The measured volume of the complement whose fixation is to be required is depicted by examining residual complement with an indicator consisting of sheep RBCs sensitized by specific haemolysin (antiserum to sheep RBC). Haemolysin vanishes and the absence of haemolysin in the test predicts the fixation of complement. Standardization of every component of the system is necessary before starting the test. CFT has been widely used since long time and it is still practiced in the diagnosis of FMD and detection of the virus involved. Special protocol is required to run this test (Buxton and Fraser, 1977). ELISA being more sensitive test has replaced the CFT but due to some intermediate problems such as the

accessibility of ELISA reader, plates and other antigens make CFT as prime choice in many laboratories (Ferris and Dawson, 1988). The test depends on serotype. It is still used in some of the transcaucasian countries near Europe and in the regional laboratories of Iran. CFT permits low cost screening of serum samples for different agents within a single assay, and is a useful tool for the serological diagnosis of acute respiratory infections. Vaccine matching tests such as virus neutralization, enzyme-linked immunosorbent assay with polyclonal antibodies and complement fixation are still performed in a relatively small number of laboratories around the world (Paton et al. 2005). The CFT is still an economical, rapid, and valid test for the diagnosis and sero-surveillance of outbreaks of FMD (Tariq, 2006).

2.1.1.0 Virus Neutralization Test (VNT)

VNT has been designed as approved test for international trade by OIE.Rweyemamu et al, 2008 suggested that VNT is mainly suitable in laboratory for detecting intra-type antigenic discrepancy. It also includes the antigenic epitopes exclusively liable for virus strain and evoking defensive antibody. In diverse neutralization test systems the antigenic relationship were found autonomous of the adopted system and also were understood to actually reflexive antigenic variation. Differentiation of FMD virus strain was brought into being suitable for micro neutralization test. Comparisons were established as matching pairs in order to minimize the variations during the test. The assessment of the significance of association obtained is done by mutual conflict of the system. Anti-sera from vaccinate animals were more specific than convalescent animals. Serum quality of CFT appeared more critical than the VNT. Serotype “O” of FMD virus isolates are differentiated using micro-serum neutralization test (Wani et al. 1997). The isolates of three type of “O” FMDV from the herds of cattle in Haryana, Karnal and Punjab in 1988 during outbreaks and in Haryana these isolates were compared accordingly with “O”

type vaccine strain. It was found through serum neutralization test that field isolates were intimately associated with reference strain. Analysis can diverge between different laboratories on the basis of end point taken. Laboratories must set up the criteria by reference to typical reagents which can be gained for FMD from the FAO. Commonly in the serum/virus mixture, a titer of 1/45 or additional of the ultimate serum dilution is taken as positive. Uncertain measurement lies in range of titer of 1/16 to 1/32 and more serum samples are subjected for testing. If the sample taken as secondary has titer of 1/16 or more then animals are regarded as positive. A titer of 1/8 or less than 1/8 is regarded as negative (OIE, 2000).

2.1.1.1. Enzyme Linked Immunosorbent Assay (ELISA)

OIE approved another test for international trade that is indirect sandwich ELISA. It is a substitute in most of the laboratories for CFT and VNT for daily screening all over the world (Ferris and Dawson, 1988). Over the past ten years it has become very popular in the diagnostic labs of veterinary. It matches fine with VNT and does not undergo biological inconsistency that is intrinsic to VNT. In this test rabbit antiserum is encrusted on unlike rows in micro-titer plates to all of the seven FMDV serotypes. These are known as “capture” sera. Suspensions of the test samples are supplied to each row and apposite controls are also integrated. In each of the FMDV serotype anti-sera of guinea pig is added followed by rabbit anti-guinea pig serum linked to an enzyme. Boundless reagents are removed through extensive washing between each step. If colored reaction appeared after the addition of enzyme substrate then it is the indication of positive reaction. This can be viewed through naked eye if strong positive reaction but it is rational to interpret the result with spectrophotometer setting suitable wavelength. A positive reaction is predicted by absorbance measurement of more than 0.1. ELISA can also be used to recognize the serotypes of FMD virus. We should confirm the values giving readings close to 0.1

by repetition of test or by intensification of antigen through tissue culture passage, after CPE has formed. ELISA is much more supplementary, particular, precise, authentic and detailed test that gives 99% realistic and answering results (Alonso et al. 1992). This sero-diagnostic method is the basic requirement for the serotype of FMDV detection and verification that infects animals for the purpose of scheming appropriate immune-prophylaxes program and successful immunotherapy (Muhammad and Ahsan, 2003-4) In the developing countries like Pakistan it is out of reach of minor stack holders because it is expensive and too pricy but the developing countries have been using it since 1971. Factors like deficiency of trained persons, knowledge about the importance of ELISA, expensive equipment and chemicals and lack of interest are the major reasons for implementation of ELISA. Different standardized and commercially accessible plates of Enzyme Linked Immunosorbent Assay were introduced by McCullough Parkinson (2004) for the purpose of their binding capability for pure FMDV antigen, ratio of binding, their variation coefficient in a plate, between different plates and in different batches of plates. A 'spot-test' was designed for this reason for quick and proficient identification of particular antibody when that antibody has low levels or infected with other intrusive proteins. It was found that the performance of the majority of polystyrene and PVC (polyvinyl chloride) plates was well maintained. Immunoplates were used to obtain the lowest variation coefficients. Antibodies specific to FMD virus are serologically evaluated by ELISA (Hamblin et al. 1984).

2.0. Cell Culture

Cell culture is cultivation of cells from animals or their embryos. It is commonly used in many laboratories of bio sciences such as immunology, biotechnology, toxicology, physiology, biochemistry, etc. Primary cell culture is labor intensive and is maintained *in vitro* only for a limited period. In contrast, continuous cell line is serially passaged for a limited number of cell

divisions (about thirty) or otherwise for unlimited generation. The continuous cell lines are transformed tumor cells. Different cell lines are derived from clinical tumors and are available all the time for laboratory. Establishment of cell culture facilities in research potentiated the advancement in research work in area of virology. Monolayer of the continuous cell line is developed under hygienic or sterile environment which ultimately improved the techniques for biochemical assays, biological titration of viruses, genomics, monoclonal antibody and production of pharmaceuticals such as insulin and interferon (Freshney, 1998). Cultivation of FMD virus in animal host species and cell culture of FMD virus serotypes can be isolated in various animal host species, and the virus is cultivated on mucous membrane of buccal mucosa and tongue of the calves or foot-pad of guinea pigs. The FMD virus is adopted and cultured on cell culture (Maitland and Maitland, 1931). Two to seven days-old un-weaned mice from inbred strain were used for the cultivation and propagation of FMDV by intramuscular or intra-peritoneal route of inoculation. The virus multiplies in heart and skeletal muscles (Skinner, 1951). A range of laboratory animals such as mice, chick embryos, tissue cultures which are susceptible to many viruses and hence are used for isolation of FMD virus (Lennete and Schmidt, 1964).

The Baby Hamster Kidney (BHK) cell line has been established since 1962 to facilitate work on isolation, characterization and vaccine production of FMD virus serotypes (MacPherson and Stoker, 1962). Primary Bovine Thyroid cells (BTY) (Snowdon, 1966) and primary lamb or calf kidney cells are sensitive systems for the isolation of FMD Virus types. FMD virus is grown initially in unweaned mice (Ferris et al. 2002) and then adopted to grow on monolayer of black goat fetal lungs cells (Shin et al. 2003). Two primary cell cultures such as Bovine Thyroid cells (BTY) and lamb kidney cells and two continuous cell lines such as BHK and IB-RS-2 are used

for culturing of adopted FMD virus serotypes (Cavanagh et al, 1978). Monolayer of BTY cells are most susceptible for culturing of FMD virus. FMD virus contains a highly immunogenic loop of VP-1 (Acharya et al. 1989). This loop has a conserved sequence Arg-Gly-Asp (RGD) that is used in its cell interaction (Fox et al. 1989: Baxt and Becker, 1990). The sequence (RGD) of FMD virus type A12 is important for cell binding and development of cytopathic effect. Point mutation or deletion mutation of the sequence makes the virus noninfectious for *in vitro* cell culture (Mason et al. 1994; McKenna et al. 1995). However, deletion in the sequence makes the virus avirulent for cattle, even at higher doses of challenge infection (McKenna et al. 1995). Binding of specific antibodies with RGD sequence (integrin, αv β3) in the pathogenic FMD virus neutralizes its infectivity in the cell culture (Berinstein et al. 1995). Bovine Kidney Cell Culture (BKCC) is superior to unweaned mouse for isolation of FMD virus from field samples from infected cattle, buffaloes, sheep, goats and yaks (Tewari et al. 1980). The samples when passaged on BKCC 2-3 times show cytopathic effects within 24-48 hours. Such BKCC adopted virus is pathogenic for unweaned mice. Syrian Baby Hamster Kidney fibroblast (and its clones) is commonly used as adherent or suspension cells for FMD virus research and its vaccine production (MacPherson and Stoker, 1962; Kalanidhi et al. 1992). Apthovirus type A22 strain550 and type O1 strain 194 has been propagated and concentrated in rabbits or BHK-21 cells (Perevozchikova et al. 1986). Apthovirus types O, A, C and Asia-1from cattle tongue sample also has been adapted on pig kidney cell lines IBRS-2 and MVPK. It means that FMD virus grows and provides higher infectivity titers on BHK-21 cells than that of IBRS-2 and MVPK (Nair, 1987).

The infectivity titer of FMD virus in clinical samples is insufficient for its molecular characterization so require its propagation on cell cultures to have sufficient amount of the

antigen. The process of virus cultivation on the primary cell culture requires several days (Ferris and Dawson, 1988). FMD virus is recovered from 134 (67%) out of 201 epithelial tissue samples through culture method on bovine thyroid or kidney cell cultures (Westbury et al. 1988). FMD virus "O" type can be adapted to BHK-21 cell line with a single passage, using 30-minute virus adsorption and 72 hours cultivation (Gugiu et al. 1989). Sometimes FMD virus persists in BHK-21 cells and may result in co-evolution of the cells and the infecting resident virus. The virulence of FMD virus for the BHK-21 cells is gradually increased and ultimately the cells become partially resistant to the virus (Escarmis et al. 1998). The samples are collected from carrier or infected animals using probing and are inoculated on primary bovine kidney (BK) and bovine thyroid cell cultures. In such cases, the cytopathic effect (CPE) is seen two days thereafter (Sakamoto et al. 2002).

2.1. Factors affecting Baby Hamster Kidney (BHK-21) cells

Various physico-chemical factors such as cells, medium, serum and physical conditions influence the growth of cells during *in vitro* culture. For this work, aliquots of frozen cell population (-136^0C) may be used. Cytofluorometric analysis of BHK-21 cell populations cultivated under normal conditions, in the presence of 10% whole serum, cells enter in G1 phase of the cell cycle after arresting their growth to transfer into the quiescent state (Kruman et al. 1981). Various fractions of bovine serum affect growth of BHK-21 cells. The fraction with molecular weight of 100000 daltons has growth-promoting ability but do not support growth up to the level of whole bovine serum. The intensity of cell proliferation is achieved in the presence of the whole serum or in the presence of combined serum fractions with molecular weight of 100,000 – 200,000 daltons and fraction with low-molecular weight of 14,000 daltons. However, neither of these two fractions shows similar effect (Kruman et al. 1984). Baby hamster kidney

cells (BHK-21) at low density on gelatin coated dishes proliferate actively in the mixture of Dulbecco's modified medium and Ham's F12 medium (1:1) containing high density lipoproteins (HDL), transferrin and fibroblast growth factor (FGF). This serum free medium combination supports cell proliferation at a rate equal to serum containing medium even at low seeding cell density i.e., 1000 cells per 35-mm dish. Epidermal growth factor (EGF), although mitogenic for BHK-21 cells, is less efficient than acidic and basic FGF. Basic FGF is 10 times more potent than acidic FGF. BHK-21 cells requirement for transferrin appears to be minimal since cells are exposed to HDL in the absence of transferrin. BHK-21 cells are grown in stirring flask containing micro-carrier show the highest cell density within 72 hours of incubation. The infectivity titer of FMD virus in BHK-21 in micro-carrier containing spinner flasks yield 10 times more than that of conventional monolayer in roller bottle culture system (Ferrari et al. 1990). There are different methods for counting the cells. MTT 3-(4, 5-dimethylthiazol-2- yl)-2,5- diphenyl tetrazolium bromide assay is a colorimetric method to assess cell viability. It is extensively used for counting biomass of proliferating lymphocytes, BHK-21 cells, Vero cells and guinea pig ventricular myocytes. Components of the growth medium and their byproducts affect the growth of Chinese Hamster Ovary (CHO) cells. Growth inhibitors are glucose and ammonia. In cell culture medium, L-glutamine is unstable in solution form and hence a main source of ammonia. It is inferred during cell multiplication in the growth medium that net amount of L-glutamine is utilized with production of net amount of ammonia. Asparagine supports CHO cell growth as a substitute for L-glutamine. Glucose fed batch culture is grown on asparagine supplemented cell culture medium. In such medium, cell concentration reaches to peak level of 3.4×10^{6} per ml due to low concentration of glucose and ammonia production. This yield is 1.8 times more than that of control experiment having 1.15g glucose initially and

0.29 g glutamine per liter of growth medium and without additional glucose fed later on (Kurano et al. 1990). Goat serum supports the growth of primary cultures of fibroblast from chicken embryo and hamster kidney. Goat serum may be preferred for its utilization in cell culture for scientific research and national economy (Castillo et al. 1991). Fetal calf serum, PEG-treated bovine serum and PEG-treated goat serum directly affect the growth of BHK cells during its *in vitro* culture. PEG-treated bovine serum is better than PEG treated goat serum and equivalent to fetal calf serum. Bovine serum treated with PEG is effectively used for the cultivation and maintenance of BHK-21 cells. Moreover, various concentrations of FBS in Eagle's minimal essential medium are prepared and supplemented to monolayer of chick cells to check their effect on multiplication of the cells. The cells supplemented with growth medium containing 5 – 30 % of FBS show increased cell yield with same density at stationary phase medium. The results show that the replication of the cells is dependent on the concentration of FBS up to 20 % (Ryan 1997). Addition of serum in basal medium serves as a source of nutritional and chemical factors for the cells. Fetal Calf Serum (FCS) is extensively used in number of synthetic media in many cell culture laboratories (Paranjape, 2004). National institute of virology, Pune, is conducting research on virus isolation and identification from clinical samples using tissue culture techniques. Fetal bovine serum (FBS) is used initially. However, it is costly and sometimes uncertainly available. Various other sera obtained from different animals such as newborn calf, horse, sheep, and human are tried. The most suitable serum is goat serum (GS) for primary cultures and various cell lines. For propagation of primary cultures from different animals such as chick embryo, monkey kidney, mouse peritoneal macrophages, etc. goat serum is used to supplement the medium. Fetal Bovine Serum (FBS) is also used in comparison to see the growth pattern, morphology and culture characteristics. Mass cultivation, quantification and

virus susceptibility is also performed. Effect of various nutrients, metabolic essentials and virus replication is also recorded. Monoclonal antibodies against Japanese encephalitis (JE) are also characterized. The results from both the media are compared and conclusion is drawn that GS support more cell proliferation (Paranjape, 2004). Encourage dependent cells adhere the substrate (glass/ plastic) with the help of adhesion molecules such as fibronectin and calcium and magnesium ions. Such cells can be detached either by adding EDTA solution or versine solution that chelate the bivalent ions and thus detach the cells. Trypsin can also be used to detach cells. It hydrolyses adhesion molecules, thus separating cells from substrate as well as from each other. Trypsin (0.25%) dissociates keratinocytes within 5 minutes.

2.2. Culture Systems

Various cell lines such as monkey kidney cell line and primary chicken embryo cell lines are propagated in micro-carrier culture flasks. The cell proliferation rate in final cell density depends on the number of micro-carrier in the cell culture medium and initial seeding density (Giard et al, 1978). It is estimated that 0.1-0.2 g of beads per liter of the cell culture medium with seeding density of 20,000 cells per cm^2 of bead surface is optimal. The micro-carrier culture system is compared with the stationary and roller culture system. The cell density obtained from roller bottles is two times more than that of stationary cultures while the cell density of micro-carrier is three times more than stationary cultures at a bead concentration of 2.5 – 1 g per liter. BHK-21 cells in micro-carriers in the bioreactor to produce a stock of Pasteur are grown for strain of rabies virus (Mered et al, 1980). Perfusion mode operation of this bioreactor produced between two- and four fold larger yields (cells/ml) than traditional stationary cell culture systems (i.e., Blake, and Roller bottles or cell factory multi-trays). The method employed harvested 281 of rabies virus in 200 h (infectivity titer 0.6 +/- 1.4 x 10^7 LD_{50} per ml) in a single operation. The

risk of contamination is thus reduced when compared with traditional stationary methods which, in order to obtain the same amount of virus, would require the operation of 285 Blake bottles, or 143 Roller bottles, or 15 Cell Factory multi-trays (10 trays). By perfusion mode operation of the bioreactor, 89% of the cell culture medium was recovered as vaccinal virus, which contrasts with the yield of only 50 -59% using traditional cell culture systems (Gallegos et al. 1995). The roller bottle culture system consists of racks for the storage of bottles containing monolayer of mammalian cells providing cell culture medium. The roller bottle culture system provides cell inoculation, media change and harvesting of cells. Development of such system supports the large scale production of mammalian cells. *In vitro* retinal cells are propagated on horizontal rotating roller drum in roller culture incubator at 36.5^0 C (Rzeczinski et al. 2006). Moreover, Hybrodoma (H9r9) cells are also grown in roller bottles to record the effect of various FCS concentrations (3 and 5%) (Sathya et al. 2008).

3.0 Control

FMD can be controlled by vaccination but this is tricky because of the existence of multiple FMD virus serotypes, Variety of host species including wildlife. Diagnostic techniques and effective vaccines significantly improve the control in both the free and the affected parts of the world (Paton et al. 2009). Moreover, well-organized animal health systems, disease surveillance, ante-mortem and post-mortem inspection of all cattle population successfully help in plummeting the risk of FMD transmission Sutmoller (2003). One of the appropriate approaches to control the disease is to prevent infected animals from entering the principal trading routes for pigs, cattle and buffaloes (Perry et al. 2002). A supplementary policy to consider is to protect livestock populations adjacent to these trading routes by vaccination. However, Epidemiological support is critical for an effective and efficient disease control or

eradication program (Torres et al. 2002). The farms where bio-security measures are not practiced more often are at risk for dissemination of the virus (Amass et al. 2004). It is investigated that a single shot of vaccination protects the cows against FMD and that probably no virus transmission occurs within a vaccinated herd (Orsel et al. 2007). In order to control the disease following strategies are adapted in endemic areas (1) evaluation of FMD status at national level; (2) animal movement control; (3) instituting vaccination and suppressing virus transmission to achieve absence of clinical disease; (4) get rid from FMD with vaccination in accordance with the OIE standards; (5) achieving freedom from FMD without vaccination in accordance with the OIE standards; and (7) maintenance of FMD free zone (Rweyemamu et al. 2008).

3.1 Vaccine and vaccination

Inactivated FMD vaccine is used in many countries including Pakistan but occurrence of many subtypes within a serotype of the virus is the main hindrance in controlling the disease through vaccination (Piatti et at. 1995). Sub level of FMD virus immunogen per dose of the vaccine is one of the causes of vaccine failure. However, a required amount of the immunogen per dose can provoke an effective immune response. The adjuvant used in the vaccine potentiate the retention time of the immunogen at the injection site that ultimately enhance antibody response of the vaccinates (Barteling, 1991). There are many other factors such as mycotoxicated feed, tick infestation, hemoparasites, bovine viral diarrhea, ephemeral fever, etc which are incriminated as cause of failure of immunoprophylaxis (Rashid et al, 2009). In Pakistan, disease is endemic and outbreaks occur throughout the year. However, intensity of the disease is high during winter season. More than 88% outbreaks of the disease are caused by serotype"O", 8% due to serotype "Asia -1" and 4% with type "A" (Khawaja et al. 2009; Zulfiqar et al. 2003;

Awan et al. 2009). Asia 1 serotype of the FMD virus causes the second largest number of disease outbreaks in India. Information about circulating viruses in the country is essential for selection of serotype for the vaccine production. Most of the FMD vaccines are prepared by inactivating the virus by BEL or acetylethylimine. Formaldehyde modulates the configuration of immunogen molecules but doesn't inactivate its RNA that may enter in the cytoplasm of the cell and cause disease outbreaks. This could be a possible reason of outbreak of the disease after vaccinating the animals with formaldehyde inactivated FMD virus vaccine (Brown, 1991). FMD virus is inactivated using Formaldehyde and Binary ethleneimine (BEI). Both the inactivants have their own mode of action and BEI is preferred because of its least interference in the configuration of the virus immunogen (Doel et al, 1993). In order to produce a safe killed FMD vaccine, BEI at concentration of 1.6 mM inactivates the virus within 8 to 10 hours. Adjuvants are grouped according to their physical characteristics and mode of action such as particulate adjuvants, oil and emulsifier based adjuvants those providing control delivery of antigen, adjuvants based on specific targeting of antigen and gel type adjuvants. They may act nonspecifically to improve an immune response to an antigen with the formation of Depot or very specifically by a delivery system (Jennings et al. 1998). Stability of vaccines adjuvanted with Montanide (water in oil emulsion) and comparative immunogenicity of vaccine formulations was studied by (Miles et al. 2004). Three oils such Montanide ISA-206, Montanide ISA-57 and Montanide ISA-50V are used for FMD formulation. Among various adjuvants (Montanide ISA-206, Montanide ISA-57 and Montanide ISA-50V), the vaccine admixed with Montanide ISA -57 gives best result in the vaccinated animals. Oil based killed FMD vaccine provides longer duration of immunity. Unlike cattle, goats show late immune response for oil adjuvanted vaccines. Universal use of oil based killed FMD vaccine controls the disease (Patil et al 2002). Recombinant vaccines are the

gateway in modern biotechnology and provide broad range protection using multivalent vaccine production. In order to enhance the efficacy of vaccine and immune response effect of chemical adjuvants on DNA vaccination is studied that shows that vaccines admixed with adjuvants provide long time immunity (Jin et al. 2004).

3.2. Antibody Response

The inactivated whole-virus vaccine containing adjuvant is used to control the disease (Barteling and Vreeswijk, 1991). The pathogen specific antibodies provide protection to the animals against infection (Muhammad and Ahsan, 2004). Such protection requires high titer of the neutralizing antibodies (McCullough et al. 1992). Oil based FMD virus vaccine is successful in endemic areas. However, it is difficult to differentiate the antibody response due to recovery or vaccination. The recovered animals despite having high titer of neutralizing antibodies may shed the virus and are potential source of infection for other susceptible animals in their vicinity. Currently, an ELISA test is used to detect anti non-structural (NS) protein antibodies that can differentiate antibody response due to infection and vaccination (Mezencio et al. 1998; Shen et al. 1999; Oem et al. 2007). T cells dependent antigenic epitopes of the virus proteins stimulate CD + and CD8 + cells specific immunity. Such T-cells dependent responses are antigen specific and are restricted by Major Histocompatibility complex (MHC) genes. These MHC genes encode for Class I and Class II for the presentation of peptides to the immune system. After infection or vaccination, T-cells mediated antiviral responses are recorded in cattle and swine. In infected animals; virus is cleared from the body through cell mediated immunity. Th2 are stimulated by IL-4 while Th1 expression is suppressed including IFNϒ (Forshubor et al. 1996; Bembridge et al. 1998). Route of vaccination, biological titration of the virus and amount of immunogen affect the immune response in the vaccinated animals. Effective immune response

depends upon the proper and timely good quality vaccine (Doel, 2005). T helper cell responses play role to protect the animal from FMD and presence of FMD virus specific MHC class II restricted responses in cattle. T cell dependent immune responses to FMD virus are recorded in the pigs. Chemically inactivated FMD vaccines require cold chain for its preservation, periodic revaccination and dissemination of virus during production process (Mason et al. 2003). The vaccine virus induces antibody response in the vaccinates (Grubman et al, 2004). The Th cells in response to the antigen produce cytokines which interact with immunocytes and synergized or antagonized effects of each other. Both Th1 and Th2 contribute to overcome the pathogen. The balance of the cytokines determines the outcome of the disease in susceptible animal. FMD virus activates Th-1 cells for production of IFNγ and IL-2; while the Th2 cells produce IL-6 and IL-10 (Barnard et al. 2005; Eble et al. 2006). FMD virus and adjuvant increase the level of Th1 and Th2 (Jin et al. 2004; Shi et al. 2005). Virus induce immunity provokes the synthesis of cytokines that is essential for triggering the antibody production. The pathway followed by cytokines helps in vaccine production against virus challenges (Ahlers et al. 2001). Chemically inactivated FMD vaccine induces production of gamma interferon (IFN-γ) and MHC-restricted Tc-cell responses (Efrian et al. 2008).

Viral activity neutralizes by specific antibodies is an important component of immunity against viruses. Neutralizing antibodies neutralizes viral epitopes and thus inactivate their infectivity. It is observed that variations among the virus serotypes in eliciting early antibody response in cattle, although neutralizing antibody response against all four serotypes are detectable as early as day 4 Ppost vaccination (PV). The duration of immunity is maintained for a longer period. The neutralizing antibody titers are maintained well above 2 $\log_{10}$ even after 6 months PV irrespective of virus serotypes. This finding suggested the possibility of two

vaccinations per year for maintaining herd immunity (Patil et al. 2002). A standard dose vaccine formulation fully protected cattle against direct challenge (Golde et al. 2005). Experiments are conducted on antibody response to FMD vaccine, and observed that repeated vaccinations were required to attain a good protective immunity level in regularly vaccinated herds (Gajendragad et al. 2005).

A mathematical model of the influence of FMD antigen stability and dose on the bovine immune response is investigate and the results suggest that vaccine stability may not have a pronounced impact on the timing of the T cell response, but will affect its magnitude and duration, stable vaccine benefits more from multiple doses, and it is possible to compensate for poor stability with increased dose. Adjuvanted FMD virus “O” type vaccine when injected to one year old buffalo calves induced detectable level of anti-FMD virus neutralizing antibodies (Muhammad et al. 2009).

CHAPTER-3

MATERIALS AND METHODS

3.1. Biomass production of Baby Hamster Kidney cells

3.1.1. Source of Baby Hamster Kidney cells and activation

Baby Hamster Kidney (BHK-21) cells were procured from Department of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore. The cryopreserved cells (-196^0 C) were revived using standard procedure (Hussain et al. 2004). Ampoule containing the frozen BHK-21 cells was placed in a water bath at 37^0 C for thawing. As soon as the content of the ampoule was liquefied, the cell suspension was mixed with 5 ml warm, filter sterilized growth medium (37^0 C). The BHK-21 cell suspension was transferred to a glass Roux flask (175 cm^2) containing 30 ml of Glasgow minimum essential medium (GMEM) with Earl's salts (Biomedical; USA). The medium was supplemented with penicillin (1000 IU/ml), streptomycin (200µg/ ml), gentamicin (50µg/ ml), amphotericin B (5µg/ml) and 5 % fetal calf serum (FCS). The Roux flask was incubated at 37 °C with 5 % CO_2 for 72 hours. The cells were examined under inverted microscope (Nikon) after every 24 hours. After having complete monolayer, the cells were harvested and transferred to four new Roux flasks (175 cm^2) to scale up the cells. These cells after propagation were transferred to roller bottles (480 cm^2: Duran, GmbH, Germany) each containing 100 ml of the medium in order to observe the factors affecting its biomass production.

3.1.2. Factors affecting the biomass production of BHK-21 cells in Roller Bottles

3.1.2.1 Seeding density

Effect of seeding density on the biomass production of BHK-21 cells was monitored in the nine roller culture flasks. Five, 10 and 15 million cells were seeded in each of the three respective sets of the bottles. Each of the bottles was having 100 ml of the growth medium and each of the nine cell seeded bottles was incubated at 37 °C for 60 hours in the incubator at 3 rpm.

3.1.2.2. Amount of growth medium

Fifteen roller bottles (480 cm^2) were divided into five groups (A, B, C, D and E), each having three bottles. Twenty five, 50, 100, 125 and 150 ml of the growth medium was transferred to each of the three bottles of group A, B, C, D and E respectively. About 10 million BHK-21 cells were seeded in each of the 15 bottles and incubated at 37 °C for 60 hours in the incubator at 3 rpm.

3.1.2.3. Concentration of the fetal calf serum

Fifteen roller bottles (480 cm^2) were divided into five groups (A, B, C, D and E), each having three bottles. Hundred ml of the maintenance medium without FCS was added in each of the 15 bottles. The FCS was added in each of the three bottles of group A, B, C, D and E at the rate of 0, 5, 7, 9 and 11 % respectively. About 10 million BHK-21 cells were seeded in each of the 15 bottles. Each of the bottles was incubated at 37 °C for 60 hours in the incubator at 3 rpm.

3.1.2.4. Incubation period

Fifteen roller bottles (480 cm^2) were divided into five groups (A, B, C, D and E), each having three bottles. One hundred ml of the growth medium supplemented with 5% FCS was added to each of the bottle. About 10 million BHK-21 cells were seeded in each of the 15 bottles. Each of the three bottles of group A, B, C, D and E was incubated at 37 °C for 24, 36, 48, 60 and 72 hours at 3 rpm, respectively.

3.1.2.5. Incubation temperature

Twelve roller bottles (480 cm^2) were divided into five groups (A, B, C and D), each having three bottles. One hundred ml of the growth medium supplemented with 5% FCS was added to each of the bottle. About 10 million BHK-21 cells were seeded in each of the 12 bottles. Each of the three bottles of group A, B, C and D was incubated at 33, 35, 37, and 39°C for 60 hours at 3 rpm, respectively.

1.1.2.6. Rolling speed

Fifteen roller bottles (480 cm^2) were divided into five groups (A, B, C, D and E), each having three bottles. One hundred ml of the growth medium supplemented with 5% FCS was added to each of the bottle. About 10 million BHK-21 cells were seeded in each of the 15 bottles. Each of the three bottles of group A, B, C, D and E was incubated at 37 °C for 72 hours at 1, 2, 3, 4 and 5 rpm, respectively.

3.1.2.7. Culture system

Nine culture vessels of either Roux flask (175 cm^2: n=3), Roller Bottles (480 cm^2: n=3) or micro-carriers containing stirring bottles (n=3) were used for this experiment. Thirty five ml of the growth medium was added in each of the three Roux flasks. One hundred ml of the growth medium was added in each of three roller bottles. However, two hundred ml of the growth medium was added in each of three stirring bottles. Ten million BHK-21 cells were seeded in each of the nine culturing vessels. Each of nine culture vessels such as Roux flask, roller bottle culture and micro-carrier stirring bottles were incubated at 37^0C in the still, roller and in incubator equipped with magnetic stirrer for 60 hours, respectively.

3.1.3 Harvesting and counting of cells

The cells from each of the culture vessel were harvested and counted (Davis, 2002). The growth medium in each of the culture vessel containing monolayer of BHK-21 cells was discarded and replaced with 3 ml warm (37^0C) trypsin solution (0.025 % : Bio west)/ Roux flask, 5 ml of the trypsin solution/ roller bottle and 7 ml in each of the vessel containing micro-carrier. Each of the culture vessels was incubated at 37^0 C for 5 minutes and processed for pipetting to separate the monolayer. The suspended cells were transferred to a test tube containing 10 ml of cold (10^0 C) growth medium. Cells thus harvested were centrifuged at 400 x g for 4 min. Pellet of cells was re-suspended in 5ml of maintenance medium. The cell suspension (0.1 ml) was mixed with the equal amount of trypan blue solution (0.4% w/v) and incubated for 5 minutes at 25^0 C. After cleaning the chambers of Neubauer Hemocytometer with 70% ethanol, it was covered with cover slip. The mixture of cells and trypan blue solution was poured on to the chamber by placing the tip of pipette at the edge. A filter paper was used to blot the excessive

fluid spread from filling the grove. The hemocytometer was placed under microscope with 10 x objective and stained and unstained cells were counted. Dead cells were stained as blue while live cells remained unstained. The cells in central and four cornered squares of the counting chamber were counted for recording the viability of cells. The cells touching the upper or left boundaries were included and the cells touching lower or right boundaries were excluded. Total concentration of the cells in original cell suspension per ml was calculated as per following formula:

Total count x 1000 x dilution factor per ml

3.1.4. Statistical Analysis

The cell count in each of the culture vessels (n=3) in each of the experiment was tabulated and each value was transformed into log value. Each of the three log values were processed for calculation of mean and standard deviation. The mean of each parameter were analyzed using ANOVA at 5% probability level (SPSS 13.0). Each of the mean value was compared with either of the other values using Duncan multiplication test (SPSS: 13.0).

3.2. Cultivation of Foot and Mouth disease virus

FMD virus serotypes ("O", "A" and "Asia-1") was obtained from Department of Microbiology, FVS, UVAS, Lahore and was cultivated on monolayer of BHK-21 cells in different culture systems. The effect of incubation temperature and FCS concentration on biological titer of FMD virus type "O" was determined. Also effect of culturing systems such as still culture, roller bottle and stirring bottles containing micro-carrier on biological titer of "O", "A" and "Asia-1" serotypes of FMD virus was determined.

3.2.1. Incubation temperature

Effect of incubation temperature on biological titer of FMD type "O" virus was observed. Twelve roller bottles after attaining complete monolayer of BHK-21 cells were divided into four groups (A, B, C and D) each having three bottles. Each of the bottles was provided with 100 ml of the maintenance medium supplemented with 1% FCS. After giving infection of FMD type "O" virus (3 ml), in each of twelve bottles, each bottle of group A, B, C and D was incubated at 33^0C, 35^0C, 37^0C and 39^0C for 48 hours, respectively. After 48 hours of the incubation, CPE of the virus was observed under inverted microscope.

3.2.2. Fetal Calf Serum

Effect of FCS concentration on biological titer of FMD type "O" virus was observed. Each of the eighteen cell culture bottles after attaining complete monolayer of BHK-21 cells were replaced with 100 ml of the maintenance medium and were divided into six groups (A, B, C, D, E and F), each group containing three bottles. Sterilized FCS was added at the rate of 0, 1, 2, 3, 4, and 5 % aseptically in each of the bottles of group A, B, C, D, E and F, respectively. Monolayer in each of the bottles was infected with 3 ml of freshly grown FMD "O" virus. Each of the bottles was incubated at 37^0C for 48 hours and CPE of the virus in each bottle was observed under inverted microscope.

3.2.3. Culture system

Effect of culturing systems such as still culture (Roux flasks), roller bottle culture and spinning bottle containing micro-carrier (each culture system has three culture vessels) on the biological titer of "O", "A" and "Asia-1" serotypes of FMD virus was observed. In each of the

vessels of the culture systems, complete monolayer of BHK-21 cells was provided with 35, 100 and 200 ml of the maintenance medium containing 1% FCS, respectively. Each of the Roux flasks, roller bottles and spinning bottle containing micro-carrier culture vessels was infected with 3, 5 and 7 ml of FMD type "O", "A" and "Asia-1" virus, respectively and incubated at 37^0C for 48 hours. Status of monolayer of the cells was noticed under inverted microscope.

3.2.4. Biological Titration of FMD Virus ($TCID_{50}$)

The virus suspension showing CPE in either of the bottles of culture system in each of the above mentioned experiments was harvested and processed for biological titration to determine tissue culture infective dose 50 ($TCID_{50}$) (Reed and Muench, 1938). Hundred ul of the growth medium was added in each well of the 96 well plates (Flat bottom). The BHK-21 cells were transferred to the plate. This culture plate was incubated at 37^0C for 48 hours until complete monolayer is formed. Using Multi channel micro pipette with sterile tips, an additional 80 µl of growth medium was added to each well of the plate. In this way, total growth medium in each well of the plate became 180 ul. Using single channel micropipette, 20 µl of each of the above mentioned virus suspension was added to each of the eight wells of column 1. In each of the eight wells of column 1, the virus was diluted as 1:10. Using multi channel micropipette, after mixing the virus suspension, 20 µl of the suspension from each well of column 1 was transferred to respective wells of the column 2, in each well of the column 2 the virus was diluted as 1:100. The dilution was kept continued up to column 10. Twenty µl from each well of the column 10 was discarded in a beaker containing 0.1 % pyodine solution. The wells of column 11 and 12 were kept as control as non infected cells. In this way each of the virus serotype of FMD was diluted as 10 folds directly on cells. The cell plates were incubated at 37^0C for 72 hours.

The complete destruction of monolayer (CPE) in each well was recorded and $TCID_{50}$ was calculated using formula described as:

PD= infectivity > 50% - 50/ infectivity > 50%- infectivity < 50%

PD stands for proportional distance.

3.2.5. Statistical Analysis

The effect of serum concentration, incubation temperature and cell culture system on biomass production/infectivity titer of the FMD virus (TCID50) was determined. Log value of each TCID50 value of the virus was calculated. Then the log values of each serum concentration, each incubation temperature and each type of culture system was processed for calculation of mean and SD values. Mean of each of the above mentioned parameters were compare using one way ANOVA followed with DMR at 5% level of probability (SPSS 13.0).

3.3. Montanide oil based trivalent FMDV vaccine

Each of the FMD virus serotype was cultivated, titrated and processed for vaccine production. This process consists of inactivation, sterility and safety testing and mixing with stabilizer and adjuvant.

3.3.1. Inactivation

Each of the serotypes of FMD virus was inactivated by using Binary Ethyleneimine (Bahneman et al. 1975). BEI was prepared by dissolving 0.1 M, 2-bromoethylamine hydrobromide in 0.2 N sodium hydroxide solution and incubating at 37^0C for one hour. BEI solution was mixed with each of the virus suspension at the rate of 0.001 M and incubated at 25^0

C for 24 hours. Contents of each culture vessels were transferred to new containers and BEI solution was again added at the same concentration as mentioned above. Each culture vessel was re incubated at 25^0C for 24 hours. Sodium Thiosulphate was added in each of the virus suspension at the rate of 2% to neutralize the residual amount of BEI molecules.

3.3.2. Sterility test

The inactivated viral suspension was subjected to sterility testing by transferring one ml of BEI inactivated virus suspension of each serotypes mixed in each of the broths such as triptose broth (10 ml), Sabouraud's broth and mycoplasma broth. Each of the inoculated broth in the test tube was incubated at 37^0C for 10 days and was observed for bacterial and fungal growth. No growth in either of the broth indicated that viral suspension is free from mycotic and bacterial contamination (OIE, 2009).

3.3.3. Safety test

The viral suspension was subjected for safety test. Three sterilized test tubes were taken in a rack and labeled as 1, 2 and 3. One ml of BEI inactivated viral suspension ("O", "A" and "Asia-1") was transferred to each of the labeled tube aseptically. At the same time, 96 well flat bottom cell culture plate of BHK-21 cells monolayer was prepared. The monolayer of each of the well of the plate was washed with sterilized PBS and supplemented with 100 ul of maintenance medium containing 1% FCS. Inactivated virus suspension was added to wells in triplicate. The plate was then incubated at 37^0 C for 72 hours. Post incubation, the wells were examined at 40 x under inverted microscope. The cytopathic effect (if any) was recorded (Altaf et al. 2012).

3.3.4. Addition of preservative

Thiomersal sodium was added in virus suspension of each serotype at the rate of 0.05% and mixed with Montanide ISA-70 oil as given below.

3.3.5. Mixing with oil (ISA-70 Montanide)

Montanide ISA-70 was mixed with virus suspension containing different levels of immunogen/infectivity titer of each serotypes of FMD virus at ratio of 3:2. Oil with virus suspension was blended at 3000 rpm for 5 minutes to prepare the uniformly homogenized vaccine. Composition of each of the vaccine is given below.

3.3.6. Vaccine Label

FMD TRIVALENT VACCINE (# 1)

Each 3 ml dose of the vaccine contains 0.1 ml of 10^7 $TCID_{50}$ unit/ml of each type "A","O" and "Asia-1" of FMD virus

Composition:		**Dosage:**	**Instructions:**
Each ml contains		Cattle/buffalo: 3ml	Shake well before use
Viral suspension	0.3 ml	**Route:**	Keep at 4-6^0 C
Montanide ISA-70 oil	1.8 ml	Deep intramuscular	Use before six months
PBS	0.9 ml		
Thiomersal sodium	0.05 %		

FMD TRIVALENT VACCINE (# 2)

Each 3ml dose of vaccine contains 0.2 ml of 10^7 $TCID_{50}$ unit/ml of each type "A","O" and "Asia-1" of FMD virus

Composition:		**Dosage:**	**Instructions:**
Each ml contains		Cattle/buffalo: 3ml	Shake well before use
Viral suspension	0.2 ml	**Route:**	Keep at 4-6^0 C
Montanide ISA-70 oil	1.8 ml	Deep intramuscular	Use before six months
PBS	0.6 ml		
Thiomersal sodium	0.05 %		

FMD TRIVALENT VACCINE (# 3)

Each 3ml dose of vaccine contains 0.3 ml of 10^7 $TCID_{50}$ unit/ml of each type "A","O" and "Asia-1" of FMD virus

Composition:		**Dosage:**	**Instructions:**
Each ml contains		Cattle/buffalo: 3ml	Shake well before use
Viral suspension	0.9 ml	**Route:**	Keep at 4-6^0 C
Montanide ISA-70 oil	1.8 ml	Deep intramuscular	Use before six months
PBS	0.3 ml		
Thiomersal sodium	0.05 %		

FMD TRIVALENT VACCINE (# 4)

Each 3ml dose of vaccine contains 0.4 ml of 10^7 $TCID_{50}$ unit/ml of each type "A","O" and "Asia-1" of FMD virus

Composition:		**Dosage:**	**Instructions:**
Each ml contains		Cattle/buffalo: 3ml	Shake well before use
Viral suspension	1.2 ml	**Route:**	Keep at 4-6^0 C
Montanide ISA-70 oil	1.8 ml	Deep intramuscular	Use before six months
PBS	0.0 ml		
Thiomersal sodium	0.05 %		

3.4. Evaluation of FMD trivalent vaccines

Twenty five experimental buffalo calves (6 months of age) were selected from Buffalo Research Institute (BRI), Patokki, District Kasur and were divided into five groups A, B, C, D and E (each group contains five calves). Each of the 5 calve of group A, B, C and D were vaccinated (3ml: deep intra muscular) using vaccine # 1, 2, 3 and 4, respectively. The calves of group E were kept as control (non-vaccinated). Each calf of each group was given first boost using the same vaccine, dose and route on 60 day post priming. The blood (5 ml) was collected from jugular vein of each of the calves on 0, 1, 2, 3 and 6 months post priming. Each of the blood samples was stored at 4^0 C for one hour followed with incubation at 37^0 C for two hours.

The serum from each blood sample was separated and transferred into properly labeled serum vials. Each of the serum samples was stored at – 40^0 C till required for monitoring anti FMD virus Complement Fixation antibody (Lobo et al. 1973).

3.5. Complement Fixation Test (CFT)

The antibody titer in each of these samples was determined by Complement Fixation Test (CFT), using multi channel micro titrating 96 well plastic plates (Kartell; Italy). For the test, amboceptor (anti sheep erythrocyte antibodies) was raised in rabbits and processed for its titration (Merchant and Packer, 1983). A 5 ml of blood was collected in a test tube containing heparin (one unit per ml of the blood) from jugular vein of an experimental lab sheep. The test tube was gently agitated to mix the anticoagulant to prevent clotting. The blood was centrifuged at 400 g for 3 minutes. The supernatant was discarded. Five ml of normal saline (0.9 % aqueous solution of sodium chloride) was added to the sedimented sheep erythrocytes (RBCs) and re-suspended by gentle inverted motion. The erythrocyte suspension was centrifuged at the same speed and time. This process of washing of the RBC was repeated till supernatant became clear. At the end, the packed RBCs were re-suspended in phosphate buffer saline (PBS) to prepare 1 and 5 % suspension. The washed sheep erythrocyte (SRBC) suspension was used to raise anti-serum (amboceptor) in the rabbits (Merchant and Packer 1983). About 0.1, 0.3, 0.5, 0.7, 0.9 and 1 ml of the RBCs suspension was injected to each of the three experimental rabbits on first, 3^{rd}, 5^{th}, 7^{th}, 9^{th} and 11^{th} day post first injection through intra venous route of the ear vein. On twenty one day post 1^{st} injection 3 ml blood was collected from ear vein of each of the three rabbits separately and transferred to each of the respective labeled test tubes. The serum collected from each vial was pooled and processed for monitoring anti sheep RBCs heamagglutinating antibodies in 96 welled round bottomed micro titration plastic plates using the technique

described by (Swayne et al, 1998). The PBS (50 ul, pH 7.2) was added in each of the twelve wells of 3 rows of the plate using 8-channeled micropipette. Amboceptor (50 µl) was added in first well and its two-fold dilution was made up to well No. 11. Washed SRBC (50 µl: one percent suspension) were added from first to well No. 12 and served as control for sheep RBCs. The plate was incubated at the room temperature (25^0 C) for 60 minutes. The heam-agglutination (HA) activity of the serum was noted. The highest dilution of the serum showing HA activity was HA units of the antibodies/ml of the serum. Suppose well # 8 is the highest dilution of the serum showing HA activity. The serum dilution in well # 8 was 256. The well # 9 was not showing the HA activity but was having sub agglutinating of the amboceptors. The serum was diluted so as to have sub agglutination level of ambocepto and mixed with equal volume of 5% SRBC suspension. The mixture of SRBC suspension was incubated at the room temperature for 10 min and subjected to centrifuge at 400x g for 3 minutes. The supernatant was removed and the packed RBCs were re-suspended in veronal buffer to have 2 percent RBCs suspension. These antibodies (Amboceptors) coated RBCs were used for titration of complement.

Blood (3ml) was collected directly from heart of each of the three male apparently healthy guinea pigs and transferred to each of the respective labeled test tubes. The blood was allowed to clot and processed for separation of its serum. The serum from each of the three guinea pigs was pooled and stored at 4^0 C for determining its hemolytic activity, veronal buffer (50ul) was added to each of the 12 wells of two rows of the above mentioned micro titration plate using multi channel micro pipette. Guinea pig serum (50 ul) was added to 1st well of each of the two rows of micro titration plate and processed further as two fold dilution up to well #11 of the plate. The sensitized RBCs (amboceptor coated RBCs: 50 ul) were added to each of the 12 wells of each row. The plate was incubated at the room temperature for 10 minutes and subjected

to centrifugation in low temperature centrifuge machine. The highest dilution of the guinea pig serum showing hemolytic (HL) activity was the HL units of the serum. Division of this dilution by 4 resulted into dilution having 4 HL units of the complements. Such diluted guinea pig serum was used in final CFT.

The CFT was performed as per recommendations of (Cruickshank, 1975: OIE 2000). The brief procedure is as following. The veronal buffer (50 µl) was added from well # 1 to well # 12 of the immuno-plate. The calf serum (50 µl) was added in well # 1 and its two-fold dilution was made up to well # 9. FMD virus type "O" (50 ul) was added from well # 1 to well # 10. The plate was incubated at the room temperature for 10 minutes. Guinea pig serum (50 µl) having 4 HL unit of the complement was added in well # l to well # 11 and the plates were incubated at 37°C for ten minutes. Finally, 50 µl of the sensitized sheep RBC (2%) were added from well # 1 to well # 12 of each row of the plate. The highest dilution of the serum showing no hemolysis of the sensitized RBCs was the titer of anti FMDV "O" CFT antibodies. In this way each of the serum samples was processed for monitoring its antibody titer against "O", "A" and "Asia-1" types of FMD virus.

3.6. Statistical Analysis

The serum antibody titer of each calf of each group on 0, 30, 60, 90 and 180 days post priming was processed for calculation of its geometric mean titer (GMT) (Villagas and Purchase, 1989). The GMT data of each group on 30, 60, 90 and 180 days post priming was processed for calculation of mean (CGMT) and SD values. These CGMT values of the antibody titer of each vaccinated group were compared using one way analysis of variance (ANOVA and subsequently Duncan multiplication test (SPSS 13.0)).

CHAPTER-4

RESULTS

4.1. Effect of nutritional and physical factors on biomass production of BHK-21 cells

4.1.1. Nutritional factors

There was positive effect of ratio of growth medium and fetal calf serum concentration on the biomass production/ growth of BHK-21 cells.

4.1.1.1. Growth medium amount

Growth medium provides all necessary elements required for the growth of cells in *in vitro* culture. Effect of different volumes of Glasgow minimum essential medium (GMEM) with 5% fetal calf serum (FCS) was monitored for the growth of BHK-21 cells in roller culture bottles. Cell density in each bottle was 10 million and the results of cell count / bottle of the medium are shown in Table 4.1 (Figure 4.1). It was observed that bottles containing 25 ml of the growth medium supported cell density up to $2.1x10^6$, $2.5x10^6$ and $2.4x10^6$ and their log values were 6.32, 6.39 and 6.38, respectively in each of the three bottles. Mean ± standard deviation of these log values was 6.36 ± 0.038. The bottles containing 50 ml of the growth medium supported cell density up to $3.8x10^6$, $3.6x10^6$ and $4.0x10^6$ and their log values were 6.57, 6.55 and 6.60 respectively in each of the three bottles. Mean ± standard deviation of these log values was 6.57 ± 0.026. The bottles containing 100 ml of the growth medium supported cell density up to $4.3x10^7$, $4.3x10^7$and $4.5x10^7$and their log values were 7.63, 7.63 and 7.65 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.63 ± 0.011. The bottles

containing 125 ml of the growth medium supported cell density up to $4.6x10^7$, $4.9 x10^7$and $4.6x10^7$ and their log values were 7.66, 7.69 and 7.66 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.67 ± 0.018. The bottles containing 150 ml of the growth medium supported cell density up to $4.5x10^7$, $5.0x10^7$ and $4.6x10^7$ and their log values were 7.65, 7.69 and 7.66 respectively in each of the three bottles. Mean± standard deviation of these log values was 7.67 ± 0.020. Mean cell density of each volume of the growth medium was significantly different from each other ($p < 0.05$). It was further noticed that minimum cell growth in bottle having 25 ml of medium was significantly less than the cell density observed in bottle having 50 ml or more amount of the medium. The cell density in bottles having medium 100 ml (7.63), 125 ml (7.67) & 150 ml (7.67) were not significantly different but each of the cell density was significantly higher than that of bottle having either 25 ml (6.63) or 50ml medium (6.57) as $p<0.05$.

4.1.1.2. Fetal calf serum

Effect of different concentrations of FCS in GMEM was monitored on biomass/ growth of BHK-21 cells in roller culture bottles. Cell density in each bottle was 0.1 million cells/ ml of the medium and the results in the form of cell count / bottle as shown in Table 4.2 (Fig 4.2). It was observed that bottles supplemented with 0 % FCS supported cell density up to 2.6×10^5, 2.5×10^5 and 2.1×10^5 /ml of the medium and their log values were 5.41, 5.39 and 5.32, respectively in each of the three bottles. Mean ± standard deviation of these log values was 5.37 ± 0.047. The bottles supplemented with 5 % FCS supported cell density up to 3.7×10^7, 3.8×10^7 and 3.7×10^7 and their log values were 7.56, 7.57 and 7.56 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.56 ± 0.005. The bottles supplemented with 7 % FCS supported cell density up to 4.7×10^7, 4.7×10^7 and 4.8×10^7 and their log values were

7.67, 7.67 and 7.68 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.67 ± 0.005. The bottles supplemented with 9 % FCS supported cell density up to $4.9x10^7$, $4.7x10^7$ and $4.8x10^7$ and their log values were 7.69, 7.67 and 7.68 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.68 ± 0.010. The bottles supplemented with 11% FCS supported cell density up to $4.9x\ 10^7$, $4.7x\ 10^7$ and $4.8\ x\ 10^7$ and their log values were 7.69, 7.67 and 7.68 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.68 ± 0.010. Mean cell density of each of the percentage of FCS was significantly different ($p>0.05$). It was further added that the bottle having 0% of FCS was significantly less than the cell density observed in bottle having 5 % FCS ($p>0.05$). The cell density in the bottles having 5 % (7.56), 7 % (7.67), 9 % (7.68) and 11 % (7.68) of FCS were not significantly different but each of the bottles, cell density was significantly higher than that of bottle with 0 %.

4.1.2. Physical factors

4.1.2.1. Initial cell density

Effect of different cell densities of initial inoculums was monitored on growth of BHK-21 cells in roller culture bottles. Inoculums of initial cell density was 5 million, 10 million and 15 million and the results in the form cell count / bottle as shown in Table 4.3 (Fig 4.3). It was observed that bottles containing 5 million cells/ bottle supported cell yield up to $2.3\ x\ 10^7$, $2.1x10^7$ and $2.4x10^7$ and their log values were 7.36, 7.32 and 7.38 respectively in each of the three bottles. Mean± standard deviation of these log values was 7.35 ± 0.030. The bottles containing ten million cells/ bottle supported cell yield up to $4.5\ x\ 10^7$, $4.3x10^7$ and $4.6x10^7$ and

their log values were 7.65, 7.63 and 7.66 respectively in each of the three bottles. Mean± standard deviation of these log values was 7.64 ± 0.015.

The bottles containing 15 million cells/ bottle supported cell yield up to 4.6 x 10^7, 4.8x$10^7$and 4.6x$10^7$and their log values were 7.66, 7.68 and 7.66 respectively in each of the three bottles. Mean± standard deviation of these log values was 7.66 ± 0.011. Mean cell density of each volume of growth medium was significantly different ($p>0.05$). It was further noted that minimum cell growth in bottles having 5 million of initial cell density was significantly less than the cell density observed in bottle having 10 million (7.64) and 15 (7.66) million ($p>0.05$). The cell density in bottles having 10 million (7.64) and 15 million (7.66), were not significantly different but each of the cell density was significantly higher than that of bottle 05 million (7.35) as $p>0.05$.

4.1.2.2. Incubation period

Effect of different times of incubation was monitored on growth of BHK-21 cells in roller culture bottles. Cell density in each bottle was 10 million irrespective of time of incubation and the results in the form of cell count / bottle was shown in Table 4.4 (Figure 4.4). It was observed that bottles provided 24 hours of incubation period supported cell density up to 2.2x10^7, 2.2x10^7 and 2.1x$10^7$and their log values were 7.34, 7.34 & 7.32 respectively in each of three bottles. Mean± standard deviation of these log values was 7.33 ± 0.011. The bottles provided 36 hours of incubation period supported cell density up to 3.4 x10^7, 3.6x$10^7$and 3.7x$10^7$and their log values were 7.53, 7.55 and 7.56 respectively in each of three bottles. Mean± standard deviation of these log values was 7.55 ± 0.015. The bottles provided 48 hours of incubation period supported cell density up to 4.7x10^7, 4.9x10^7& 4.7x$10^7$and their log values were 7.67, 7.69 & 7.67 respectively

in each of three bottles. Mean± standard deviation of these log values was 7.67 ± 0.011. The bottles provided 60 hours of incubation period supported cell density up to $4.7x10^7$, $4.9x10^7$& $4.9x10^7$and their log values were 7.67, 7.69 & 7.69 respectively in each of three bottles. Mean± standard deviation of these log values was 7.68 ± 0.011. The bottles provided 24 hours of incubation period supported cell density up to 1.3 $x10^7$, $1.2x10^7$ and $1.2x10^7$and their log values were 7.11, 7.07 and 7.07 respectively in each of three bottles. Mean± standard deviation of these log values was 7.07 ± 0.023. Mean cell density of each time period of incubation was different as $p>0.05$. It was further noted that minimum cell growth in bottle provided 72 hours of incubation time was significantly less than the cell density observed in bottle provided with 60 hours of incubation ($p>0.05$). The cell density in bottles endow with 48 hours (7.67) and 60 hours (7.68) were not significantly different but each of the cell density was significantly higher than that of bottle incubated either for 24 hours (7.33) or 72 hours (7.07) as $p>0.05$. The cell density in bottles endows with 24 hours (7.33) and 36 hours (7.55) were not significantly different but each of the cell density was significantly lesser than that of bottle incubated either for 60 hours (7.68).

4.1.2.3. Incubation temperature

Effect of different incubation temperature was monitored on growth of BHK-21 cells in roller culture bottles. Cell density in each bottle was 10 million cells per bottle irrespective of its incubation temperature and the results in the form cell count / bottle are shown in Table 4.5. Keeping in view all the other nutritional (GMEM & FCS) and physical parameters (time & revolution speed) constant, the temperature of incubation was variable as 33^0C, 35^0C ,37 ^{0}C and 39^0C and the results in the form cell count / bottle as shown in Table 4.5 (Figure 4.5). Mean cell density of each volume of growth medium was significantly different as $p>0.05$. It was observed that the bottles incubated at 33^0C supported cell growth up to 1.3 x 10^7, 1.5 x 10^7 and 1.6 x 10^7

and their log values were 7.11, 7.17 and 7.20 respectively in each of three bottles. Mean± standard deviation of these log values was 7.16 ± 0.046. The bottles incubated at 35^0C supported cell growth up to 3.3 x 10^7, 3.7 x 10^7 and 3.8 x 10^7 and their log values were 7.51, 7.56 and 7.57 respectively in each of three bottles. Mean± standard deviation of these log values was 7.54 ± 0.032. The bottles incubated at 37^0C supported cell growth up to 4.8 x 10^7, 4.9 x 10^7 and 4.9 x 10^7 and their log values were 7.68, 7.69 & 7.69 respectively in each of three bottles. Mean± standard deviation of these log values was 7.68 ± 0.005. The bottles incubated at 39^0C supported cell growth up to 1.2 x 10^3, 1.2 x 10^3 and 1.1 x 10^3 and their log values were 3.07, 3.07 and 3.05 respectively in each of three bottles. Mean± standard deviation of these log values was 3.06 ± 0.010. It was further noted that minimum cell growth in bottle incubated at 39^0C (3.06) was significantly less than the cell density observed in bottle incubated at 37^0C (7.68) and 35^0C (7.54) as ($p>0.05$). The cell density in bottles provided 35^0C (7.54) and 37^0C (7.68) were not significantly different but each of the cell density was significantly higher than that of bottle incubated at 39^0C. Remarkably different result was observed in bottles incubated at 39^0C. Mean ± standard deviation of these antilog values was 3.06 ± 0.010.

4.1.2.4. Revolution speed

Effect of different revolution speed was monitored on growth of BHK-21 cells in roller culture bottles. Cell density in each bottle was 10 million cells per bottle irrespective of its speed of revolution and the results in the form cell count / bottle as shown in Table 4.6 (Figure 4.6). It was observed that bottles revolving at speed 01 rpm and supported cell density up to 2.3x10^4, 2.5x10^4 and 2.6x10^4 and their log values are 4.36, 4.39 and 4.41 respectively in each of three bottles. Mean± standard deviation of these log values was 4.39 ± 0.025. The bottles revolving on speed 02 rpm and supported cell density up to 6.8 x10^4, 6.9 x10^4 and 7.0 x10^4 and their log

values were 4.86, 4.83 and 4.84 respectively in each of three bottles. Mean± standard deviation of these log values was 4.83 ± 0.015. The bottles revolving on speed 03 rpm and supported cell density up to 4.8 $x10^7$, 4.7 $x10^7$and 4.9 $x10^7$and their log values were 7.68, 7.67 and 7.69 respectively in each of three bottles. Mean± standard deviation of these log values was 7.68 ± 0.010. The bottles revolving on speed 04 rpm and supported cell density up to $2.2x10^3$, $2.1x10^3$and $2.2x10^3$and their log values were 3.34, 3.32 and 3.35 respectively in each of three bottles. Mean± standard deviation of these log values was 3.33 ± 0.015. The bottles revolving on speed 05 rpm and supported cell density up to 3.5 $x10^2$, 3.0 $x10^2$and 2.8 $x10^2$ and their log values were 2.54, 2.47 and 2.44 respectively in each of three bottles. Mean± standard deviation of these log values was 2.49 ± 0.051. Mean cell density of each revolution speed was significantly different as $p>0.05$. It was further noted that minimum cell growth in bottle revolving at 05 rpm was significantly less than the cell density observed in bottle revolving at 03 rpm ($p>0.05$). The cell density in bottles rotated at 01(4.39) and 02 (4.83) rpm were not significantly different but each of the cell density was significantly lesser than that of bottle rotated at 03 rpm (7.68).

4.1.2.5 Culture system

Filter sterilized GMEM supplemented with 5% FCS was added in each of the three Roux flask (35 ml/ flask), Roller bottle (100 ml/bottle) and Micro-carrier culture vessel (200 ml/bottle). BHK-21 cells were added in each of the flask/bottle at the rate of 10 million cells/ ml. Cells of each culture vessel after incubation at 37^0C for 60 hours were counted and displayed in Table 4.7 and Figure 4.7. It was observed that the cells grew in the form of monolayer and reached to the cell density of 1.9 $x10^7$, 2.4 x 10^7 and 1.7 $x10^7$ in each of the Roux flask and log values of the each cell count of each of the Roux flasks were 7.27, 7.38 and 7.23. Mean ± standard deviation of these log values was 7.29 ± 0.07. The cells grew in the form of

monolayer on inner surface of the rolling bottles and reached to the cell density of 4.5 $x10^{7,}$ 4.9 x 10^{7} and 4.4 $x10^{7}$ in each of the Roller bottle. Log values of the cell count of each of the three Roller bottles were 7.65, 7.69 and 7.64. Mean ± standard deviation of these log values was 7.66 ± 0.02. The cells grew on the inner surface of the cell culture bottle as well as on surface of micro-carriers and cell density per 200 ml of the growth medium in each of the three micro-carrier culture vessels were 1.8 $x10^{8}$, 2.4 x 10^{8} and 2.5$x10^{8}$ cells. The log values of the cell count of each of the three micro-carrier culture vessels were 8.25, 8.38 and 8.39. Mean ± standard deviation of these log values was 8.34 ± 0.07. The cell count in each of the culture vessel was significantly different from each other ($p<0.05$). There was minimum cell count in Roux flask and maximum cell count in micro-carrier culture vessels (Table 4.7).

Table 4.1: Effect of growth medium quantity on scale up of BHK-21 cells in roller bottles

* Growth Medium (volume)	Cell count ($\log_{10}$)			
	Bottle 1	**Bottle 2**	**Bottle 3**	****Mean±Std.dev**
25 ml	$2.1x10^6$(6.32)	$2.5x10^6$(6.39)	$2.4x10^6$(6.38)	$6.36^a \pm 0.038$
50 ml	$3.8x10^6$(6.57)	$3.6x10^6$(6.55)	$4.0x10^6$(6.60)	$6.57^b \pm 0.026$
100 ml	$4.3x10^7$(7.63)	$4.3x10^7$(7.63)	$4.5x10^7$(7.65)	$7.63^c \pm 0.011$
125 ml	$4.6x10^7$(7.66)	$4.9x10^7$(7.69)	$4.6x10^7$(7.66)	$7.67^c \pm 0.018$
150 ml	$4.5x10^7$(7.65)	$5.0x10^7$(7.69)	$4.6x10^7$(7.66)	$7.67^c \pm 0.020$

*Glasgow minimal essential medium (GMEM) **Mean & standard deviation of log values

Seeding density was 10 million cells per bottle (n =3), 5 % serum was added in the Glasgow minimum essential medium (GMEM) and the bottles were incubate at 37°C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.1. Log of each value determined and was depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p< 0.05$).

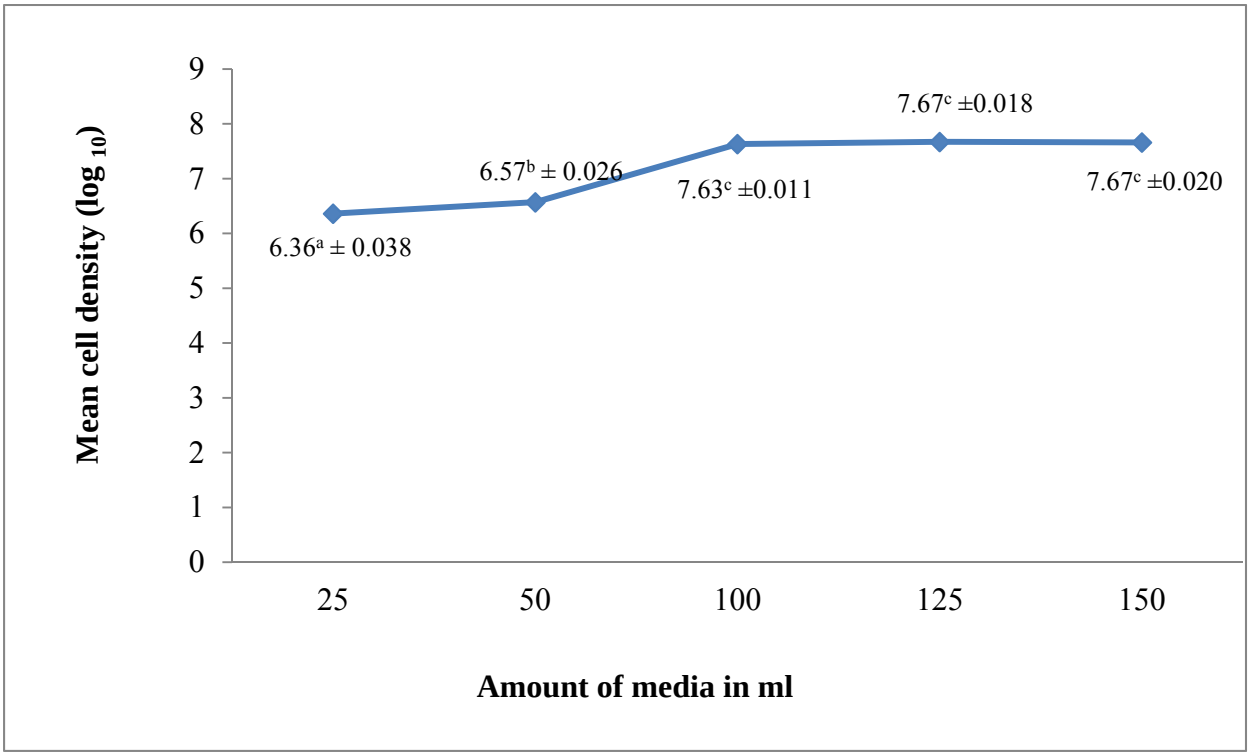

Figure 4.1: Growth rates of BHK-21 cells cultured in roller bottles at different volumes of growth medium

Seeding density was 10 million cells per bottle (n =3), 5 % serum was added in the Glasgow minimum essential medium and the bottles were incubate at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.1. Mean Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p< 0.05$).

Table 4.2: Effect of serum concentration on the yield of BHK-21 cells cultured in roller bottles

*Serum concentration (%)	Cell count ($\log_{10}$)			
	Bottle 1	Bottle 2	Bottle 3	**Mean ± std.dev
0 %	2.6×10^5 (5.41)	2.5×10^5 (5.39)	2.1×10^5 (5.32)	$5.37^a \pm 0.047$
5 %	3.7×10^7 (7.56)	3.8×10^7 (7.57)	3.7×10^7 (7.56)	$7.56^b \pm 0.005$
7 %	4.7×10^7 (7.67)	4.7×10^7 (7.67)	4.8×10^7 (7.68)	$7.67^b \pm 0.005$
9 %	4.9×10^7 (7.69)	4.7×10^7 (7.67)	4.8×10^7 (7.68)	$7.68^b \pm 0.010$
11 %	4.9×10^7 (7.69)	4.7×10^7 (7.67)	4.8×10^7 (7.68)	$7.68^b \pm 0.010$

*FCS (Fetal calf serum) **Mean ± standard deviation of log values

Seeding density was 10 million cells per bottle, 100 ml of M-199 medium was added in each bottle and the bottles were incubated at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.2. Log of each value determined and was depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

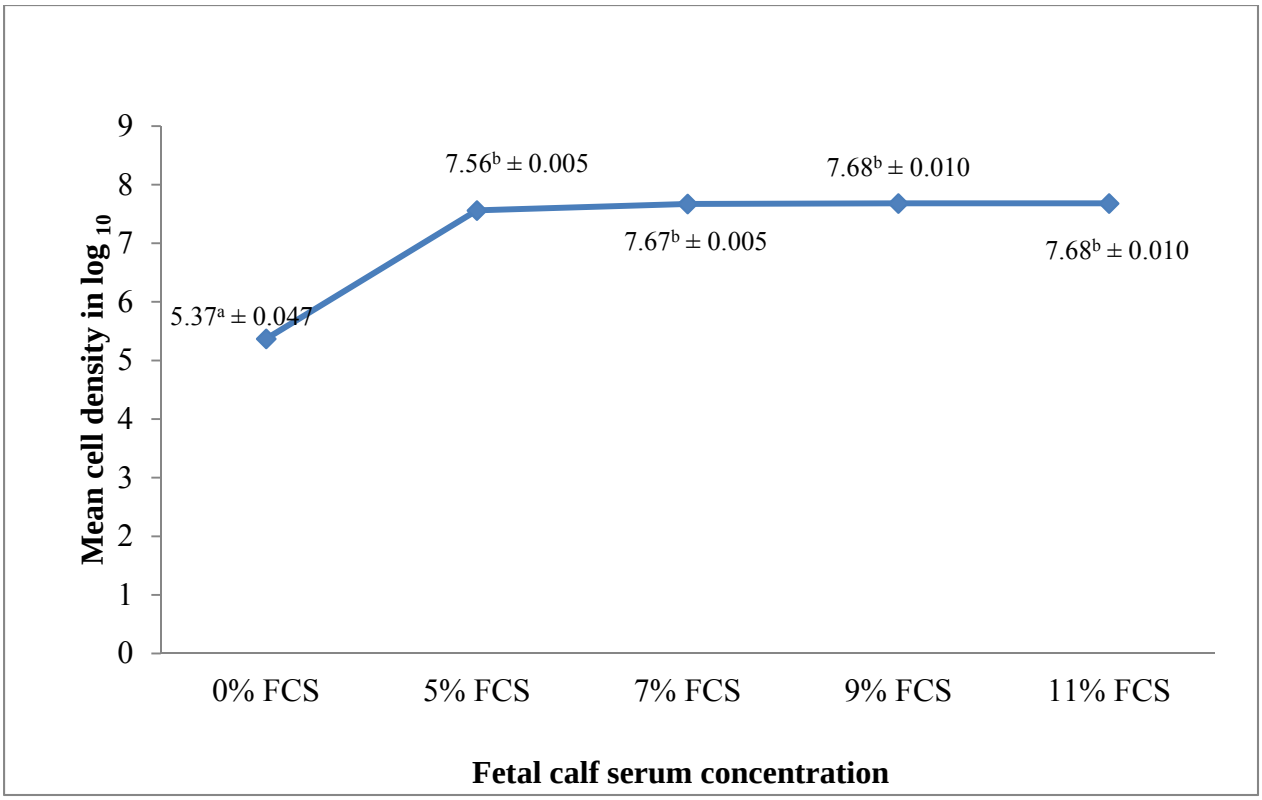

Figure 4.2: Growth rate of BHK-21 cells cultured in roller bottles at various concentrations of Fetal Calf Serum

Seeding density was 10 million cells per bottle, 100 ml of GMEM was added in each bottle and the bottles were incubated at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.2. Mean and Std. dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

Table 4.3: Effect of seeding density on yield of BHK-21 cells cultured in the roller bottles

*Amount of cells (millions)	Cell count (log $_{10}$)			
	Bottle 1	**Bottle 2**	**Bottle 3**	****Mean±Std.Dev**
5	$2.3x10^7$ (7.36)	$2.1x10^7$ (7.32)	$2.4x10^7$ (7.38)	$7.35^a \pm 0.030$
10	$4.5x10^7$ (7.65)	$4.3x10^7$ (7.63)	$4.6x10^7$ (7.66)	$7.64^b \pm 0.015$
15	$4.6x10^7$ (7.66)	$4.8x10^7$ (7.68)	$4.6x10^7$ (7.66)	$7.66^b \pm 0.011$

*Initial cell density **Mean ± standard deviation of log values

100 ml of the GMEM supplemented with 5 % serum was added in each bottle and the bottles were incubated at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.3. Log of each value determined and was depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

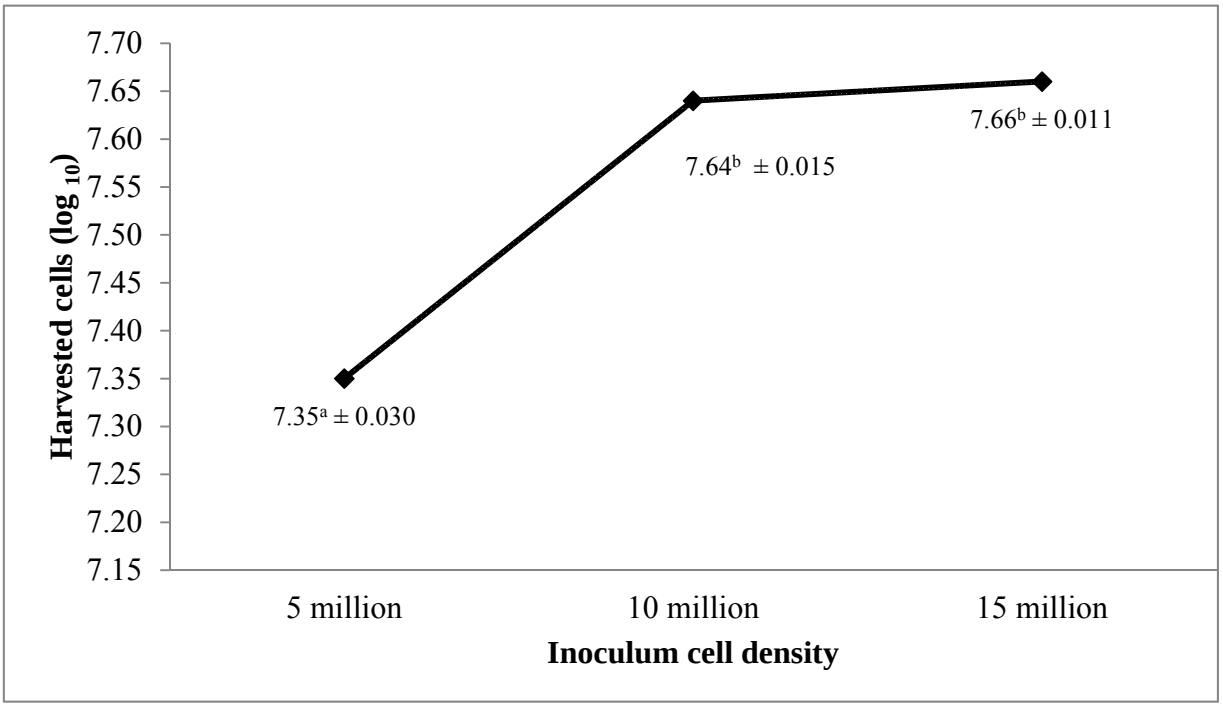

Figure 4.3: Growth rate of BHK-21 cells cultured in roller bottles at various seeding density

One hundred ml of the GMEM supplemented with 5 % serum was added in each bottle and the bottles were incubated at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.3. Mean and Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

Table 4.4: Effect of incubation period on yield of BHK-21 cells in roller bottles

Incubation time (Hours)	Cell count (log $_{10}$)			
	Bottle 1	**Bottle 2**	**Bottle 3**	***Mean± Std.Dev**
24	$2.2x10^7$ (7.34)	$2.2x10^7$ (7.34)	$2.2x10^7$ (7.34)	$7.33^a \pm 0.011$
36	$3.4x10^7$ (7.53)	$3.6x10^7$ (7.55)	$3.6x10^7$ (7.55)	$7.55^a \pm 0.015$
48	$4.7x10^7$ (7.67)	$4.9x10^7$ (7.69)	$4.8x10^7$ (7.67)	$7.67^b \pm 0.011$
60	$4.7x10^7$ (7.67)	$4.9x10^7$ (7.69)	$4.8x10^7$ (7.68)	$7.68^b \pm 0.011$
72	$1.3x10^7$ (7.11)	$1.2x10^7$ (7.07)	$1.2x10^7$ (7.07)	$7.07^c \pm 0.023$

*Mean ± standard deviation of log values

100 ml of the Glasgow minimal essential medium supplemented with 5 % serum was added in each bottle and the bottles were incubate at 37 °C. Seeding density was 10 million cells per bottle (n =3). Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.4. Log of each value determined and was depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

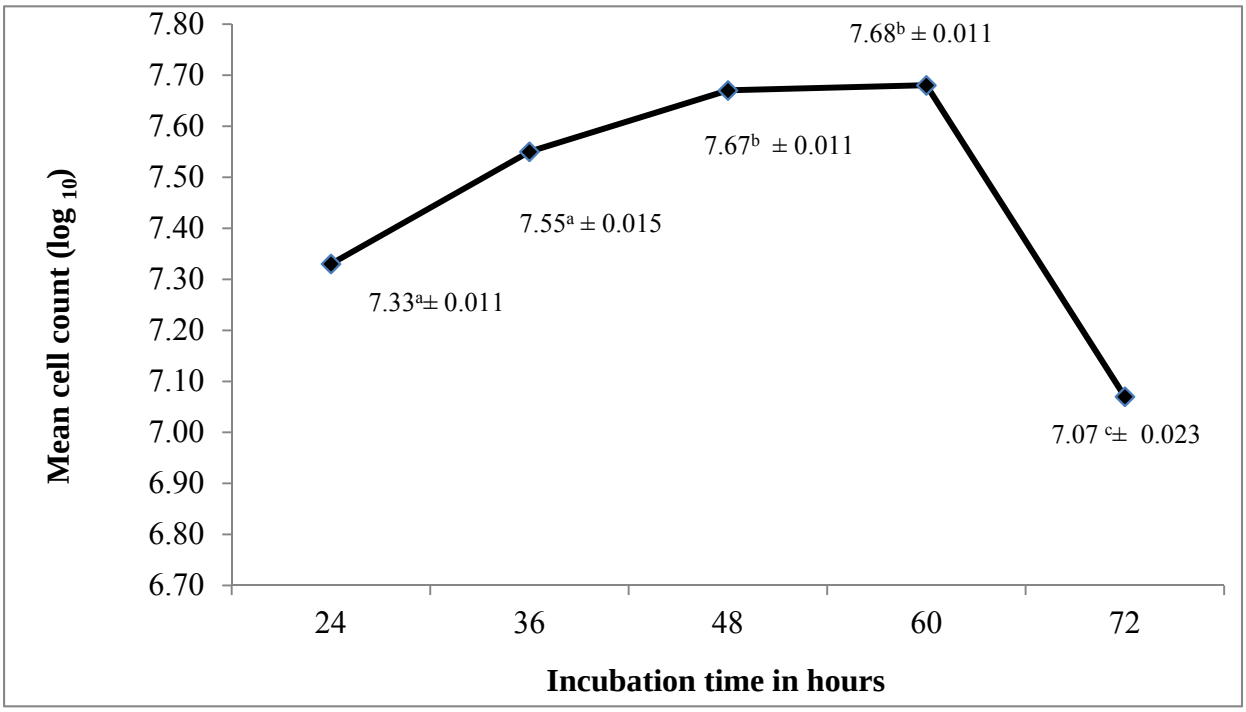

Figure 4.4: Growth rate of BHK-21 cells cultured in roller bottles at various time intervals

100 ml of the Glasgow minimal essential medium supplemented with 5 % serum was added in each bottle and the bottles were incubate at 37 °C. Seeding density was 10 million cells per bottle (n =3). Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.4. Mean and Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$.

Table 4.5: Effect of incubation temperature on yield of BHK-21 cells cultured in the roller bottles

Incubation temperature	Cell count ($\log_{10}$)			
	Bottle 1	Bottle 2	Bottle 3	*Mean ± std.dev
33°C	$1.3x10^7$ (7.11)	$1.5x10^7$ (7.17)	$1.6x10^7$ (7.20)	$7.16^a \pm 0.046$
35°C	$3.3x10^7$ (7.51)	$3.7x10^7$ (7.56)	$3.8x10^7$ (7.57)	$7.54^b \pm 0.032$
37°C	$4.8x10^7$ (7.68)	$4.9x10^7$ (7.69)	$4.9x10^7$ (7.69)	$7.68^b \pm 0.005$
39°C	$1.2x10^3$ (3.07)	$1.2x10^3$ (3.07)	$1.1x10^3$ (3.05)	$3.06^c \pm 0.010$

*Mean ± standard deviation of log values

100 ml of the GMEM supplemented with 5 % serum was added in each bottle and the bottles were incubated at various temperatures (^{0}C) for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.5. Log of each value determined and was depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

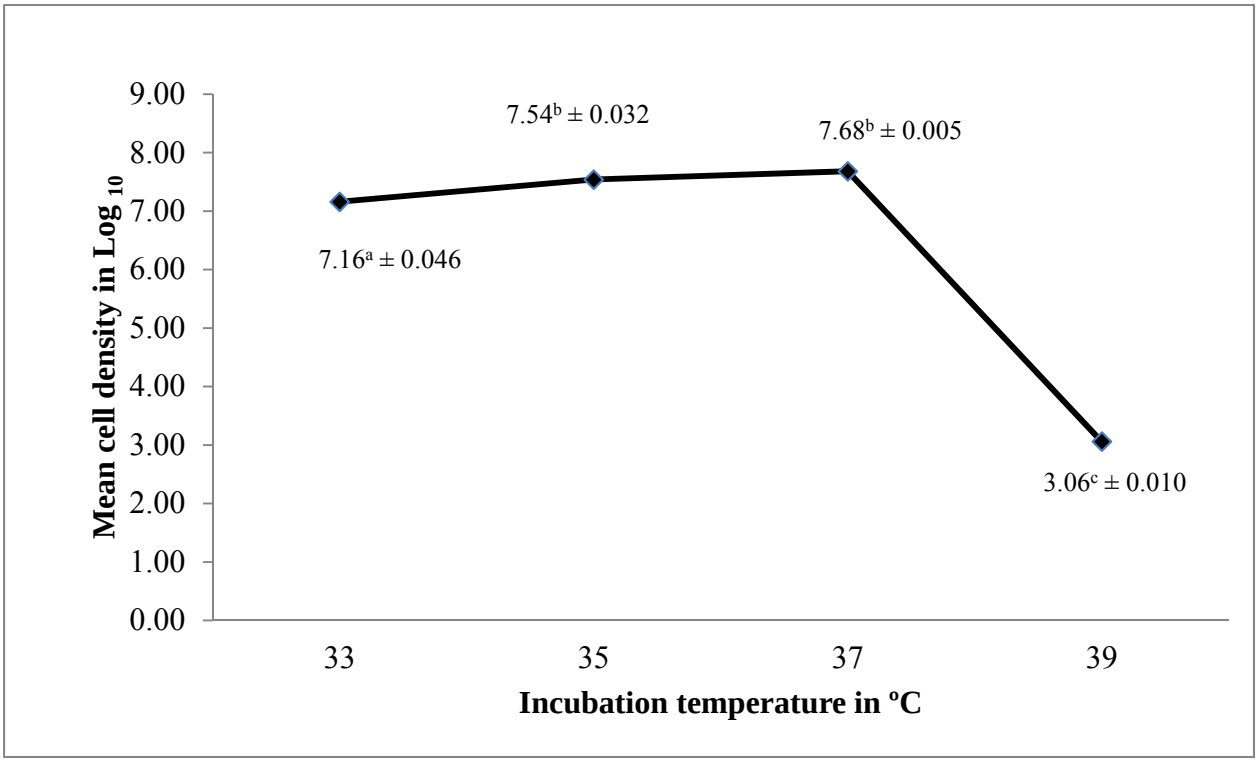

Figure 4.5: Effect of temperature on multiplication of BHK-21 cells cultured in roller bottles

100 ml of the GMEM supplemented with 5 % serum was added in each bottle and the bottles were incubated at various temperatures (^{0}C) for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.5. Mean and Std.dev of the three log values was calculated and was shown in respective box. The mean values having similar superscript are not significantly different ($p > 0.05$).

Table 4.6: Effect of rolling speed on BHK-21 cells proliferation in the roller culture system

*Revolution Speed (rpm)	Cell count ($\log_{10}$)			
	Bottle 1	**Bottle 2**	**Bottle 3**	****Mean± Std.Dev**
01	$2.3x10^4$ (4.36)	$2.5x10^4$ (4.39)	$2.6x10^4$ (4.41)	4.39^a ±0.025
02	$6.8x10^4$ (4.86)	$6.9x10^4$ (4.83)	$7.0x10^4$ (4.84)	4.83^b ± 0.015
03	$4.8x10^7$ (7.68)	$4.7x10^7$ (7.67)	$4.9x10^7$ (7.69)	7.68^c ± 0.010
04	$2.2x10^3$ (3.34)	$2.1x10^3$ (3.32)	$2.2x10^3$ (3.35)	3.33^d ± 0.015
05	$3.5x10^2$ (2.54)	$3.0x10^2$ (2.47)	$2.8x10^2$ (2.44)	2.49^e ± 0.051

*Speed in revolution per minute (rpm) **Mean ± standard deviation of log values

Seeding density was 10 million cells per bottle (n =3), 5 % serum was added in the Glasgow minimum essential medium and the bottles were incubated at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.6. Log of each value was determined and depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. None of the mean values are similar, each is having different superscript indicates that all the values are significantly different ($p > 0.05$).

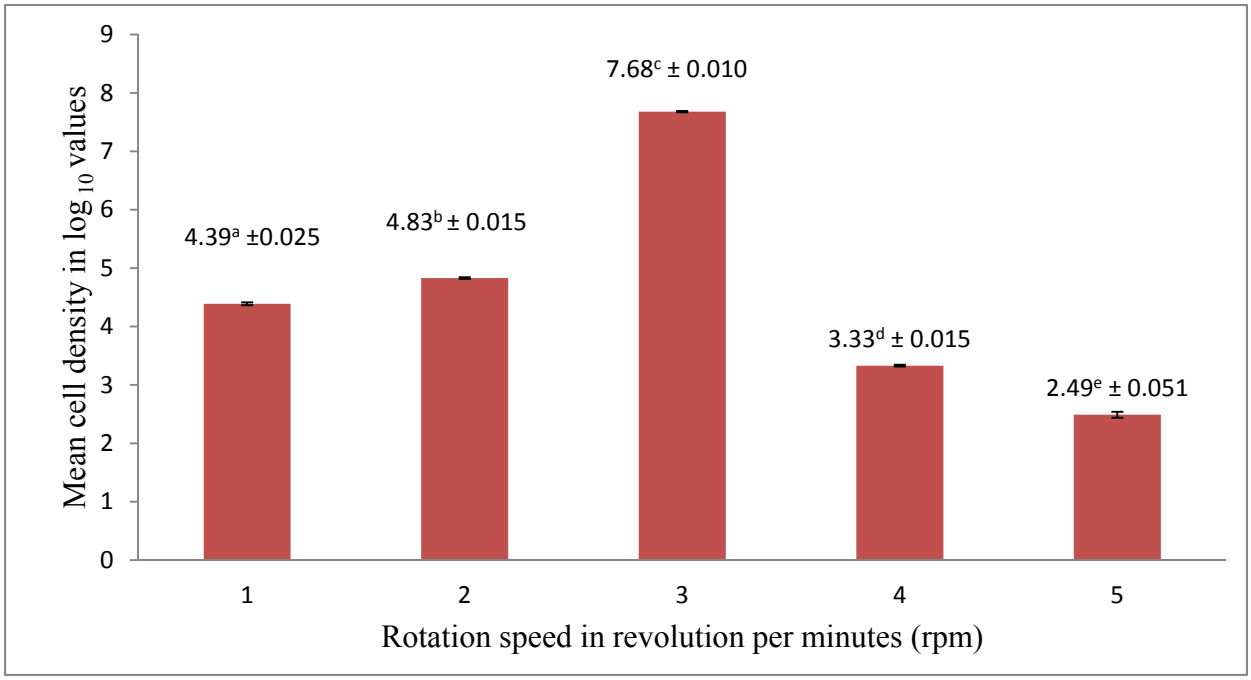

Figure 4.6: Growth rate of BHK-21 cells cultured in roller bottles at various rolling speed.

Seeding density was 10 million cells per bottle (n =3), 5 % serum was added in the Glasgow minimum essential medium and the bottles were incubated at 37 °C for 48 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.6. Log of each value was determined and depicted in parenthesis, then mean Std.dev of the three log values was calculated and was shown in respective box. None of the mean values are similar, each is having different superscript indicates that all the values are significantly different ($p > 0.05$).

Table 4.7 Biomass production of BHK-21 cells using different culture systems

Cell Culture System	Cell count (log_{10})			
	Bottle 1	**Bottle 2**	**Bottle3**	**Mean± Std.dev**
Roux Flask	1.9×10^7 (7.27)	2.4×10^7 (7.38)	1.7×10^7 (7.23)	$7.29^a \pm 0.07$
Roller Bottle	4.5×10^7(7.65)	4.9×10^7 (7.69)	4.4×10^7 (7.64)	$7.66^b \pm 0.02$
Micro-carrier	1.8×10^8 (8.25)	2.4×10^8 (8.38)	2.5×10^8 (8.39)	$8.34^c \pm 0.07$

Ten million cells (BHK-21) were added in each of the three culture vessels/ bottles (n =3) of conventional (35 ml GMEM/ Roux flask), Roller bottle (100 ml GMEM/bottle and Micro-carrier culture (200 ml GMEM/bottle) were seeded. The Glasgow minimal essential medium (GMEM) was supplemented with 5 % serum. Each of the culture vessels of each of the culture system was incubated at 37 °C for 60 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Table 4.7. Log of each value determined and was depicted in parenthesis. Mean and Std.dev of each of the three log values was calculated and was shown in respective box. The mean values having different superscript are significantly different ($p<0.05$).

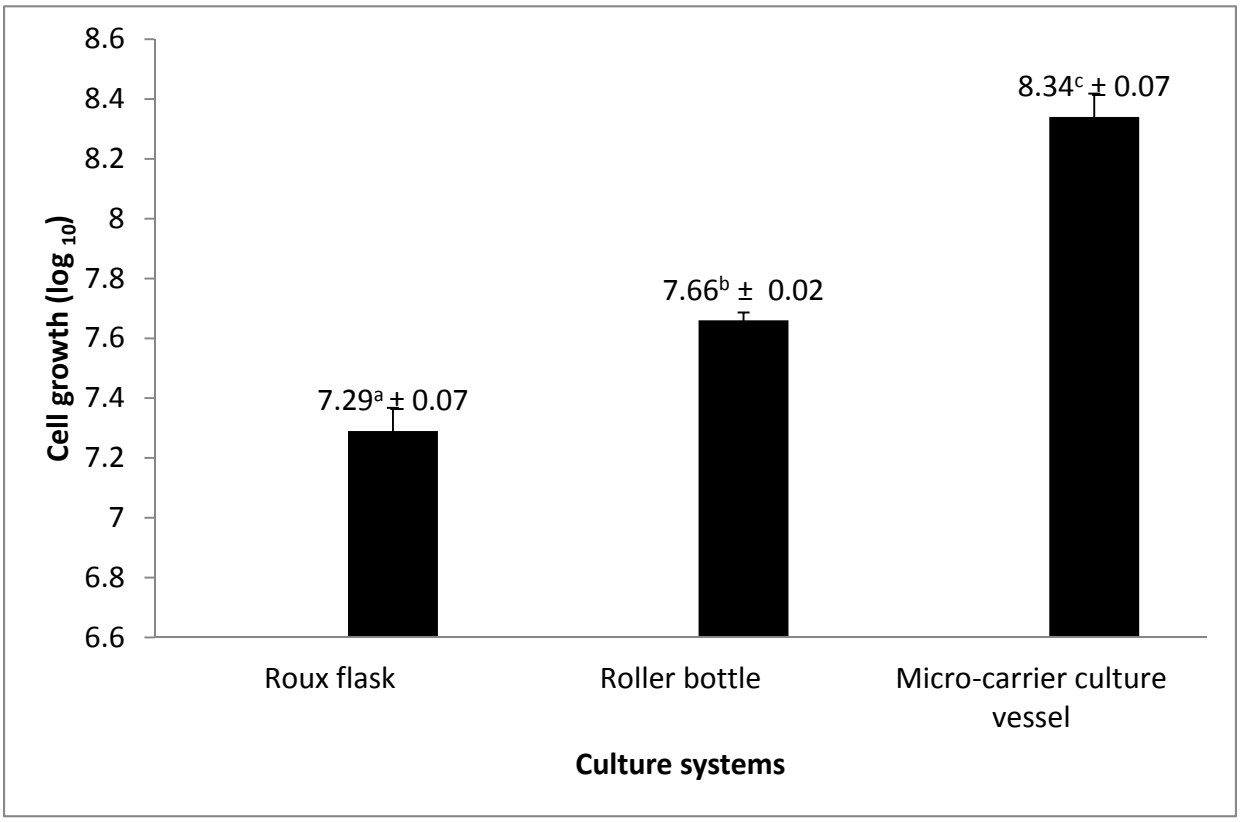

Figure 4.7 Biomass productions of BHK-21 cells using different culture systems

Ten million cells (BHK-21) were added in each of the three culture vessels/ bottles (n =3) of conventional (35 ml GMEM/ Roux flask), Roller bottle (100 ml GMEM/bottle and Micro-carrier culture (200 ml GMEM/bottle) were seeded. The Glasgow minimal essential medium (GMEM) was supplemented with 5 % serum. Each of the culture vessels of each of the culture system was incubated at 37 °C for 60 hours. Cells were harvested from each bottle and counted. The cell count was shown in the respective box of the Figure 4.7. Mean and Std.dev of each of the three log values was calculated and was shown in respective box. The mean values having different superscript are significantly different ($p<0.05$).

4.2 Factor affecting the biomass production (Biological titer) of FMD virus serotypes ("O", "A" and "Asia-1")

4.2.1. Culture system

The FMD virus serotypes ("O","A" and "Asia-1") were inoculated in each of the three bottles/flasks of each of the three culture system containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of each type of the virus was determined. The Log values of the biological titer were shown in parenthesis. The mean and standard deviation of three Log values of the same row was shown in the last box of the respective row (Table 4.8 and Figure 4.8: Table 4.9 and Figure 4.9: Table 4.10 and Figure 4.10).

4.2.1.1 Roux flask

It was observed that the virus type "O" harvested from the monolayer of BHK-21 cells in each of the Roux flask reached to biological titer ($TCID_{50}$) of $10^{6.6}$, $10^{6.3}$ and $10^{6.5}$ and $\log_{10}$ values of the each biological titer of each of the Roux flasks were 6.6, 6.3 and 6.5. Mean ± standard deviation of these log10 values was 6.46± 0.15 (Table 4.8).

The virus type "A" harvested from the monolayer of BHK-21 cells grown in Roux flask reached to biological titer ($TCID_{50}$) of $10^{6.3}$, $10^{5.9}$ and $10^{6.0}$ and $\log_{10}$ values of the each biological titer of each of the Roux flasks were 6.3, 5.9 and 6.0. Mean ± standard deviation of these log values was 6.06± 0.2 (Table 4.9).

The virus type "Asia-1" harvested from the monolayer of BHK-21 cells grown in each of the Roux flask reached to biological titer ($TCID_{50}$) of $10^{5.9}$, $10^{6.2}$ and $10^{6.4}$ and $\log_{10}$ values of the

each biological titer of each of the Roux flasks were 5.9, 6.2 and 6.4. Mean ± standard deviation of these log values was 6.1± 0.2 (Table 4.10).

4.2.1.2. Roller Bottle

The FMD "O" virus harvested from the monolayer of BHK-21 cells in Roller Bottles reached to biological titer ($TCID_{50}$) of $10^{7.2}$, $10^{7.4}$ and $10^{7.0}$ in each of the Roller bottle and log_{10} values of the each biological titer count of each of the Roller Bottle were 7.2, 7.4 and 7.0. Mean ± standard deviation of these log values was 7.20 ± 0.20 (Table 4.8). The FMD"A "virus harvested from the monolayer of BHK-21 cells grown in Roller Bottles reached to biological titer ($TCID_{50}$) of $10^{7.1}$, $10^{7.4}$ and $10^{7.2}$ in each of the Roller bottle and log_{10} values of the each biological titer count of each of the Roller Bottle were 7.1, 7.4 and 7.2. Mean ± standard deviation of these log_{10}values was 7.20 ± 0.15 (Table 4.9). The FMD "Asia-1" virus harvested from the monolayer of BHK-21 cells in Roller Bottles reached to biological titer ($TCID_{50}$) of $10^{6.8}$, $10^{7.0}$ and $10^{7.2}$ in each of the Roller bottle and log_{10} values of the each biological titer count of each of the Roller Bottle were 6.8, 7.0 and 7.2. Mean ± standard deviation of these log_{10}values was 7.20 ± 0.20 (Table 4.10).

4.2.1.3. Micro-carrier culture vessel

The FMD "O" virus harvested from the monolayer of BHK-21 cells in Micro-carrier culture vessels reached to biological titer (TCID50) of $10^{8.3}$, $10^{8.0}$ and $10^{8.1}$ and log_{10} values of the each biological titer count of each of the Micro-carrier culture vessels were 8.3, 8.0 and 8.1. Mean ± standard deviation of these log_{10} values was 8.13 ± 0.15 (Table 4.8). The FMD "A" virus harvested from the monolayer of BHK-21 cells in micro-carrier culture vessels reached to biological titer (TCID50) of $10^{8.2}$, $10^{8.3}$ and $10^{8.2}$ and log values of the each biological titer of

each of the Micro-carrier culture vessels were 8.2, 8.3 and 8.2. Mean ± standard deviation of these log values was 8.2 ± 0.05 (Table 4.9). The FMD "Asia-1" virus harvested from the monolayer of BHK-21 cells in Micro-carrier culture vessels reached to biological titer ($TCID_{50}$) of $10^{7.7}$, $10^{8.2}$ and $10^{8.3}$ and log values of the each biological titer of each of the Micro-carrier culture vessels were 7.7, 8.2 and 8.3. Mean ± standard deviation of these log_{10} values was 8.01 ± 0.3 (Table 4.10).

4.2.2. Incubation temperature

Effect of different incubation temperature was monitored on biological titer ($TCID_{50}$) of FMD type "O" virus in roller culture bottles. Cell density in each bottle was 10 million irrespective of its incubation temperature and the results in the form biological titer ($TCID_{50}$) / ml of medium are shown in Table 4.11 (Figure 4.11). It was observed that the bottles incubated at 33^0C supported biological titer ($TCID_{50}$) up to $10^{6.1}$, $10^{6.1}$ and $10^{6.2}$ and their log values were 6.1, 6.1 and 6.2 respectively in each of the three bottles. Mean± standard deviation of these log values was 6.1 ± 0.05. The bottles incubated at 35^0C supported biological titer ($TCID_{50}$) up to $10^{6.8}$, $10^{6.5}$ and $10^{6.6}$ and their antilog values were 6.8, 6.5 and 6.6 respectively in each of the three bottles. Mean± standard deviation of these log values was 6.6 ± 0.1. The bottles incubated at 37^0C supported biological titer ($TCID_{50}$) up to $10^{7.1}$, $10^{6.8}$ and $10^{7.2}$ and log values were 7.1, 6.8 & 7.2 respectively in each of three bottles. Mean± standard deviation of these antilog values was 7.0 ± 0.2. The bottles incubated at 39^0C supported biological titer ($TCID_{50}$) up to $10^{3.6}$, $10^{3.8}$ and $10^{3.1}$ and log values were 3.6, 3.8 and 3.1 respectively in each of three bottles. Mean± standard deviation of these log values was 3.5 ± 0.3. It was further added that minimum biological titer in bottle incubated at 39^0C (3.5) was significantly less than the titer observed in bottle incubated at 37^0C (7.0) and 35^0C (6.6) as ($p<0.05$).

4.2.3. Fetal Calf Serum

Effect of different concentrations of fetal calf serum in GMEM was monitored on the biological titer ($TCID_{50}$) of FMD type "O" virus on BHK-21 cells in roller culture bottles. Cell density in each bottle was 10 million irrespective of its FCS concentration and the results in the form cell count / ml of medium as shown in Table 4.12 (Fig 4.12). It was observed that bottles supplemented with 0 % FCS supported biological titer ($TCID_{50}$) up to $10^{3.3}$, $10^{3.5}$ and $10^{3.0}$ and log values were 3.3, 3.5 and 3.0 respectively in each of the three bottles. Mean ± standard deviation of these log values was 3.2 ± 0.025. The bottles supplemented with 1 % FCS supported biological titer ($TCID_{50}$) up to $10^{7.1}$, $10^{6.9}$ and $10^{7.3}$ and log values were 7.1, 6.9 and 7.3 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.1 ± 0.020. The bottles supplemented with 2 % FCS supported biological titer ($TCID_{50}$) up to $10^{6.8}$, $10^{7.2}$ and $10^{7.0}$ and log values were 6.8, 7.2 and 7.0 respectively in each of the three bottles. Mean ± standard deviation of these log values was 7.0 ± 0.020. The bottles supplemented with 3 % FCS supported biological titer ($TCID_{50}$) up to $10^{6.2}$, $10^{6.5}$ and $10^{6.1}$ and log values were 6.2, 6.5 and 6.1 respectively in each of the three bottles. Mean ± standard deviation of these log values was 6.2 ± 0.020. The bottles supplemented with 4 % FCS supported biological titer ($TCID_{50}$) up to $10^{5.5}$, $10^{6.1}$ and $10^{5.7}$ and log values were 5.5, 6.1 and 5.7 respectively in each of the three bottles. Mean ± standard deviation of these log values was 5.7 ± 0.030. The bottles supplemented with 5 % FCS supported biological titer ($TCID_{50}$) up to $10^{5.1}$, $10^{5.5}$ and $10^{5.1}$ and log values were 5.1, 5.5 and 5.1 respectively in each of the three bottles. Mean ± standard deviation of these log values was 5.2 ± 0.023.

Table 4.8: Biological titer of FMD "O" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

Cell Culture System	$TCID_{50}$ 0f FMD virus type "O" using various culturing methods (log_{10})			
	Bottle 1	Bottle 2	Bottle3	Mean± Std.dev
Roux Flask	$10^{6.6}$ (6.6)	$10^{6.3}$(6.3)	$10^{6.5}$(6.5)	6.46 ± 0.15
Roller Bottle	$10^{7.2}$(7.2)	$10^{7.4}$ (7.4)	$10^{7.0}$(7.0)	7.20 ± 0.20
Micro-carrier	$10^{8.3}$(8.3)	$10^{8.0}$(8.0)	$10^{8.1}$(8.1)	8.13 ± 0.15

The FMD "O" virus was inoculated in each of the three bottles/flasks containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of the virus was determined and shown in respective box of the above mentioned Table. The Log_{10} values of the cell counts were shown in parenthesis. The mean and standard deviation of three Log_{10} values of the same row was shown in the last box of the respective row.

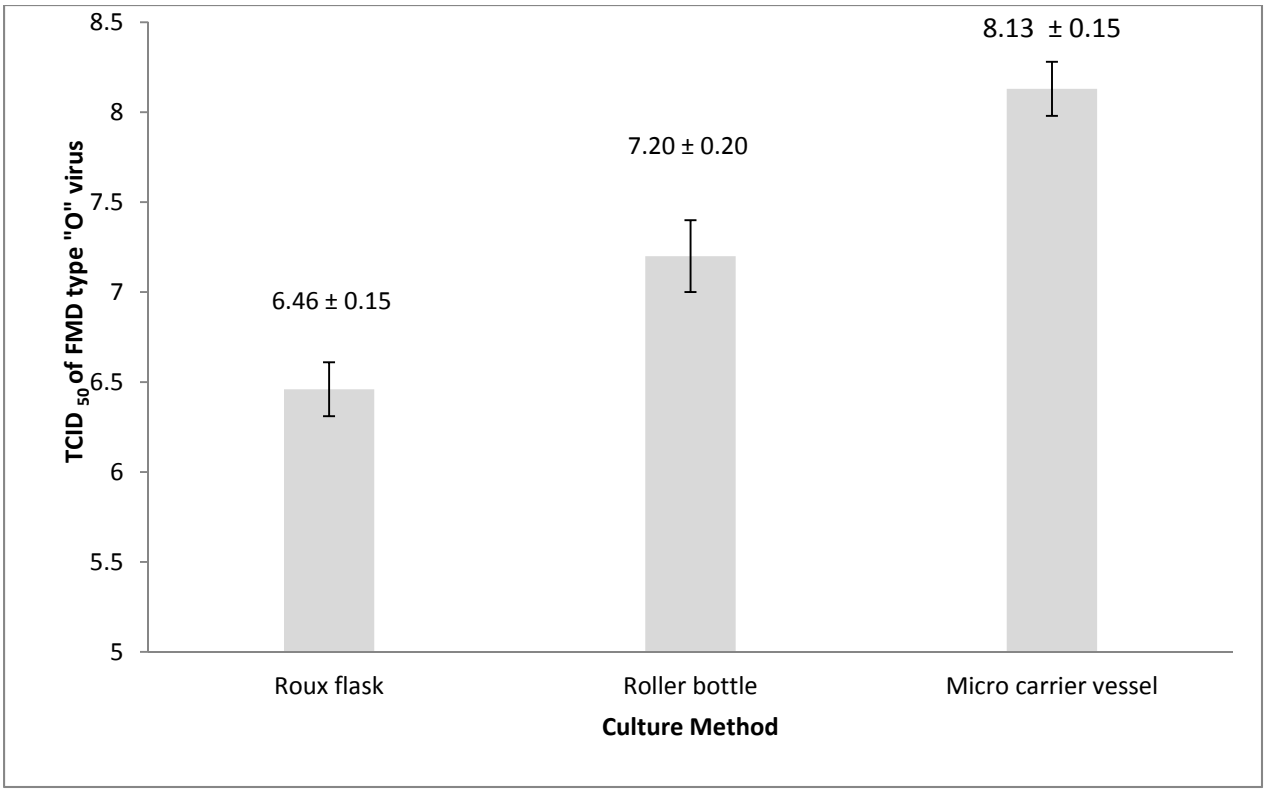

Figure 4.8: Biological titer of FMD "O" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

The FMD "O" virus was inoculated in each of the three bottles/flasks containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of the virus was determined. The mean and standard deviation of three log values of the same row was shown in the box.

Table 4.9: Biological titer of FMD "A" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

Cell Culture System	$TCID_{50}$ 0f FMD virus type "A" using various culturing methods.			
	Bottle 1	Bottle 2	Bottle3	Mean± Std.dev
Roux Flask	$10^{6.3}$(6.3)	$10^{5.9}$(5.9)	$10^{6.0}$(6.0)	6.067 ± 0.20
Roller Bottle	$10^{7.1}$(7.1)	$10^{7.4}$(7.4)	$10^{7.2}$(7.2)	7.233 ± 0.15
Micro carrier Bottle	$10^{8.2}$(8.2)	$10^{8.3}$(8.3)	$10^{8.2}$(8.2)	8.233 ± 0.05

The FMD "A" virus was inoculated in each of the three bottles/flasks containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of the virus was determined and shown in respective box of the above mentioned Table. The Log_{10} values of the cell counts were shown in parenthesis. The mean and standard deviation of three Log_{10} values of the same row was shown in the last box of the respective row.

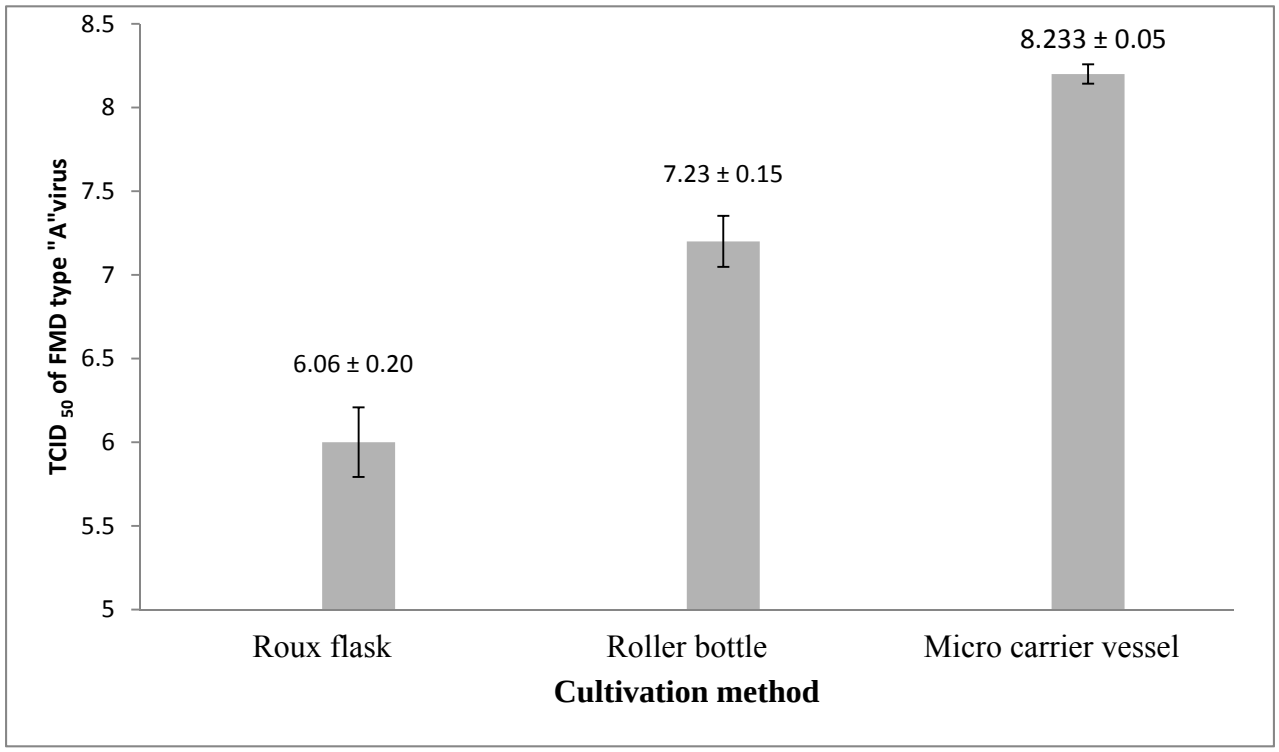

Figure 4.9 Biological titer of FMD "A" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

The FMD "A" virus was inoculated in each of the three bottles/flasks containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of the virus was determined and shown in respective box of the above mentioned Table. The mean and standard deviation of three log values are mentioned in the box.

Table 4.10: Biological titer of FMD "Asia-1" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

Cell Culturing System	**$TCID_{50}$ of FMD virus type "Asia-1" using various culturing methods.**			
	Bottle 1	Bottle 2	Bottle3	Mean± Std.dev
Roux Flask	$10^{5.9}$ (5.9)	$10.^{6.2}$(6.2)	$10^{6.4}$(6.4)	6.1 ± 0.2
Roller Bottle	$10^{6.8}$(6.8)	$10^{7.0}$(7.0)	$10^{7.2}$(7.2)	7.0 ± 0.2
Micro-carrier	$10^{7.7}$(7.7)	$10^{8.2}$(8.2)	$10^{8.3}$(8.3)	8.0 ± 0.3

The FMD "Asia-1" virus was inoculated in each of the three bottles/flasks containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of the virus was determined and shown in respective box of the above mentioned Table. The Log_{10} values of the cell counts were shown in parenthesis. The mean and standard deviation of three Log_{10} values of the same row was shown in the last box of the respective row.

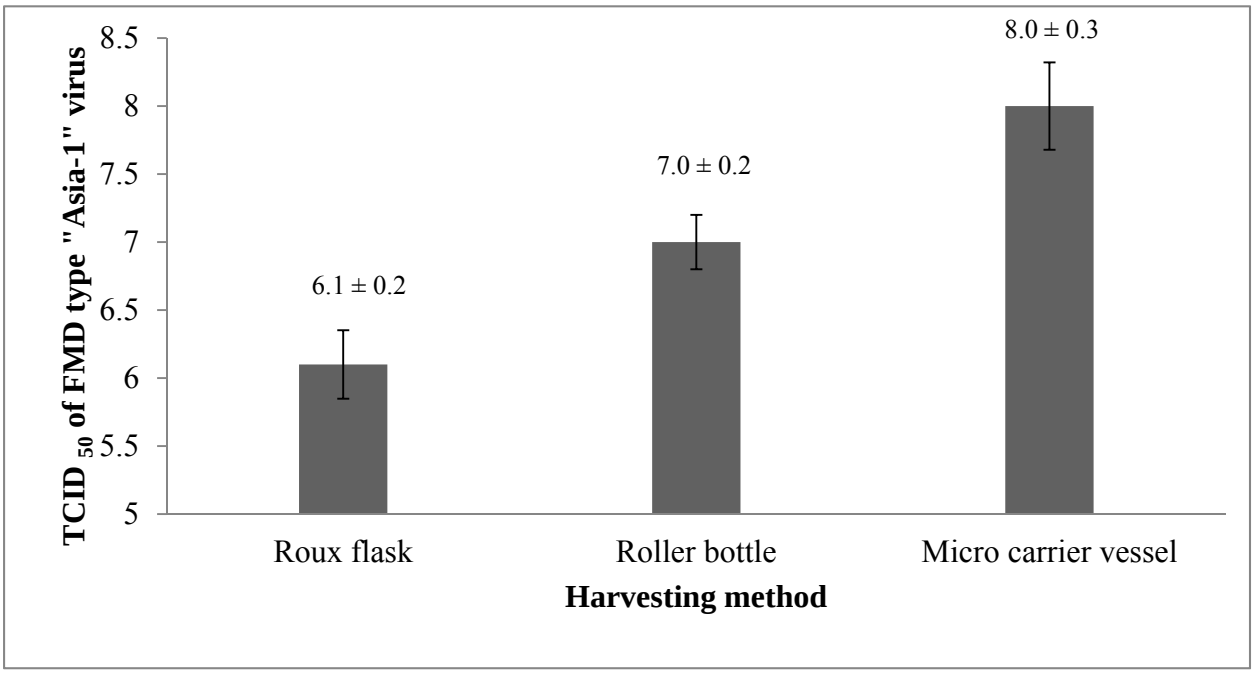

Figure 4.10 Biological titer of FMD "Asia-1" virus cultivated in Roux flask, Roller Bottle and Micro- carrier culture system

The FMD "Asia-1" virus was inoculated in each of the three bottles/flasks containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer of the virus was determined and shown in respective box. The mean and standard deviation of three log values of the same row was shown in the box.

Table 4.11: Effect of Incubation temperature on biomass production of FMD "O" virus ($TCID_{50}$)

Incubation temperature	$TCID_{50}$ of FMD virus type "O" (log 10)			
	Bottle 1	Bottle 2	Bottle 3	Mean ± std.dev
33 °C	$10^{6.1}$ (6.1)	$10^{6.1}$ (6.1)	$10^{6.2}$ (6.2)	6.1 ± 0.05
35 °C	$10^{6.8}$ (6.8)	$10^{6.5}$ (6.5)	$10^{6.6}$ (6.6)	6.6 ± 0.1
37 °C	$10^{7.1}$ (7.1)	$10^{6.8}$ (6.8)	$10^{7.2}$ (7.2)	7.0 ± 0.2
39 °C	$10^{3.6}$ (3.6)	$10^{3.8}$ (3.8)	$10^{3.1}$ (3.1)	3.5 ± 0.3

FMD type "O" virus was inoculated to roller bottles having monolayer of BHK-21 cells. 100 ml of the Glasgow minimal essential medium supplemented with 1 % serum was added in each bottle and the bottles were incubated at 33 °C, 35 °C, 37 °C and 39 °C. Seeding density of BHK-21 cells was 10 million (n =3). After incubation of 48 hours, virus was harvested and biological titer ($TCID_{50}$) from each of the three bottles was shown in Table 4.11. Log of each value determined and was depicted in parenthesis, Mean and Std.dev of the three log values was calculated and was shown in respective box.

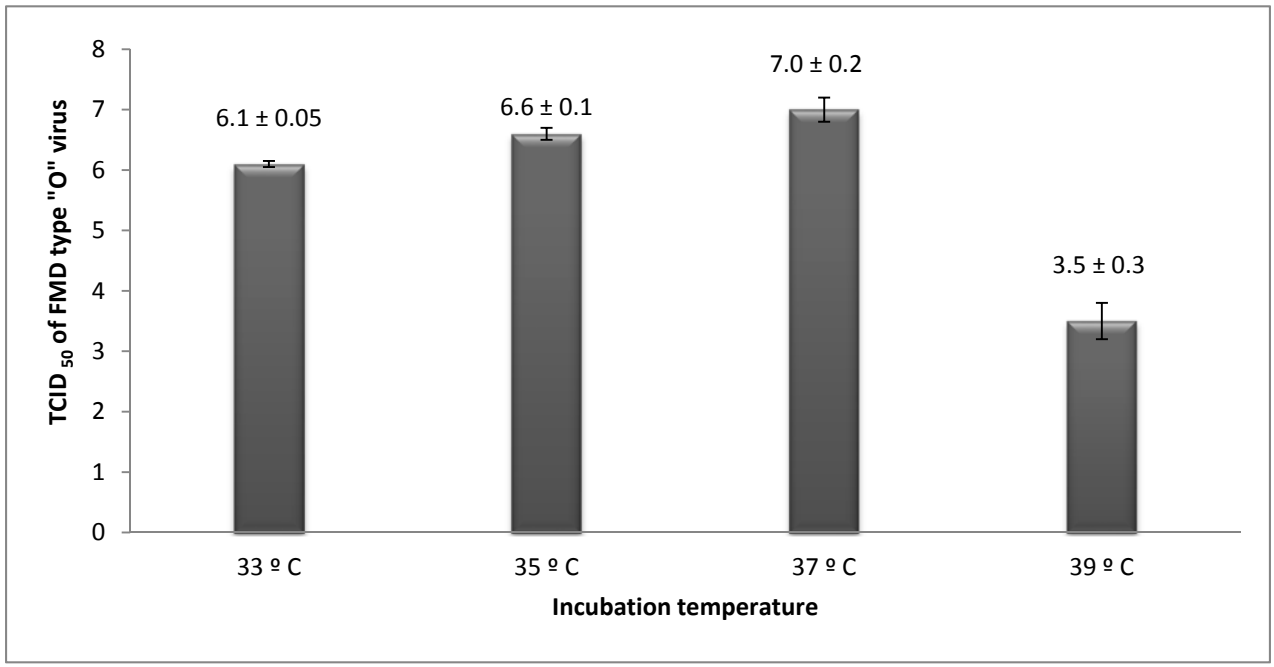

Figure 4.11: Effect of Incubation temperature on biomass production of FMD "O" virus ($TCID_{50}$)

FMD type "O" virus was inoculated to roller bottles having monolayer of BHK-21 cells. 100 ml of the Glasgow minimal essential medium supplemented with 1 % serum was added in each bottle and the bottles were incubated at 33 °C, 35 °C, 37 °C and 39 °C. Seeding density of BHK-21 cells was 10 million (n =3). After incubation of 48 hours, virus was harvested. Mean and Std.dev of the three log values was calculated and was shown in respective box.

Table 4.12: Effect of Fetal Calf Serum concentration on biomass production of FMD "O" virus ($TCID_{50}$)

Fetal Calf serum Concentration	TCID $_{50}$ FMD virus type "O"(log $_{10}$)			
	Bottle 1	Bottle 2	Bottle 3	Mean ± std.dev
0 %	$10^{3.3}$ (3.3)	$10^{3.5}$(3.5)	$10^{3.0}$(3.0)	3.2 ± 0.25
1 %	$10^{7.1}$ (7.1)	$10^{6.9}$(6.9)	$10^{7.3}$(7.3)	7.1 ± 0.20
2 %	$10^{6.8}$ (6.8)	$10^{7.2}$(7.2)	$10^{7.0}$(7.0)	7.0 ± 0.20
3 %	$10^{6.2}$(6.2)	$10^{6.5}$(6.5)	$10^{6.1}$(6.1)	6.2 ± 0.20
4 %	$10^{5.5}$(5.5)	$10^{6.1}$(6.1)	$10^{5.7}$(5.7)	5.7 ± 0.30
5 %	$10^{5.1}$(5.1)	$10^{5.5}$(5.5)	$10^{5.1}$(5.1)	5.2 ± 0.23

Effect of different concentrations Fetal Calf serum was monitored. The FMD type "O" virus was inoculated in each of the three bottles containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer ($TCID_{50}$) of the virus was determined and shown in respective box of the above mentioned Table. The Log_{10} values of the cell counts were shown in parenthesis. The mean and standard deviation of three Log_{10} values of the same row was shown in the last box of the respective row.

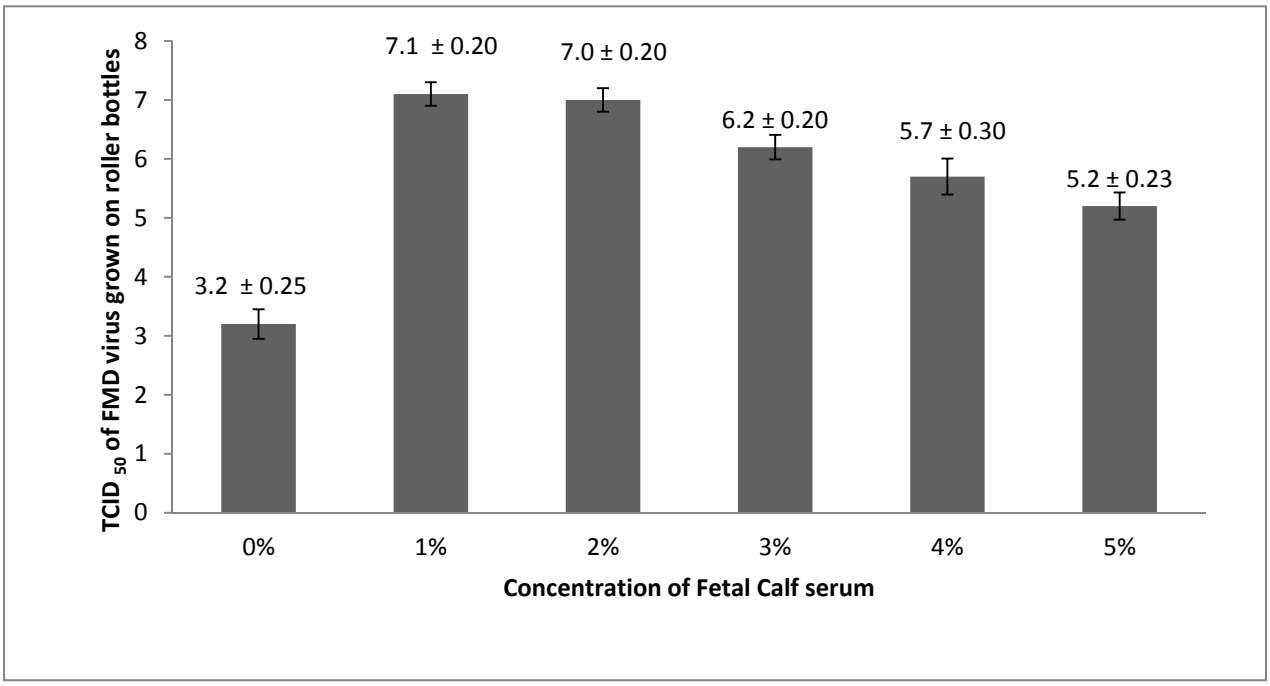

Figure 4.12: Effect of Fetal Calf Serum concentration on biomass production of FMD "O" virus ($TCID_{50}$)

Effect of different concentrations of Fetal Calf serum was monitored. The FMD type "O" virus was inoculated in each of the three bottles containing BHK-21 cells. After incubation of these culture vessels at 37^0C for 48 hours, the biological titer ($TCID_{50}$) of the virus was determined. The mean and standard deviation of three log values of the same row was shown in the last box.

4.3.1 Effect of Immunogen level on antibody response of buffalo calves to FMD trivalent vaccine

Montanide ISA-70 based Foot and Mouth Disease (FMD) "O","A" and "Asia-1" virus vaccine was prepared containing either 0.1 ml , 0.2 ml ,0.3 ml and 0.4 ml of each of the serotype of the virus / dose of the vaccine (3 ml). One ml suspension of each serotype of the virus was containing 10^7 units of $TCID_{50}$ titer.

4.3.1.1 FMD virus "Type O"

Each of the vaccines when injected to each of the five buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotype "O" of the virus (Table 4.7 and Figure 4.7). The anti-FMD "O" type virus CFT antibody titer of the calves in control group (non vaccinated calves) on 30 day was 0, 0, 0, 0 and 0. The CFT antibody titer on 60 day was 0, 0, 0, 0 and 0. The CFT antibody titer on day 90 was 0, 0, 0, 0 and 0. The CF antibody titer on day 180 was 0, 0, 0, 0 and 0. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 0, 0, 0, 0 and 0 respectively. The CGMT ± standard deviation was 0.0 ± 0.0. The anti-FMD "O" type virus CFT antibody titer of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) on 30 day was 2, 2, 2, 2 and 2. The CFT antibody titer on 60 day was 2, 2, 4, 2 and 4. The CFT antibody titer on day 90 was 4, 4, 4, 2 and 4. The CF antibody titer on day 180 was 2, 2, 4, 2 and 2. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 1, 1.4, 1.8 and 1.2 respectively. The CGMT ± standard deviation was 1.3 ± 0.4435. The anti-FMD "O" type virus CFT antibody titer of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension)

on 30 day was 4, 4, 4, 4 and 4. The CFT antibody titer on 60 day was 4, 4, 8, 4 and 8. The CFT antibody titer on day 90 was 4, 4, 4, 4 and 8. The CF antibody titer on day 180 was 4, 4, 4, 8 and 8. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 2, 2.4, 2.2 and 2.4 respectively. The CGMT ± standard deviation was 2.2 ± 0.20. The anti-FMD "O" type virus CFT antibody titer of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) on 30 day was 8, 8, 8, 8 and 8. The CFT antibody titer on 60 day was 32, 32, 16, 32 and 32. The CFT antibody titer on day 90 was 32, 64, 64, 64 and 64. The CF antibody titer on day 180 was 256, 256, 256, 256 and 256. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 3, 4.8, 5.8and 7.8 respectively. The CGMT ± standard deviation was 5.3 ± 2.0. The anti-FMD "O" type virus CFT antibody titer of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) on 30 day was 8, 8, 8, 16 and 8. The CFT antibody titer on 60 day was 32, 32, 32, 32 and 32. The CFT antibody titer on day 90 was 64, 64, 64, 64 and 64.. The CF antibody titer on day 180 was 256, 256, 256, 256 and 256. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 3.2, 5.0, 6.0 and 8.0 respectively. The CGMT ± standard deviation was 5.5 ± 1.53.

4.3.1.2. FMD virus "Type A"

Each of the vaccines when injected to each of the five buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotype "A" of the virus (Table 4.8 and Figure 4.8). The anti-FMD "A" type virus CFT antibody titer of the calves in control group (non vaccinated calves) on 30 day was 0, 0, 0, 0 and 0. The CFT antibody titer on 60 day was 0, 0, 0, 0 and 0The CFT antibody titer on day 90 was 0, 0, 0, 0 and 0. The CF

antibody titer on day 180 was 0, 0, 0, 0 and 0. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 0, 0, 0, 0 and 0 respectively. The CGMT ± standard deviation was 0.0 ± 0.0. The anti-FMD "A" type virus CFT antibody titer of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) on 30 day was 2, 2, 4, 2 and 2.. The CFT antibody titer on 60 day was 2, 2, 2, 2 and 4. The CFT antibody titer on day 90 was 2, 4, 4, 4 and 2. The CF antibody titer on day 180 was 4, 4, 4, 2 and 4 The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 1.2, 1.2, 1.6, and 1.8 respectively. The CGMT ± standard deviation was 1.45 ± 0.300. The anti-FMD "A" type virus CFT antibody titer of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) on 30 day was 4, 4, 2, 4 and 4.. The CFT antibody titer on 60 day was 4, 4, 4, 4 and 4.The CFT antibody titer on day 90 was 8, 4, 8, 4 and 4.. The CF antibody titer on day 180 was 4, 4, 4, 4 and 8. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 1.8, 2.0, 2.4 and 2.2, respectively. The CGMT ± standard deviation was 2.1 ± 0.2517. The anti-FMD "A" type virus CFT antibody titer of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) on 30 day was 8, 8, 8, 8, and 8. The CFT antibody titer on 60 day was 16, 8, 16, 8 and 32. The CFT antibody titer on day 90 was 16, 64, 64, 64 and 64 The CF antibody titer on day 180 was 64, 64, 64, 64 and 64. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 3, 3.8, 5.8 and 6.0 respectively. The CGMT ± standard deviation was 4.4 ± 1.86. The anti-FMD "A" type virus CFT antibody titer of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) on 30 day was 8, 8, 8, 8 and 8. The CFT antibody titer on 60 day was 8, 16, 8, 8 and 8. The CFT antibody titer on

day 90 was 64, 64, 64, 32 and 64.. The CF antibody titer on day 180 was 64, 64, 64, 64 and 64. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 3, 3.2, 5.8 and 6.8 respectively. The CGMT ± standard deviation was 4.4 ± 1.86.

4.3.1.3. Type Asia-1 virus

Each of the vaccines when injected to each of the five buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotype"Asia-1" of the virus (Table 4.9 and Figure 4.9). The anti-FMD "Asia-1" type virus CFT antibody titer of the calves in control group (non vaccinated calves) on 30 day was 0, 0, 0, 0 and 0. The CFT antibody titer on 60 day was 0, 0, 0, 0 and 0. The CFT antibody titer on day 90 was 0, 0, 0, 0 and 0. The CF antibody titer on day 180 was 0, 0, 0, 0 and 0. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 0, 0, 0, 0 and 0 respectively. The CGMT ± standard deviation was 0.0 ± 0.0. The anti-FMD "Asia-1" type virus CFT antibody titer of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) on 30 day was 2, 2, 2, 2 and 2. The CFT antibody titer on 60 day was 4, 4, 8, 8 and 8. The CFT antibody titer on day 90 was 4, 4, 8 and 4. The CF antibody titer on day 180 was 4, 4, 4, 8 and 8. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 1, 2.6, 2.2, and 2.4 respectively. The CGMT ± standard deviation was 2.0 ± 0.71 The anti-FMD "Asia-1" type virus CFT antibody titer of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) on 30 day was 4, 4, 8, 4 and 8. The CFT antibody titer on 60 day was 8, 8, 8, 16 and 16.The CFT antibody titer on day 90 was 16, 16, 16, 16 and 16. The CF antibody titer on day 180 was 8, 8, 8, 16 and 8. The GMT of the anti FMD "Asia-1" CFT antibody titer of

calves of this group on 30, 60, 90 and 180 days post priming were 2.4, 3.4, 4.0, and 3.2 respectively. The CGMT ± standard deviation was 3.4 ± 0.83. The anti-FMD "Asia-1" type virus CFT antibody titer of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) on 30 day was 4, 8, 8, 8, and 8. The CFT antibody titer on 60 day was 8, 16, 16, 16 and 16. The CFT antibody titer on day 90 was 64, 64, 128, 16 and 128 The CF antibody titer on day 180 was 128, 128, 256, 256 and 128. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 2.8, 3.8, 6 and 7.4 respectively. The CGMT ± standard deviation was 5.0 ± 2.15. The anti-FMD "Asia-1" type virus CFT antibody titer of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) on 30 day was 8, 8, 8, 8 and 8. The CFT antibody titer on 60 day was 16, 16, 32, 16 and 32The CFT antibody titer on day 90 was 64, 64, 64, 64 and 128. The CF antibody titer on day 180 was 128, 128, 128, 256 and 256.. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 3, 4.4, 6.2 and 7.4 respectively. The CGMT ± standard deviation was 5.2 ± 1.85.

4.3.2. Effect of boosting on antibody response of buffalo calves to oil based FMD trivalent ("O", "A" and "Asia-1") vaccine

4.3.2.1. Boosting response of Control group (non vaccinated)

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus (Table 4.16 and Figure 4.16 : Table 4.17 and Figure 4.17 : Table 4.18 and Figure 4.18 : Table 4.19 and Figure 4.19). Each of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) was boosted on day 60 post priming. The anti-FMD type "O" virus CFT antibody titer of the calves in control group (non vaccinated

calves) on 30 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on day 60 after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on day 90 after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 180 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 0, 0, 0, 0 and 0 respectively. The anti-FMD type "A" virus CFT antibody titer of the calves in control group (non vaccinated calves) on 30 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on day 60 after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on day 90 after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 180 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 0, 0, 0, 0 and 0 respectively. The anti-FMD type "Asia-1" virus CFT antibody titer of the calves in control group (non vaccinated calves) on 30 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on day 60 after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on day 90 after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 180 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming were 0, 0, 0, 0 and 0 respectively.

4.3.2.2. Boosting response of Group 1

The anti-FMD type "O" virus CFT antibody titer of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) on 0 day after priming was 0,0

and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 2, 2 and 2 while of boosting was 2, 2 and 2. The CFT antibody titer of the calves on day 60 after priming was 2, 2 and 4 while of boosting was 4, 4 and 2. The CFT antibody titer of the calves on day 90 after priming was 8, 4 and 8 while of boosting was 16, 8 and 8. The CFT antibody titer of the calves on 180 day after priming was 4, 8 and 8 while of boosting was 16, 64 and 64. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 1, 1.3, 2.6 and 2.6 and 0, 1, 1.6, 3.3 and 5.3 respectively. The anti-FMD type "A" virus CFT antibody titer of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 2, 2 and 2 while of boosting was 2, 2 and 2. The CFT antibody titer of the calves on day 60 after priming was 2, 2 and 4 while of boosting was 4, 4 and 2. The CFT antibody titer of the calves on day 90 after priming was 8, 4 and 8 while of boosting was 16, 8 and 8. The CFT antibody titer of the calves on 180 day after priming was 4, 8 and 8 while of boosting was 16, 64 and 64. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 1, 1.3, 2.6 and 2.6 and 0, 1, 1.6, 3.3 and 5.3 respectively. The anti-FMD type "Asia-1" virus CFT antibody titer of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 2, 2 and 2 while of boosting was 2, 2 and 2. The CFT antibody titer of the calves on day 60 after priming was 2, 4 and 2 while of boosting was 4, 4 and 4. The CFT antibody titer of the calves on day 90 after priming was 4, 8 and 8 while of boosting was 16, 16 and 16. The CFT antibody titer of the calves on 180 day after priming was 8, 8 and 4

while of boosting was 64, 32 and 32. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 1, 1.3, 2.6 and 2.6 and 0, 1, 2, 4 and 5.3 respectively.

4.3.2.3. Boosting response of Group 2

The anti-FMD type "O" virus CFT antibody titer of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 4, 4 and 4 while of boosting was 4, 4 and 4. The CFT antibody titer of the calves on day 60 after priming was 4, 8 and 4 while of boosting was 4, 8 and 4. The CFT antibody titer of the calves on day 90 after priming was 8, 8 and 16 while of boosting was 32, 32 and 32. The CFT antibody titer of the calves on 180 day after priming was 32, 16 and 32 while of boosting was 64, 128 and 64. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 2, 2.3, 3.3 and 4.6 and 0, 2, 2.3, 5 and 6.3 respectively. The anti-FMD type "A" virus CFT antibody titer of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 4, 4 and 2 while of boosting was 4, 4 and 4. The CFT antibody titer of the calves on day 60 after priming was 4, 2 and 4 while of boosting was 4, 4 and 4. The CFT antibody titer of the calves on day 90 after priming was 8, 8 and 8 while of boosting was 32, 32 and 16. The CFT antibody titer of the calves on 180 day after priming was 16, 64 and 16 while of boosting was 128, 128 and 128. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 1.6, 1.6, 3 and 4.6 and 0, 2, 2, 4.6 and 6 respectively. The anti-FMD type "Asia-1" virus CFT antibody titer of the

calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 4, 4 and 4 while of boosting was 4, 4and 8. The CFT antibody titer of the calves on day 60 after priming was 8, 8 and 4 while of boosting was 4, 8 and 8. The CFT antibody titer of the calves on day 90 after priming was 32, 32 and 32 while of boosting was 32, 64 and 64. The CFT antibody titer of the calves on 180 day after priming was 64, 32 and 32 while of boosting was 128, 128 and 128. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 2, 2.6, 5 and 5.3 and 0, 2.3, 2.4, 5.6 and 6 respectively.

4.3.2.4. Boosting effect on group 3

The anti-FMD type "O" virus CFT antibody titer of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 8, 8 and 8 while of boosting was 8, 8 and 8. The CFT antibody titer of the calves on day 60 after priming was 16, 16 and 32 while of boosting was 16, 16 and 16. The CFT antibody titer of the calves on day 90 after priming was 64, 128 and 64 while of boosting was 128, 128 and 128. The CFT antibody titer of the calves on 180 day after priming was 128, 128 and 256 while of boosting was 64, 128 and 64. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 256, 512, 256 and 180 days post priming and boosting were 0, 3, 4.3, 6.3 and 7.3 and 0, 3, 4, 7 and 8.3 respectively. The anti-FMD type "A" virus CFT antibody titer of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 8, 8 and 8 while of boosting was 8, 8 and

8. The CFT antibody titer of the calves on day 60 after priming was 8, 16 and 16 while of boosting was 16, 16 and 16. The CFT antibody titer of the calves on day 90 after priming was 64, 64 and 32 while of boosting was 64, 128 and 128. The CFT antibody titer of the calves on 180 day after priming was 16, 128 and 128 while of boosting was 512, 256 and 256. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 3, 3.6, 5.6 and 6.6 and 0, 3, 4, 6.6 and 8.3 respectively. The anti-FMD type "Asia-1" virus CFT antibody titer of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 8, 8 and 16 while of boosting was 8, 8 and 8. The CFT antibody titer of the calves on day 60 after priming was 16, 8 and 16 while of boosting was 16, 32 and 32. The CFT antibody titer of the calves on day 90 after priming was 64, 64 and 128 while of boosting was 128, 128 and 128. The CFT antibody titer of the calves on 180 day after priming was 256, 256 and 256 while of boosting was 512, 512 and 256. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 3.3, 3.6, 6.3 and 8.0 and 0, 3, 4.6, 7 and 8.6 respectively.

4.3.2.5. Boosting effect on group 4

The anti-FMD type "O" virus CFT antibody titer of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 8, 8 and 8 while of boosting was 8, 8 and 8. The CFT antibody titer of the calves on day 60 after priming was 16, 32 and 32 while of boosting was 16, 32 and 32. The CFT antibody titer of the calves on day 90 after priming was 64, 64 and 32 while of boosting was 128, 128 and

128. The CFT antibody titer of the calves on 180 day after priming was 64, 128 and 256 while of boosting was 64, 128 and 64. The GMT of the anti FMD "O" CFT antibody titer of calves of this group on 256, 256, 256 and 180 days post priming and boosting were 0, 3, 4.3, 5.6 and 6.6 and 0, 3, 4.6, 7 and 8 respectively. The anti-FMD type "A" virus CFT antibody titer of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 8, 8 and 8 while of boosting was 8, 8 and 8. The CFT antibody titer of the calves on day 60 after priming was 4, 16 and 8 while of boosting was 8, 8 and 16. The CFT antibody titer of the calves on day 90 after priming was 16, 64 and 64 while of boosting was 64, 64 and 64. The CFT antibody titer of the calves on 180 day after priming was 64, 128 and 128 while of boosting was 128, 256 and 256. The GMT of the anti FMD "A" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 3, 3, 5.3 and 6.6 and 0, 3, 3.3, 6 and 7.6 respectively. The anti-FMD type "Asia-1" virus CFT antibody titer of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) on 0 day after priming was 0,0 and 0 while of boosting was 0, 0and 0. The CFT antibody titer of the calves on 30 day after priming was 8, 8 and 16 while of boosting was 8, 8 and 8. The CFT antibody titer of the calves on day 60 after priming was 16, 32 and 16 while of boosting was 16, 8 and 8. The CFT antibody titer of the calves on day 90 after priming was 64, 64 and 16 while of boosting was 64, 64 and 64. The CFT antibody titer of the calves on 180 day after priming was 128, 256 and 256 while of boosting was 256, 256 and 256. The GMT of the anti FMD "Asia-1" CFT antibody titer of calves of this group on 30, 60, 90 and 180 days post priming and boosting were 0, 3.3, 4.3, 5.3 and 7.6 and 0, 3, 3.3, 6 and 8 respectively.

Table13: Antibody response of buffalo calf to different levels of immunogen of each serotype of Foot and Mouth Disease virus in trivalent vaccine

Immuogen amount /dose n=5	Serotype of FMD virus	Anti FMD virus Compliment fixation antibody titer on days post priming					
		0	**30**	**60**	**90**	**180**	**CGMT (Log_2) ±Std.Dev**
$10^{6.2}$	"O"	0,0,0,0,0 (0)	2,2,2,2,2 (1)	2,2,4,2,4 (1.4)	4,4,4,2,4 (1.8)	2,2,4,2,2 (1.2)	1.3^{a} ± 0.4
	"A"	0,0,0,0,0 (0)	2,2,4,2,2 (1.2)	2,2,2,2,4 (1.2)	2,4,4,4,2 (1.6)	4,4,4,2,4 (1.8)	1.4 ^{a}±0.30
	"Asia-1"	0,0,0,0,0 (0)	2,2,2,2,2 (1)	4,4,8,8,8 (2.6)	4,4,4,8,4 (2.2)	4,4,4,8,8(2.4)	2.0^{a}± 0.71
$2x10^{6.2}$	"O	0,0,0,0,0 (0)	4,4,4,4,4(2)	4,4,8,4, 8 (2.4)	4,4,4,4,8 (2.2)	4,4,4,8,8 (2.4)	2.2 ^{b}± 0.2
	"A"	0,0,0,0,0 (0)	4,4,2,4,4 (1.8)	4,4,4,4,4 (2)	8,4,8,4,4 (2.4)	4,4,4,4,8 (2.2)	2.1^{b}± 0.25
	"Asia-1"	0,0,0,0,0 (0)	4,4,8,4,8 (2.4)	8,8,8,16,16(3.4)	16,16,16,16, 16 (4)	8,8,8,16,8 (3.2)	3.4^{b}± 0.83
$3x10^{6.2}$	"O"	0,0,0,0,0 (0)	8,8,8,8,8 (3)	32,32,16,32, 32 (4.8)	32,64,64,64, 64(5.8)	256,256,128, 256,256 (7.8)	5.3^{c} ± 2.0
	"A"	0,0,0,0,0 (0)	8, 8, 8, 8, 8 (3)	16,,8,16,8,3 2 (3.8)	16,64,64,64, 64 (5.6)	64,64,64,64,6 4(6)	4.6^{c}± 1.88
	"Asia-1"	0,0,0,0,0 (0)	4,8,8,8,8(2. 8)	8,16,16,16,1 6(3.8)	64,64,128,16 ,128(6)	128,128,256, 256,128(7.4)	5.0^{C}± 2.15
$4x10^{6.2}$	"O"	0,0,0,0,0 (0)	8,8,8,16,8 (3.2)	32,32,32,32, 32 (5)	64,64,64,64, 64 (6)	256,256,256, 256,256 (8)	5.5^{c} ± 1.5
	"A"	0,0,0,0,0 (0)	8, 8, ,8 ,8 ,8 (3)	8,16,8,8,8 (3.2)	64,64,64,32, 64(5.8)	64,64,32,64, 64 (5.8)	4.4 ^{c}± 1.86
	"Asia-1"	0,0,0,0,0 (0)	8,8,8,8,8, (3)	16,16,32,16, 32(4.4)	64,64,64,64, 128(6.2)	128,128,128, 256,256(7.4)	5.2^{C} ±1.85

Montanide ISA-70 based trivalent Foot and Mouth Disease (FMD) “O”,”A” and “Asia-1” virus vaccine was prepared containing either 0.1 ml , 0.2 ml ,0.3 ml and 0.4 ml of each of the serotype of the virus / dose of the vaccine (3 ml). Each of the vaccine was injected (3 ml: deep intramuscular: neck) to each of the five buffalo calves (6 months of age). Blood samples (3 ml) from each of the calves of each group were collected on 0, 30, 60, 90, and 180 days post priming. The serum from each blood sample was separated , stored in properly labeled vials at -80 ^{0}C and processed for monitoring anti-FMD “Asia 1” type virus complement fixing (CFT) antibodies. The geometric mean (GMT) antibody titer of the virus is shown in the Table 4.13. The cumulative geometric mean (CGMT) antibody titer and Std.Dev is shown in respective box. The mean values having similar superscript are not significantly different ($p< 0.05$).

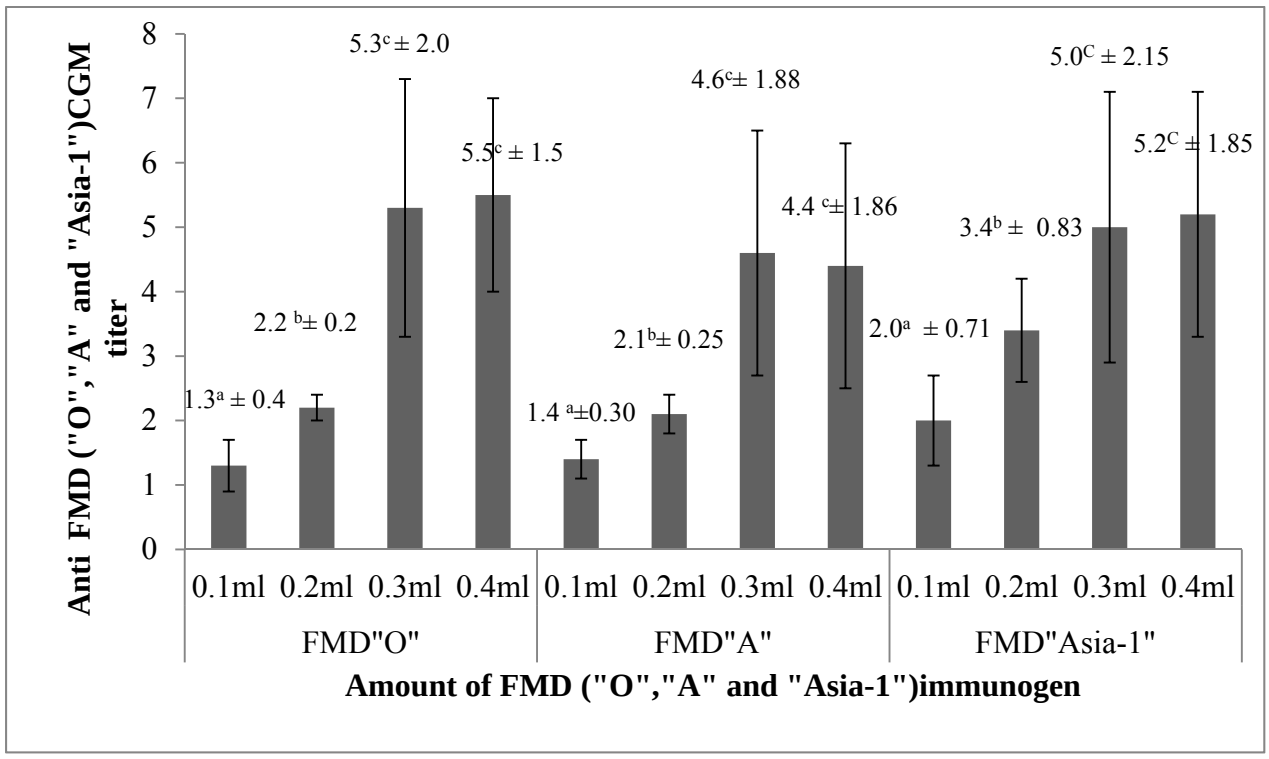

Figure 4.13: Antibody response of buffalo calf to different levels of immunogen of each serotype of Foot and Mouth Disease virus in trivalent vaccine

Montanide ISA-70 based trivalent Foot and Mouth Disease (FMD) "O","A" and "Asia-1" virus vaccine was prepared containing either 0.1 ml , 0.2 ml ,0.3 ml and 0.4 ml of each of the serotype of the virus/dose of the vaccine (3 ml). Each of the vaccine was injected (3 ml: deep intramuscular: neck) to each of the five buffalo calves (6 months of age). Blood samples (3 ml) from each of the calves of each group were collected on 0, 30, 60, 90, and 180 days post priming. The serum from each blood sample was separated , stored in properly labeled vials at -80 ^{0}C and processed for monitoring anti-FMD "Asia 1" type virus complement fixing (CFT) antibodies. The geometric mean (GMT) antibody titer of the virus is shown in the Fig. 4.13. The mean values having similar superscript are not significantly different ($p< 0.05$).

Table 4.14: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Days Post-priming and *boosting	**Anti FMD virus Compliment fixing antibody response of buffalo calves to trivalent oil based FMD vaccine (group 1)**					
	Type "O" FMD		**Type "A" FMD**		**Type "Asia-1" FMD**	
	Priming (GMT)	Boosting (GMT)	Priming (GMT)	Boosting (GMT)	Priming (GMT)	Boosting (GMT)
0	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)
30	2,2,2 (1)	2,2,2 (1)	4,2,2 (1.3)	2,2,2 (1)	2,2,2 (1)	2,2,2 (1)
60 (Boosting)	2,2,4 (1.3)	4,4,2 (1.6)	2,4,4 (1.6)	4,4,2 (1.6)	2,4,2 (1.3)	4,4,4 (2)
90	8,4,8 (2.6)	16,8,8 (3.3)	4,4,4 (2)	16,16,16 (4)	4,8,8 (2.6)	16,16,16 (4)
180	4,8,8 (2.6)	16,64,64 (5.3)	4,8,4 (2.3)	16,32,32 (4.6)	8,8,4 (2.6)	64, 32,32 (5.3)

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

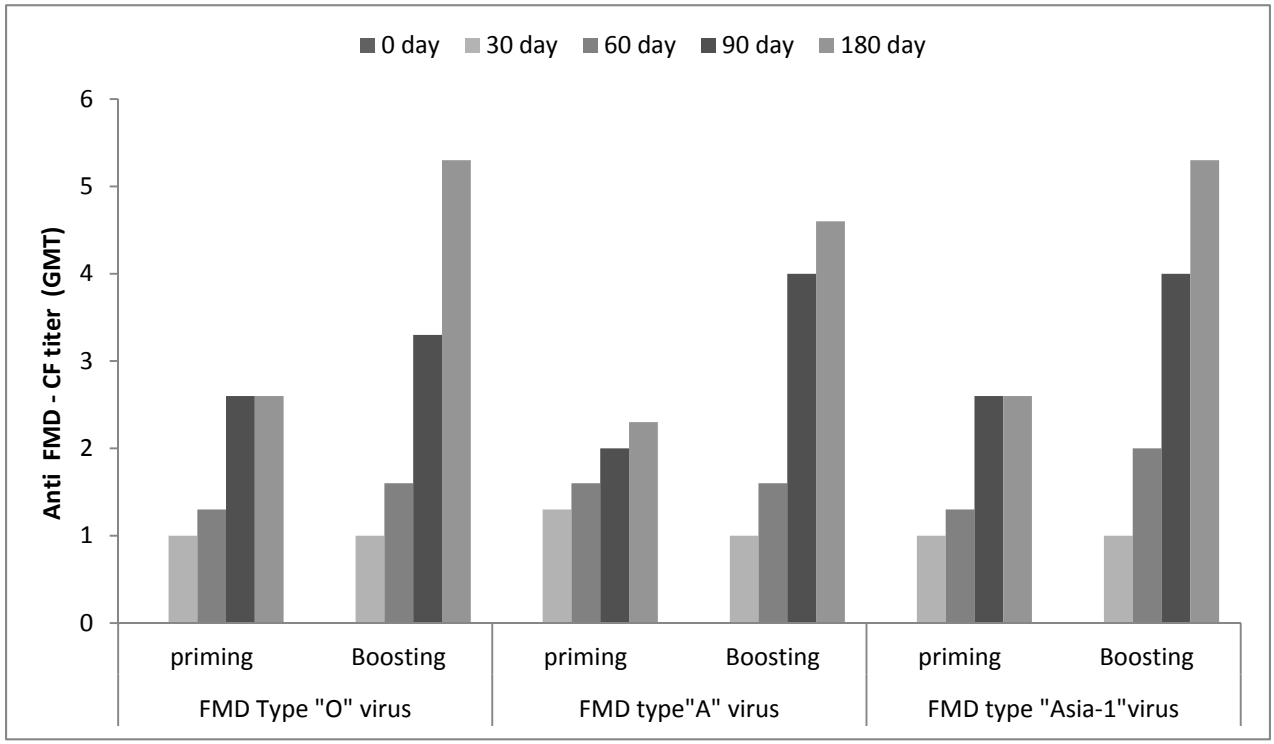

Figure 4.14: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 1 (3 ml vaccine dose contained 0.1 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

Table 4.15: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $2x10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Days Post-priming and *boosting	**Anti FMD virus Compliment fixing antibody response of buffalo calves to trivalent oil based FMD vaccine (group 2)**					
	Type "O" FMD		**Type "A" FMD**		**Type "Asia-1" FMD**	
	Priming (GMT)	**Boosting (GMT)**	Priming (GMT)	**Boosting(GMT)**	Priming (GMT)	**Boosting (GMT)**
0	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)
30	4,4,4 (2)	4,4,4 (2)	4,4,2 (1.6)	4,4,4 (2)	4,4,4 (2)	4,4,8 (2.3)
60 (Boosting)	4,8,4 (2.3)	4,8,4 (2.3)	4,2,4 (1.6)	4,4,4 (2)	8,8,4 (2.6)	4,8,8 (2.6)
9	8,8,16 (3.3)	32,32,32 (5)	8,8,8 (3)	32,32,16 (4.6)	32,32,32 (5)	32,64,64 (5.6)
180	32,16,32 (4.6)	64,128,64 (6.3)	16,64,16 (4.6)	128,128,128 (6)	64,32,32 (5.3)	128,128,128 (6)

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

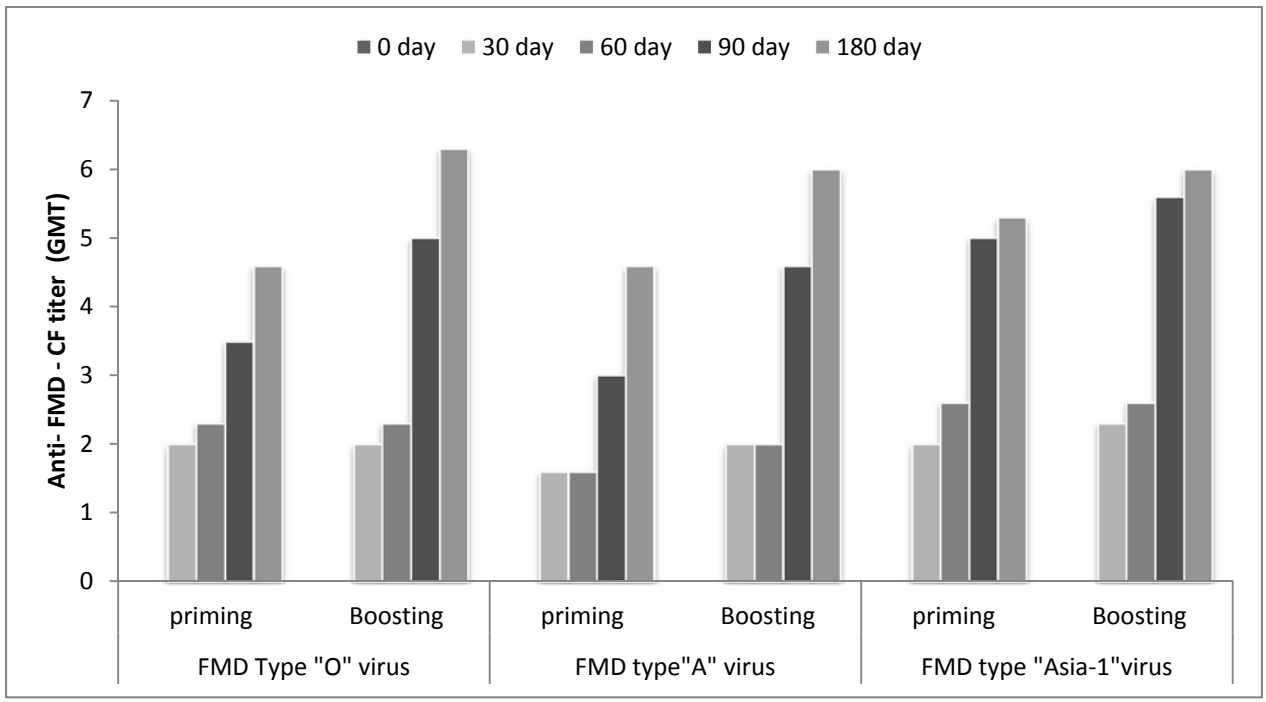

Figure 4.15: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $2x10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 2 (3 ml vaccine dose contained 0.2 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

Table.4.16: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $3x10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Days Post-priming and *boosting	**Anti FMD virus Compliment fixing antibody response of buffalo calves to trivalent oil based FMD vaccine (group 3)**					
	Type "O" FMD		**Type "A" FMD**		**Type "Asia-1" FMD**	
	Priming (GMT)	**Boosting (GMT)**	Priming (GMT)	**Boosting (GMT)**	Priming (GMT)	**Boosting (GMT)**
0	0, 0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)
30	8,8,8 (3)	8,8,8 (3)	8,8,8 (3)	8,8,8 (3)	8,8,16 (3.3)	8,8,8 (3)
60 (Boosting)	16,16,32 (4.3)	16,16,16 (4)	8,16,16 (3.6)	16,16,16 (4)	16,8,16 (3.6)	16,32,32 (4.6)
90	64,128,64 (6.3)	128,128,128 (7)	64,64,32 (5.6)	64,128,128 (6.6)	64,64,128 (6.3)	128,128,128 (7)
180	128,128,256 (7.3)	256,512,256 (8.3)	64,128,128 (6.6)	512,256,256 (8.3)	256,256,256 (8)	512,512,256 (8.6)

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

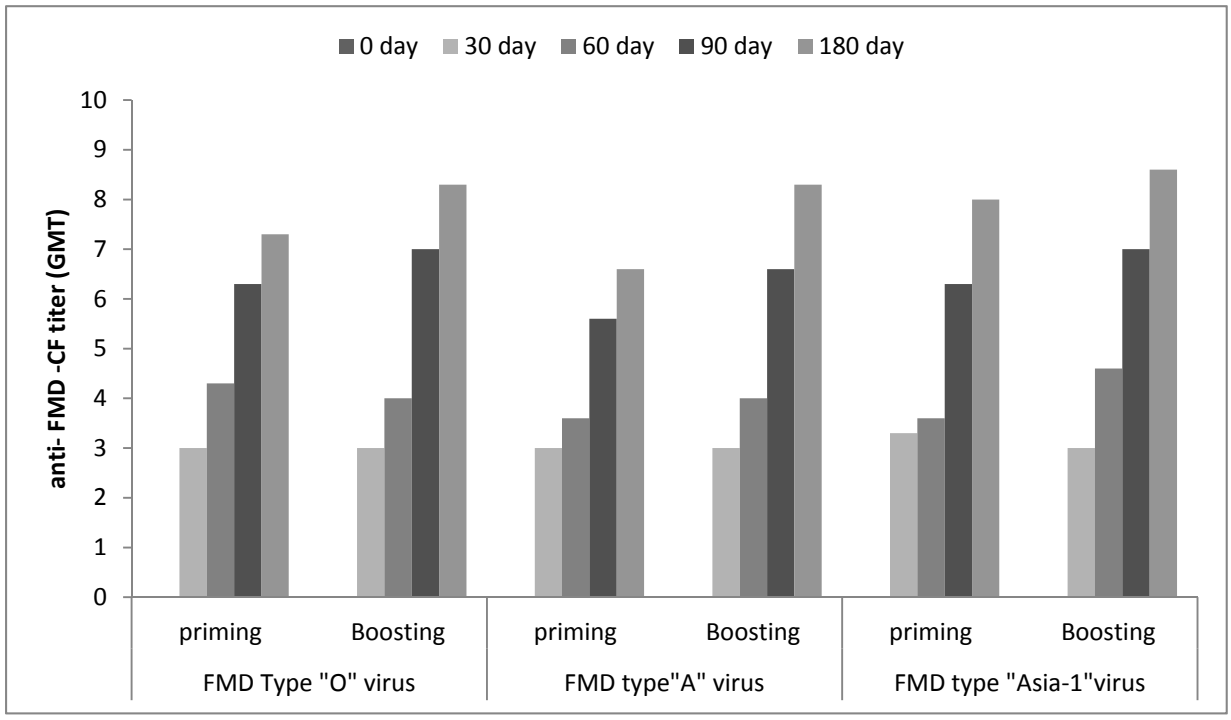

Figure 4.16: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $3x10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 3 (3 ml vaccine dose contained 0.3 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

Table 4.17: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $4x10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Days Post-priming and *boosting	**Anti FMD virus Compliment fixing antibody response of buffalo calves to trivalent oil based FMD vaccine (group 4)**					
	Type "O" FMD		**Type "A" FMD**		**Type "Asia-1" FMD**	
	Priming (GMT)	**Boosting (GMT)**	Priming (GMT)	**Boosting (GMT)**	Priming (GMT)	**Boosting (GMT)**
0	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)	0,0,0 (0)
30	8,8,8 (3)	8,8,8 (3)	8,8,8 (3)	8,8,8 (3)	8,8,16 (3.3)	8,8,8 (3)
60 (Boosting)	16,16,32 (4.3)	16,32,32 (4.6)	4,16,8 (3)	8,8,16 (3.3)	16,32,16 (4.3)	16,8,8 (3.3)
90	64,64,32 (5.6)	128,128,128 (7)	16,64,64 (5.3)	64,64,64 (6)	64,64,16 (5.3)	64,64,64 (6)
180	64,128,128 (6.6)	256,256,256 (8)	64,128,128 (6.6)	128,256, 256 (7.6)	128,256, 256 (7.6)	256,256,256 (8)

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD–complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus. Each of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

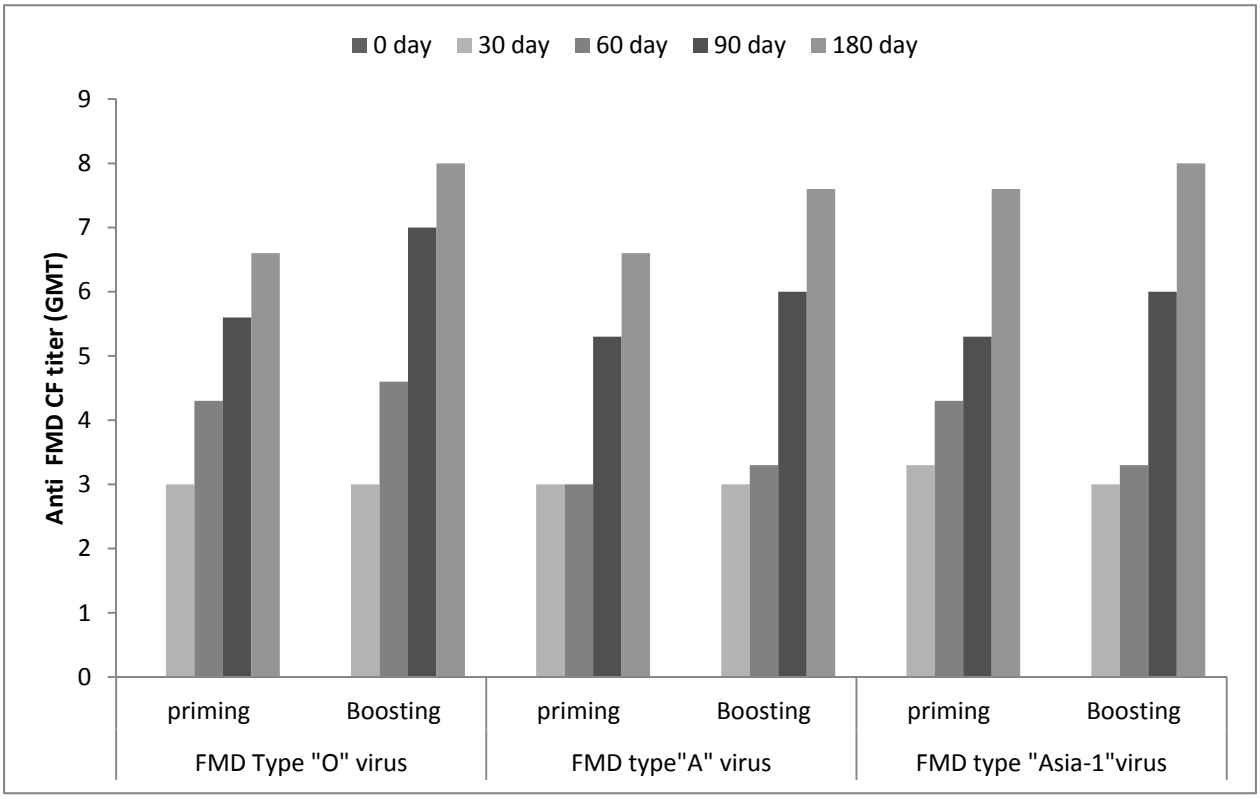

Figure 4.17: Effect of priming and boosting on antibody response of buffalo calves to oil based trivalent vaccine containing $4x10^{6.2}$ units of immunogen of "O", "A" and "Asia-1" serotypes of Foot and Mouth Disease virus

Each of the vaccines when injected to each of the three buffalo calves (6 months of age) induced anti FMD – complement fixing (CFT) antibody titer against serotypes "O","A" and "Asia-1" of FMD virus (Figure 4.19). Each of the calves in group 4 (3 ml vaccine dose contained 0.4 ml of each serotype of the virus suspension) was boosted on day 60 post priming.

CHAPTER-5

DISCUSSION

Baby hamster kidney (BHK-21) cell line (adherent cells) grew well in glass/plastics culture vessels such as Roux flask, roller bottles (reagent bottle) and micro-carrier culture system. The cells grew, adhered and formed monolayer on the surface of Roux flask, inner surface of roller bottle and on surface of micro-carriers. BHK-21 cells routinely grow and adhere on the plastic and glass surfaces. Adherent cells secrete adhering molecules such as fibronectin; plays a major role in the adhesion of many cell types such as fibroblasts, hepatocytes, macrophages, etc., on glass or plastic surface and also form cell to cell adhesion. Cations such as Ca++ and Mg++ are found to attach on glass surface from one side and bind to fibronectin from other side (Urushihara et al. 1977; Freshney, 1998). Cell attachment therefore can be disrupted by using chelating agent such as versin or trypsin solution or both of these (versin trypsin solution). These chemicals can hydrolyze the fibronectin molecules and detach the cells from the substrate and also dissociate from the neighboring cells. Some adherent cells lose their adherent property due to lack of their own surface adhesion molecules (knock out gene expression) or change in the environment. These cells are known as non adherent cells and can be grown in suspension form (Ferrari et al. 1990). There are several nutritional and physical factors that affect growth and viability of BHK-21 cells. The growth media provide nutrients to the cells needed for their survival. There are number of growth media (M-199, DMEM, GMEM, etc.), that are now commercially available for propagation of cells. A growth medium contains carbohydrates, amino acids, lipids and fatty acids, proteins and peptides, inorganic salts, vitamins, serum, supplements (L. Glutamine, Sodium pyruvate and non-essential amino acids), etc. The carbohydrates are glucose, galactose, maltose or fructose. Media with higher amount of sugars

support the growth of a wider range of cell types such as high concentration of glucose in GMEM favors the rapid multiplication of BHK-21 cells. It was observed that increasing the amount of growth medium from 25 ml to 100 ml, increased the cell count significantly ($p<0.05$) while no such increase was observed by further increase in amount of the medium quantity (Table 4.1). Increasing amount of growth medium (GMEM) supports the growth of BHK-21 cells. Further increase in amount of medium in the bottle didn't show any effect on the cell density. This could be attributed to the limitation of space available for the cell attachment and their proliferation (Kurano et al. 1990). Amount of the growth medium (volume) has augmenting effects on the growth of adherent cells in still culture (Sathya et al. 2008) and non-adherent cells in the spinner flasks (Ryan et al. 1997).

It was observed that medium without additive enrichment didn't support the cell growth. Commonly used enrichment additive in the maintenance cell culture medium are Fetal Calf Serum (FCS), goat serum, horse serum, allontoic fluid, colostral whey, oval albumin, etc (Rehman et al. 2007). Addition of 5 % FCS showed optimum results. The medium supplemented with 7%, 9 % showed no significant effect of the cell density (Table-4.2). Sometimes source of the serum is contaminated with bovine diarrheal virus, mycoplasma, chlamydia, etc., infectious agents that has detrimental effect on the growth and maintenance of the cell line. Serum in the medium is an important source of growth factors such as vitamins, minerals anti trypsin agents, adherent molecules, growth hormones, etc. in cell culture. The vitamins are precursors for various co-factors. Many vitamins especially B group (riboflavin, thiamine, biotin, etc.) are necessary for cell growth and their proliferation. Trace elements are zinc, copper, selenium and tricarboxylic acid intermediates. Selenium is a strong detoxifier and helps in removing oxygen

free radicals **(**Paranjape 2004). It is therefore important to screen batches of serum for their ability to support the growth of cells.

There are several physical factors (seeding density, time and temperature of incubation, rolling speed, etc.) affecting the growth of BHK-21 cells and its viability. Scaling up the cell density is the primary target in either of the method as quantity of cell is directly linked with initial cell density (Sathya et al. 2008). Seeding density significantly affected the growth properties of the cells. It is clear from Table- 4.3 and Figure 4.3 that double amount of cells were harvested after 48 hours of incubation by increasing the cell density in the inoculum from 5 million to 10 million. High yield of hybridoma cells is obtained by increasing the seeding cell density (Sadettin et al. 1990). However, low seeding cell density with longer incubation showed confluent monolayer of the cells. Cell attachment is dependent on the suitable and consistent substrate provided by the vessel/culture bottles. For still culture plastic/ glass Roux flasks are used. Space is the limiting factor for obtaining high cell density. Roux flask (175 cm^2) yielded $1.5x10^7$ cells per bottle/ 35 ml of the growth medium while roller bottles (480 cm^2) yielded $4.6x10^7$ cells per bottle/ 100ml of the growth medium (Table 4.3). The roller bottles slowly rotate (1-3 rpm) on motorized racks or drums are widely used for producing large quantities of cells also glass bottles and provide good substrate for attachment of the cells. Incubation temperature is important for cell growth. Incubation temperature (37° C) showed optimum cell growth while the reduced growth was obtained by keeping the flasks at lower temperatures (Table 4.5). Low temperature suppresses the cell growth (Evelyn et al. 2006). Additionally, the cells grown at lower temperatures have reduced metabolic activities as demonstrated by reduced interferon production by the CHO cells (Alireza et al. 2006). The rapid decline in the cell yield by keeping cells at high temperature in the present study might be due to the fact that enzymatic activities of

the mammalian cells seizes at a temperature slightly higher than the optimum temperature. Optimum incubation period is one of the critical requirements of the cells to support the cell growth. Maximum cell density can be achieved within 60 hours of incubation while an abrupt decline in the harvested cell counts was recorded after another incubation of 12 hours (Table 4.4). The decline in cell yield after achieving confluence could be due to the accumulation of metabolic wastes or shortage of the vital ingredients like glutamine in the spent medium (Radlett et al. 1971). Many anchorage dependent cells are adapted to grow on micro-carriers to achieve high cell density. While considering the cell propagation as monolayer culture, the roller bottles are thought to be a better choice (Kunitake et al. 1997; Kretzmer 2002). The major advantage of roller bottles over flat surface flasks is that it provides increased surface area for the attachment of cells (Sadettin et al. 1990). However, rolling speed is a critical factor for the cell growth. The maximum amount of cells were harvested at a rolling speed of 3 rpm while the cell yield was relatively less at low or high rolling speeds (Table 4.6, Fig 4.6). Glasgow minimal essential medium was found to sustain optimum proliferation of BHK-21 cells. Medium insufficiency at low speed and attachment failure at high speed could be the most probable reasons for low cell yield.

BHK-21 cell line supports the replication of Foot and Mouth disease (FMD) virus which is the cause of FMD in cloven footed animals. The virus belongs to Apthovirus of Picornaviridae. The virus has single stranded, positive-sense, non-enveloped RNA genome of 8.5 kb. The RNA of FMDV contains codes for structural and non-structural proteins (Forss et al., 1984; Racaniello, 2006). The RNA genome is surrounded by the capsid of 60 copies of each of the four structural proteins such as VP-1, VP-2, VP-3 and VP-4; of which VP1-3 are external and VP-4 is completely internal (Fox et al. 1989). Other than structural proteins there are nine non-

structural proteins. The antigenic properties of structural proteins of FMDV capsid play important role in the classification of FMD virus into seven distinct serotypes namely “O”, “A”, “Asia-1”, “C” and SAT I-III (Domingo et al. 2002; Mittal et al. 2005). Various antigenic sites have been identified in different serotypes of FMD virus. In FMD type “O” virus four antigenic sites (two located on VP-1, 3rd on VP-2 and forth on VP-3) are identified (Kiston et al. 1990: Crowther et al, 1993). VP-1 is considered to contain protein for most significant virus attachment and serotype specificity. FMD type “Asia-1” virus has four trypsin sensitive and neutralizing sites. VP-1 is reported as the major antigenic site Gurumurthy et al, 2002). Two major antigenic sites have been identified in FMD type “A” virus; one on VP-1 is trypsin sensitive and the other on VP-3 is trypsin insensitive. Two minor sites are located near C-terminus of VP-1 (Thomas et al. 1988). FMD virus grew well on BHK-21 cell line (Table 4.8). Virus has ligant molecules and host cells have specific receptors. The virus therefore binds with the host cells and genome (RNA) of FMDV then enters into cytoplasm (Carolina and Graham, 2012). The virus genome directly binds on ribosome of the host cell and starts its primary translation of structural and non structural proteins (Barton and Flanegan, 1997; Li and Baltimore, 1988). After translation, assembly of virus takes place and cells show clear cyto-pathic effects like clumping, aggregation and detachment (Awan et al. 2009). FMD virus serotypes from field do not grow directly on monolayer of BHK-21 cells. This could possibly be due to coating of secretary antibodies on receptors of the cells (Jackson et al. 1996). The BHK- 21 cell line seems to be less sensitive than Bovine Thyroid cells (BTY) due to low expression of cell integrin (Berinstein et al. 1995: Khuwaja et al. 2009). There has been many cell line including BHK-21 cells, lamb kidney cells and bovine thyroid (BTY) cells used but BTY cells showed the best sensitivity for field strains of FMD virus (Ferris et al. 2002). FMD virus can directly grow on primary monolayer of BTY cell

then after 2nd, 3rd passages can adapt on BHK-21 cells (Khuwaja et al. 2009). First time growth of FMDV on BHK-21 cells after passages on BTY gives 10^3 units of $TCID_{50}$/ml. However, repeated passages on BTY cells increase $TCID_{50}$ of FMD up to 10^6 units of $TCID_{50}$/ml (Khuwaja et al. 2009). FMD virus showed poor growth in BHK-21 cell line having cell culture medium without FCS. 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 at the rate of 0, 1 and 2 % concentration proportionally increased the infectivity titer of FMDV, i.e., 10^3 units of $TCID_{50}$/ml, 10^7 units of $TCID_{50}$/ml, 10^7 units of $TCID_{50}$/ml, respectively (Table, 4.12 and Figure 4.12). However, further increase of 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 a 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 virus (Gallina et al. 2001). 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 a possible reason of having poor infectivity titer of the virus at above mentioned temperature. Incubation temperature of 35^0C and 37^0C supported the multiplication and maintenance of BHK-21 cells that might had supported the replication of FMD virus. This might be a possible reason of high infectivity titer of $10^{6.6}$ units of $TCID_{50}$/ml and $10^{7.2}$ units of $TCID_{50}$/ml at 35^0C and 37^0C respectively (Razdan et al. 1996). The replication/infectivity titer of virus is directly proportional to number of cells. In

the still culture (Roux flask) 75cm^2 yielded10^6 units of infectivity titer per ml. Monolayer of BHK-21 cells in roller bottle culture system (480 cm^2) showed 10^7 infectivity titer per ml. BHK-21 cells on micro-carrier suspension in stirring bottle containing 200 ml of GMEM growth medium showed 10^8 units/ml of infectivity titer of the virus (Table 4.11 and Figure 4.11). Higher the density of the cells, higher is the infectivity titer of the virus (Altaf et al. 2012; Khuwaja et al. 2009; Salivac et al. 2006). FMD virus doesn't attain attenuated form, so live attenuated form of vaccine is not available. Variety of physical factors and chemical agents are used to inactivate the virus for vaccine production (Awan et al. 2009; Ghori et al. 2011). Formaldehyde binds with amine group of viral capsid proteins thus alters its configuration so these ligand molecules do not bind with receptors of host cells and cannot produce the disease and becomes inactivated (Bahnemann et al. 1975). However, the RNA of FMD virus is a positive sense nucleic acid that can directly enter from formaldehyde inactivated viral suspension into the cytoplasm of host cells and may start its replication. FMD outbreaks are not therefore, uncommon even in the animals vaccinated with gel based formaldehyde inactivated FMD virus vaccine. This is why, the FMD virus was inactivated using Binary Ethyleneamine (BEI) that might have inactivated viral nucleic acid and inhibited its replication in the host cells. Such BEI inactivated FMD virus suspension qualified safety and sterility test. The BEI molecules do not bind with the capsid protein so may not decrease its immunogenicity. Moreover, residual amount of BEI can be neutralized with sodium thiosulphate, eliminating its toxicity in the vaccine. The BEI inactivated FMDV suspension absorbs quickly from inoculation sites of the vaccinated animals and does not elicit the antibody response. The viral suspension is therefore, adsorbed on aluminium hydroxide gel or admixed with mineral oil such as Montanide ISA-70. The adjuvant enhanced its retention time and induced mild form of granuloma at the inoculation site. The antigen is removed through

antigen presenting cells (APC) for production of cell mediated as well as humoral immune responses (Tizzard, 1998; Graves 1971; Kimura et al. 1978; Chowdhery et al. 1996; Jennings et al. 1998 and OIE manual 2011). The humoral response of the buffalo calves is measured by potency testing such as virus notarization test (VNT) and complement fixation test (CFT) (Tizzard, 1998; Altaf et al. 2012) and ELISA (Ferris and Dawson, 1988). It was observed that higher was the immunogen level of FMD virus per dose of the vaccine, higher was the anti FMDV-CFT antibody titer of vaccine (Table 4.13 – 4.16 and Figure 4.13 – 4.16). The antibody response of the vaccinated animals is directly proportional to biological titer of the immunogen per dose of the vaccine (Muhammad et al. 2011). It was observed in the present study that the required level of antibody titer is achieved by incorporating 0.2 ml of the viral suspension having infectivity titer of 10^7 per dose of the vaccine. The volume of the immunogen can be reduced to 0.1 ml of the viral suspension by increasing two times infectivity titer of the virus. This will open a gateway for production of multivalent vaccine against common diseases of cattle and buffalo. Multivalent vaccines are commonly prepared against infectious diseases of bovine, caprine, canine and poultry (Altaf et al. 2012; Palanisamy et al. 1992). FMD vaccinated animals can resist the field exposure of virulent infections through any of the following methods 1) Antibodies bind with ligand molecules of the newly entering virus and inhibiting its attachment and replication in the host cell (Tizzard, 1998). 2) Specific immunoglobulin (IgG) binds with the virus particles and leads to its clearance through phagocytosis by macrophages/neutrophils mediates phygocytosis. 3) Immunoglobulins (IgM and IgG) bind with the specific ligand of the virus and bind with the phagocytic cells through activated C3b leading to its clearance from circulation/tissue through phagocytosis. 4) Binding of the IgG with the virus and its clearance through natural killer cells. 5) T- cell dependent immunogens of FMD virus elicits cytokines

production such as IL-2, IL-4, IL-5 and INF-α, ultimately enhances the potential antiviral activity of natural killer cells and cytotoxic T-cells (Zhang et al, 2011). These activated cells recognize the virus specific antigens presented along with MHC class-1 and mediate lysis of such virus infected cells (Alireza et al. 2000; Efrain et al. 2008).

CHAPTER-6

CONCLUSIONS

It is concluded that conventional system (Roux flask: 75 cm2 surface area) support the growth of $1.7x10^7$ BHK-21 cells/ flask/35 ml growth medium and yield of $10^{6.2}$ units of biological/ infectivity titer of the virus per ml of the virus suspension. In this way biological titer of the virus is 35 x 10^6 or 3.5 $x10^7$ units of FMD virus/bottle. Physico-chemical factors such as seeding density (10 million BHK-21 cells/ bottle), growth medium (100 ml/ bottle), fetal calf serum (5%), rolling speed (3rpm), incubation temperature (37^0C) and incubation period (60 hours) for growth of BHK-21 cells in roller bottles (480 cm^2 surface area) yielded more than $4.5x10^7$ cells/ bottle and 10^7 units of infectivity titer of the virus/ml of the medium. In this system total virus yield was therefore, 100x 10^7 or 1x 10^9 units of infectivity titer of the virus per bottle. The roller bottle culture system therefore yields 65 times more infectivity titer of the virus than that of still culture. The adaptation of this type of culture system in biological production units may enhance more than 15 times biological production potential.

CHAPTER-7

RECOMMENDATION

It was observed that high cell density can be achieved by growing adherent cells in roller bottle culture system or micro-carrier in stirring vessels. In this way, many times more infectivity titer of the virus can be achieved than that of Roux flasks. However, many times higher cell density can be achieved by growing BHK-21 cells on micro-carriers in stirring culture vessels and subsequently many folds higher infectivity titer of the virus can be attained by growing the virus serotypes in the stirring culture system. It is therefore recommended to investigate the factors effecting BHK-21 cell cultivation on micro-carrier and suspension culture system. Moreover, factors augmenting infectivity titer of FMD virus on adherent cells on micro-carrier and suspension cultures in spinner flask/biofermentor for improving vaccine production potential of biologics production units.

CHAPTER-8

SUMMARY

Foot and Mouth Disease (FMD) is endemic in Pakistan and killed virus vaccines against prevalent serotypes are used for the control of disease. Baby Hamster Kidney (BHK-21) cells as monolayer culture are routinely used for the propagation of FMD viruses. Various nutritional factors: amount of growth medium and serum concentration as well as physical conditions: seeding density, rolling speed, growth time, and incubation temperature for the propagation of BHK-21 cells on roller culture bottles were optimized. The roller culture bottles having surface area of 480 cm^2 were used for the propagation of cells. Feeding of cells with 100 ml of growth medium per bottle and supplementation of 5% serum supported the growth of the cells in optimum way. Seeding of ten million cells per bottle resulted into the development of complete monolayer with maximum cell density within 48 hours. The cultured cells remained confluent up to 60 hours while a rapid decline in the number of harvested cells was recorded after 72 hours of incubation. Growth rate of the cells was slower at 33° C that increases at 35° C, reaching to its maximum at 37° C while cells could not tolerate the temperature of 39° C. The bottles kept at rolling speed of 3 rpm yielded maximum amount of cells while higher or lower rotation speed negatively affected the cell proliferation.

Antibody response of buffalo calves to different levels of FMD virus immunogen in trivalent vaccine was investigated. The vaccine containing $10^{6.2}$ units of immunogen/$TCID_{50}$ of each of the three serotypes of FMD virus induced $\log_2(1.3\pm 0.4)$ units of anti-FMD "O" Complement Fixing Cumulative Geometric Mean antibody (FMD"O" CFT-CGM) titer, $\log_2(1.4\pm0.3)$ units of anti-FMD"A" CFT-CGM titer and $\log_2(2.0\pm0.7)$ units of anti-FMD"Asia-

1" CFT-CGM titer. The vaccine containing $2x10^{6.2}$ units of immunogen of each of the three serotypes of FMD virus induced $\log_2(2.2\pm0.2)$ units of anti- FMD"O" CFT-CGM titer, $\log_2(2.1\pm0.25)$ units of anti- FMD"A" CFT-CGM titer and $\log_2(3.4\pm0.8)$ units of anti-FMD"Asia-1" CFT-CGM titer. The vaccine containing $3x10^{6.2}$ units of $TCID_{50}$ of each of the three serotypes of FMD virus induced $\log_2$ (5.3 ± 2.0) units of anti-FMD"O" CFT-CGM titer, $\log_2$ (4.6±1.9) units of anti-FMD"A" CFT-CGM titer and $\log_2$ (5.0±2.2) units of anti-FMD"Asia-1" CFT-CGM titer. The vaccine containing 4 x $10^{6.2}$ units of $TCID_{50}$ of each of the three serotypes of FMD virus induced $\log_2(5.5\pm1.5)$ units of anti-FMD"O" CFT-CGM titer, $\log_2(4.4\pm1.9)$ units of anti-FMD"A" CFT-CGM titer and $\log_2(5.2\pm1.9)$ units of anti-FMD"Asia-1" CFT-CGM titer. Moreover, buffalo calves (n=3) which were primed and boosted with 60 days interval using vaccine containing $2x10^{6.2}$units of immunogen of each serotype of FMD virus, showed $\log_2$5.0 and $\log_2$6.3 units of anti FMD"O"-CFT-GMT antibody titer, $\log_2$4.6 and $\log_2$ 6.0 units of anti FMD"A"-CFT GMT antibody titer, $\log_2$5.6 and $\log_2$6.0 units of anti FMD"Asia-1"-CFT GMT antibody titer, on 30 and 120 days post boosting.

Each serotype of the virus grew well on BHK-21 cell line. The virus showed poor $TCID_{50}$ (log $10^{3.2\pm0.2}$) in BHK-21 cells having Glasgow Minimal Essential Medium (GMEM) without Fetal Calf Serum (FCS). Addition of FCS in the medium at the rate of 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 $\log10^{6.6\pm0.1}$ and $\log10^{7.0\pm0.2}$units of virus $TCID_{50}$, respectively. Each serotype of FMD virus showed $\log10^{6.29\pm0.07}$ units of virus $TCID_{50}$ in the stationary monolayer of BHK-21 cells in Roux flask ($75cm^2$), $\log10^{7.66\pm 0.02}$ units of virus $TCID_{50}$ in roller bottles (490 cm^2) and $\log10^{8.34 \pm 0.07}$ 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.

CHAPTER-9

LITERATURE CITED

Ahmad MM, Khan NA. 1988. Typographical studies of foot and mouth disease virus in Pakistan. Conference on Meeting of counterparts scientists on livestock under SAARC, Islamabad, Pak. Published by PARC, Islamabad, Pak. P 55-59

Acharya R, Fry E, Stuart D, Fox G, Rowlands D and Brown F. 1989. The three dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature. 337: 709-716.

Ajmal M, Arshad M and Ahmad MD. 1989. Epidemiology of major livestock diseases in Pakistan. Fifth Annual Report. Pakistan Agriculture Research Council, Islamabad. p. 49-53.

Alonso A, Martins MA, Gomes P, Allende R and Sondahl MS. 1992. Foot-and-mouth disease virus typing by complement fixation and enzyme-linked immunosorbent assay using monovalent and polyvalent antisera. J Vet Diagn Invest. 4: 249-53.

Alireza H, Wauben MH, Grosfeld MCS and Hensen EJ. 2000. Selection of T-cell epitopes from Foot and Mouth Disease Virus reflects the binding affinity to different cattle MHC class II molecules. Immunogenetics, 51: 733-42.

Ahlers JD, Belyakov IM, Matsui S, Berzofsky JA. 2001. Mechanisms of cytokine synergy essential for vaccine protection against viral challenge. Int Immunol. 13(7):897-908.

Adam KH and Marquardt O. 2002. Differentiation of type A, Asia-1 and O Foot and Mouth Disease Virus Variants, amplified by the same system, by sequencing of the capsid protein genes. J. Virol. Methods. 104:117-123.

Alexanderson S, Zhang Z, Donaldson AI and Garland AJM. 2003. The pathogenesis and diagnosis of foot and mouth disease. J. Comp. Path. 129: 1-36.

Amass SF, Mason PW, Pacheco JM, Miller CA, Ramirez A, Clark LK, Ragland D, Schneider JL and Kenyon JS. 2004. Procedures for preventing transmission of foot-and-mouth disease virus (O/TAW/97) by people. Vet. Microbiol. 103: 143-149.

Alireza Z, Khodayar O and Hassan B. 2006. Lower culture-temperature effects on recombinant Ifn-alpha production in chinese hamster ovary (CHO) cells. Int. J. Agri. Biol. 3: 378-381.

Awan FN, Khan MA, Muhammad K, Rabbani M, Younus M and Khawaja RM. 2009. Epidemiological investigation of foot and mouth disease in districts of Punjab. Pakistan J. Zool. Suppl. 9: 179-185.

Altaf I, Saddique M, Irshad M, Khan MZ, Anjum AA and Kamran M. 2012. Antibody response of Rabbits to combined Hemorrhagic Septicemia and Foot and mouth disease virus vaccine. The journal of Animal & Plant sciences. 22(2): 501-504.

Bahnemann HG. 1975. Inactivation of viruses in serum with binary ethyleneimine. Clin Microbiol. 3(2): 209-210.

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

Bundo M.K and Morita KB. 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.

Baxt B and Becker Y. 1990. The effect of peptides containing the argininеglycine-aspartic acid sequence on the adsorption of foot and mouth disease virus to tissue culture cells. Virus genes 4:73-83.

Barteling SJ and Vreeswijk J. 1991. Developments in foot-andmouth disease vaccines. Vaccine 9: 75–88.

Berinstein A, Roivainen M, Hovi T, Mason P and Baxt B. 1995. - Antibodies to the vitronectin receptor (integrin aVß3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J. Virol. 69: 2664-2666.

Barton DJ and Flanegan JB. 1997. Synchronous replication of poliovirus RNA: initiation of negative-strand RNA synthesis requires the guanidine-inhibited activity of protein 2C. J. Virol. 71: 8482–8489.

Bembridge GP, Lopez JA, Cook R, Melero JA and Taylor G. 1998. Recombinant vaccinia virus coexpressing the F protein of respiratory syncytial virus (RSV) and interleukin-4 (IL-4) does not inhibit the development of RSV-specific memory cytotoxic T lymphocytes, whereas priming is diminished in the presence of high levels of IL-2 or gamma interferon. Journal of Virology. 72: 4080–4087.

Brown F, Benkirane N, Limal D, Halimi H, Newman JF, Van Regenmortel MH, Briand JP and Muller S. 1991. Delineation of a neutralizing subregion within the immunodominant epitope (GH loop) of foot-and-mouth disease virus VP1 which does not contain the RGD motif. Vaccine. 18: 50–56.

Butler M. 2003. *In* Animal cell culture and techonology, 2nd Edi, pp: 10-27. T.J. internl. Padstow, Cornwall. U.K

Brown F. 2003. The history of research in foot and mouth disease. Virus Research 91:3-9.

Barnard AL, Arriens A, Cox S, Barnett P, Kristensen B, Summerfield A and McCullough KC. 2005. Immune response characteristics following emergency vaccination of pigs against foot-and-mouth disease. Vaccine. 23: 1037–1047.

Cowan KM and Trautman R. 1967. Immunochemical studies of foot and mouth disease. 1. Complement fixation reaction with isolated antigenic components. J Immunol. 99: 729-736.

Cruickshank R, Coghlan JD, Collee JG and Duguid JP. 1975. Int Medical Microbiology. 12th ed. Churchill Livingstone. London. P. 94-95.

Cavanagh D, Rowlands DJ and Brown F. 1978. Early events in the interaction between foot-and mouth disease virus and primary pig kidney cells. J Gen Virol. 41: 255-264.

Castillo AA, Morier LD, Perez FV and Durruthy MC. 1991. Use of goat serum as a substitute for calf serum for growing various primary cultures from vertebrates. Rev. Cubana. Med. Trop. 43: 89-92.

Crowther JR, Farias S, Carpenter WC and Samuel AR. 1993. Identification of a fifth neutralizable site on type O foot and mouth disease virus following characterization of single and quintuple monoclonal antibody escape mutants. J. Gen. Virol. 74, 1533-1547.

Curry S, Abrams CC, Fry E, Crowther JC, Belsham GI, Stuart DI and King AM. 1995. Viral RNA modulates the acid sensitivity of foot-and-mouth disease virus capsid. J Virol. 69: 430-438.

Chowdhury SM, Rahman MF, Rahman MB and Rahman MM. 1996. Strains of foot and mouth disease virus in different districts of Bangladesh. Austral. J. Anim. *Sci*. 9: 315-317.

Callens M and ClereqKDe. 1997. Differentiation of the seven serotypes of foot and mouth disease virus by reverse transcriptase polymerase chain reaction. J.Virol. Methods 67: 35-44.

Cox SJ, Voyce C, Parida S, Reid SM, Hamblin PA, Paton DJ and Barnett P V. 2005. Protection against direct-contact challenge following emergency FMD vaccination of cattle and the effect on virus excretion from the oropharynx. Vaccine. 23: 1106-1113.

Cottam EM, Haydon DT, Gloster J, Wilesmith JW, Ferris NP, Hutchings GH and King DP. 2006. Molecular epidemiology of the foot and mouth disease virus outbreak in the United Kingdom in 2001.J. of Virology 80: 11274-11282.

Carolina AS and Graham B. 2012. Detection of foot and mouth disease virus RNA in pharyngeal epithelium biopsy samples obtained from infected cattle: investigation of possible sites of virus replication and persistence. Vet Micrbiol. 154: 230-239.

Domanski R and Fitko 1959. Disturbances of the pituitary and other hormonal glands in cows after foot and mouth disease. Proceedings of the 16th International Veterinary Congress, Madrid, pp. 421.

Donaldson AI, Herniman KAJ, Parker J and Sellers RF. 1970. Further nvestigations on the airborne excretion of foot and mouth disease virus. J. Hyg. Camb. 68: 557-564.

Donaldson AI. 1983. Quantitative data on air borne foot and mouth disease virus: its production, carriage and deposition. Phil. Trans. R. Soc. Lond. (B) 302: 529-534.

Doel-Amaral CMF, Owen NE, Ferris NP, Kitching RP and Doel TR. 1993. Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preprations by the polymerase chain reaction. Vaccine. 11: 415-421.

Doll K. 2001. Clinical picture and differential diagnosis of foot-and-mouth disease in cattle. Dtsch Tierarztl Wochenschr. 108: 494-498.

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

Domingo E, Baranowski E, Escarmis C and Sobrino F. 2002. Foot-and-mouth disease virus Comparative Immunology. Microbiology and Infectious Diseases. 25: 297–308.

Doel TR. 2005. Natural and vaccine induced immunity to FMD.springer 288: 103-131.

Escarmis C, Carrillo EC, Ferrer M, Arriaza JF, Lopez N, Tami C, Verdaguer N, Domingo E and Franze FMT. 1998. Rapid selection in modified BHK-21 cells of a Foot and Mouth Disease virus variant showing alteration in cell tropism. J. Virol. 72 (12):10171-10179

Eble PL, de Bruin MG, Bouma A and Van Hemert-Kluitenberg F. 2006. A. Dekker Comparison of immune responses after intra-typic heterologous and homologous vaccination against foot-and-mouth disease virus infection in pigs. Vaccine. 24: 1274–1281.

Evelyn T, Katharina F, Silke S, Kornelia S, Christine L, Kunert R and Muller D. 2006. Effect of DOT, pH, and Temperature on the Performance of Epo-Fc expressing CHO Cells Cultivated in Controlled Batch Bioreactors. Biotechnology and bioengineering. 94: 1033-1044.

Efrain G, Geraldine T, Bryan C, Michael A and Shirley AE. 2008. An MHC-restricted $CD8^+$ T-cell response is induced in cattle by foot-and-mouth disease virus (FMDV) infection and also following vaccination with inactivated FMDV. J Gen Virol. 89: 667-675.

Economic Survey of Pakistan. 2011-12. Economic Affair Division, Government of Pakistan, Islamabad.

Forss S, Strebel K, Beck E and Schaller H. 1984. Nucleotide sequence and genome organisation of foot-and-mouth disease virus. Nucleic Acids Res. 12: 6587-6601.

Ferris NP and Dawson M. 1988. Routine application of Enzyme Linked Immunosorbant Assay in comparison with complement fixation for the Diagnosis of Foot and Mouth and Swine Vesicular Diseases. Vet. Microbiol. 16: 201-209.

Fox G, Parry NR, Barnett PV, McGinn B, Rowlands D and Brown F. 1989. The cell attachment site on Foot and mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J. gen. Virol. 70: 625-637.

Ferrari M, Losio MN, Gualandi GL and Bugnetti MC. 1990. Cultivation of Foot-and-Douth Disease virus in BHK21 cells grown in microcarrier culture system. Micro. 13: 161–163.

Fenner FJ, Gibbs EPT, Muri FA, Rott R, Studdert MJ and White DO. 1993. Veterinary Virology. 2nd Ed. Academic Press Inc. New York. pp 408-416.

Freedman D, Wang G, Kadouri A. 1996. Method and Apparatus for Anchorage and suspension Cell Culture. New Brunswick Scientific Co. Inc. US Patent #5,501,971.

Forshubor T, Yip HCand Lehmann PV. 1996. Induction of TH1 and TH2 immunity in neonatal mice Science. 271: 1728–1730.

Freshney RI. 1998. In: Culture of Animal Cells: A Manual of Basic Technique, 2nd Edi, pp: 1–3. Alan R. LISS, Inc. N.Y

Ferris NP, Hutchings GH, Moulsadle HJ, Golding J and Clarke JB. 2002. Sensitivity of primary cells immortalized by oncogene transfection for the detection and isolation of foot-and-mouth disease viruses. Vet. Microbio. 84: 307-316(Year corrected)

Graves JH. 1971. Contact transmission of foot and mouth disease from infected to susceptible cattle. J. Infect. Dis. 123: 386-391.

Giard DJ, Thilly WG, wang DI and Levine DW. 1978. Virus production with newly developed microcarrier system. Applie and envr. Micro. 34(6): 668-72.

Gugiu I, Stirbu C, Danes M, Gruia M, Malinetescu M, Gradinaru D, Gugiu M, Anton T, Chiciudean I and Marin D. 1989. Improvements in manufacturing a vaccine against foot and outh disease from cell culture virus. Pasteur. 18: 57-64.

Gallegous RM, Espinosa ELL, Romas LR, Kretschmer RS and Aguilear AS. 1995. Rabies veterinary virus vaccine produced in BHK-21 cells grown on microcarriers in a bioreactor. Arch Med Spring. 26: 59-6.

Gallina FNM, Rosana L, Regina M, Moura FO and Soraia AC. 2001. Higher production of rabies virus in serum-free medium cell cultures on microcarriers. Journal of biotechnology. 92: 67–72.

Gurumurthy CB, Sanyal A, Venkataramanan R, Tosh C, George M and Hemadri D. 2002. Genetic diversity in the VP1 gene of foot-and mouth disease virus serotype Asia1. Arch. Virol. 147:85-102.

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

Gajendragad MR, Venkatesha MS, Prabhudas K, Subodh K, Gopalakrishna S. 2005. Control of foot-and-mouth disease by vaccination. Ind. J. Vet. Pathol. 29: 7-9.

Golde WT, Pacheco JM, Duquea H, Doel T, Penfold B, Ferman GS, Gregga DR, Rodriguez LL. 2005. Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: Use in emergency outbreak response. Vaccine. 23: 5775–5782.

Ghori MT, Muhammad K and Rabbani M. 2011. Physical factors affecting *in vitro r*eplication of foot and mouth disease virus (serotype "O"). Pak Vet J. 31(4): 313-316.

Hamblin C, Armstrong RM, Hedger RS. 1984. A rapid enzyme-linked immunosorbent assay for the detection of foot and mouth disease virus in epithelial tissues. Vet Microbiol. 9: 435-443.

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

Ishimaru D, Sá-Carvalho D and Silva LJ. 2004. Pressure-inactivated FMDV: a potential vaccine. Vaccine. 22: 2334–2339.

Iqbal H, Ahmad I, Farooq T, Nayyar A and Khan AR. 2011. Prevalence of foot-and-mouth disease in cattle and buffaloes in Central Punjab, Pakistan. Int J Agro Vet and Med Sci. 5: 444-446.

Jung M, Brunner S and Dobec M. 1995. The complement fixation reaction: a traditional, updated method for the detection of antibodies in the diagnosis of infections. Lijec Visen. 117: 39-46.

Jackson T, Ellard F, Abu-Ghazaleh R, Brooks SM, Blackmore WE, Corteyen AH, Stuart D, Newman J and King A. 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.

Jennings R, Simms JR and Heath AW. 1998. Adjuvants and delivery systems for viral vaccines: mechanisms and potentials. Develop. Biol. Stand. 92: 19-28.

Jin HL, Li YJ, Ma ZH, Zhang FC, Xie QG, Gu DF and Wang B. 2004. Effect of chemical adjuvants on DNA vaccination. Vaccine. 29: 2925-2935.

Jamal MS, Ahmed S, Hussain M and Ali Q. 2010. Status of foot-and-mouth disease in Pakistan. Arch Virol. 155: 1487-1491.

Jamal MS, Ferrari G, Ahmed S, Normann P, Curry S and Belsham JG. 2011. Evolutionary analysis of serotype A foot-and-mouth disease viruses circulating in Pakistan and Afghanistan during 2002-2009. J Gen Virol. doi: 10.1099/vir.0.035626-0.

Kimura J, Nariuchi H, Watanabi T, Matuhasi T, Okayasu I and Hatakeyama S. 1978. Studies on the adjuvant effect of water-in-oil-in-water (w/o/w) emulsion of sesame oil. 1. Enhanced and persistent antibody formation by antigen incorporated into the water-in-oil-in-water emulsion. Japanese J. exp. Med. 48: 149-54.

Kruman II, Ostapenko NF, Gavriliuk BK, Gordon RI and Karnaukhov VN. 1981. Arrest of BHK-21 cell culture growth and its stabilization in a resting state as affected by serum fractions. Tsitologiia. 23: 937–943.

Kruman II, Miakisheva SN and Gavriliuk BK. 1984. Age-related changes in the growth-stimulating effect of serum on BHK-21 cells. Tsitologiia. 26: 1043–1047 .

Kitson JDA, McCahon D and Belsham GJ. 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot-and-mouth disease virus: evidence for the involvement of the three surface exposed capsid proteins in four antigenic sites. Virology. 179: 26-34.

Kurano NC, Messi F, Kurano S and Fiechter A. 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.

Kalanidhi AP, Nagaiah K, Palanisamy R and Srinivasan VA. 1992. Screening of Indian elephants. Cattle, and sheep for antibodies to foot and mouth disease virus infection associated antigen. Ind. Vet. J. 69: 390-392.

Kitching RP and Salt JS. 1995. The interference by maternally derived antibody with active immunization of farm animals against foot-and-mouth disease. Br Vet J. 151: 379-89.

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

Kesy A. 2002. Global situation of foot-and-mouth disease (FMD)–a short review. Pol J Vet Sci. 5**:** 283-287.

Kretzmer G. 2002. Industrial processes with animal cells Published online: 11 June 2002 © Springer. Verlag. 59(2-3):135–142.

Klein J. 2008. Understanding the molecular epidemiology of foot-and-mouth disease virus. Infection, Genetics and Evolution. 9:153-161.

Khawaja RM, Muhammad K, Shahzad W, Hussain I, Choudhry ZI, Awan FN, Bukhari A, Altaf I and Hanif A. 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

Loeffler F and Frosch P. 1897. Referred by I. A. Merchant and R. A. Packer in Veterinary Bacteriology and Virology, 6th ed. Iowa State University Press,Ames, Iowa

Lennete EH and Schmidt NJ. 1964. Diagnostic Procedures for Viral and Rickettsial Diseases. 3rd Ed. American Public Health Association, New York. pp 723-26.

Lobo CA, Cowan KM and Hanson RP. 1973. Complement-Fixation analysis of four subtypes of foot-and-mouth disease virus type A. Appl. Microbiol. 26(4): 570-573.

Li JP and Baltimore D. 1988. Isolation of poliovirus 2C mutants defective in viral RNA synthesis. J Virol. 62: 4016–4021.

Maitland MC and Maitland HB. 1931. Cultivation of Foot and Mouth Disease Virus. J. Comp. Path. 44: 106.

Macpherson I and Stoker M. 1962. Baby Hamster Kidney Fibroblast Cells (BHK-21). Virol. 16: 147–151.

McKercher PD and Gailuinas P. 1969. Response of swine to inactivated foot and mouth disease vaccines. Duration of immunity and local tissue reactions. Arch. Ges. Virusforsch 28: 165.

Mered B, Albrecht P and Hopps E. 1980. Cell growth optimization in microcarrier culture J. Virol. 5: 859-865.

Merchant IA and Packer RA. 1983. Veterinary Bacteriology and virology. Cytolysis and Complement Fixation. 7th Ed. CBS Publisher and Distributers India. P. 163-172.

McCullough KC, Bruckner L, Schaffner R, Fraefel W, Müller HK and Kihm U. 1992. Relationship between the anti-FMD virus antibody reaction as measured by different assays, and protection in vivo against challenge infection. Vet Microbiol. 30: 99-112.

McCullough KC, De Simone F, Brocchi E, Capucci L, Crowther JR and Kihm U. 1992. Protective immune response against foot-and-mouth disease. J Virol. 66: 1835–1840.

Mason PW, Rieder E and Baxt B. 1994. RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody dependent enhancement pathway. Proc. Natl. Acad. Sci. USA. 91: 1932-1936.

Murphy ML, Meyer RF, Mebus C, Schudel AA and Rodriguez M. 1994. Analysis of sites of foot and mouth disease virus persistence in carrier cattle via the polymerase chain reaction. Arch Virol. 136: 299-307.

McKenna TS, Lubroth J, Rieder E, Baxt B and Mason PW. 1995. Receptor binding site deleted foot-and-mouth disease (FMD) virus protects cattle from FMD. J. Virol. 69: 5787-5790.

Mezencio JM, Babcock GD, Meyer RF, Lubroth J, Salt JS, Newman JF and Brown F. 1998. Differentiating foot-and-mouth disease virus-infected from vaccinated animals with baculovirusexpressed specific proteins. Vet Q 20 (Suppl. 2), S11–S13.

Murphy FA, Gibbs EPJ, Horzinek MC and Studdert MJ. 1999. Veterinary Virology. 3rd Ed. Academic Press, New York. pp 521-527.

Meyer RF and Knudsen RC. 2001. Foot-and-mouth disease: A review of the virus and the symptoms. J Environ Health. 64: 21-23.

Merck and Co. Inc. The Merck Veterinary Manual. 2002. Eighth Edition, Review

Mason PW, Chinsangaram J, Moraes MP, Mayr GA and Grubman MJ. 2003. Engineering better vaccines for foot-andmouth disease. Dev Biol (Basel) 114: 79–88.

Muhammad K and Ahsan Z. 2003-2004. Immuno-prophylaxis of foot-and-mouth disease in bovines; 1st Annual Technical and Financial Report. University of Veterinary and Animal Sciences, Lahore

Miles AP, McClellan HA, Rausch KM, Zhu D, Long CA and Saul A. 2004. Montanide ISA 720 vaccines: Quality control of emulsions, Stability of formulated antigens, and comparative immunogenicity of vaccine formulations J.vaccine. 13: 781-784.

McCullough K and Sobrino F. 2004. Immunology of Foot-and-Mouth Disease. In Foot-and-Mouth Disease. 173-222.

Mittal M, Tosh C, Hemadri D, Sanyal A and Bandyopadhyay SK. 2005. Phylogeny, genome evolution and Antigenic variability among endemic foot and mouth disease virus type A isolates from India. Arch Virol. 150: 911-928.

Muhammad K, Tariq AM, Altaf I, Rabbani M, Hanif A, Khawaja RM, Riaz A. 2009. Antibody response of buffalo calves to foot and mouth disease virus vaccine. Pak. J. Zoology In press

Muhammad K, Chaudhry IZ, Rabbani M, Altaf I, Tariq AM, Anees M. 2011. In process quality control factors affecting potency of foot and mouth disease. Pak J Zool. 43: 249-254.

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

Nair SP. 1987. Studies on the susceptibility and growth pattern of foot and mouth disease virus vaccine strains in two pig kidney cell lines. Indian J. Comp. Microbiol., Immunol. and Inf. Dis. 8 (2): 76-81.

Nazir F. 2006. The impact of community based animal health services delivery system on epidemiological parameters and farmer's economic sustainability in Central Punjab. Ph.D thesis. Uni. of Vet and Anim Sci. Lahore, Pakistan

Office International des Epizooties. 2000. Foot-and-mouth disease. OIE Manual of Standards for Diagnostic Tests and Vaccines. OIE, Paris, France, p. 77-92.

Oem JK, Chang BS, Joo HD, Yang MY, Kim GJ, Park JY, Ko YJ, Kim YJ, Park JH. and Joo YS. 2007. Development of an epitope-blocking-enzyme-linked immunosorbent assay to differentiate between animals infected with and vaccinated against foot-andmouth disease virus. J Virol Methods. 142: 174–181.

OIE. 2009. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Pereira HG. 1981. Foot and Mouth Disease virus. In Gibbs, R.P.G. (ed.), Virus Diseases of Food Animals.Vol.2. Academic Press, New York, NY. pp: 333-363.

Perevozchikova NA, Falina GM, Satina TA, Perevozchikov VA, Burdov AN, Ivanyushchenkov VN, Vasilenko SK, Onishchenko AM, Netesov SV and Sandakhchiev LS. 1986. Isolation of apthovirus RNA. Veterinariya, Moscow, USSR. 5: 31- 34.

Palanisamy R, Kalanidhi AP, Nagaiah K and Srinivasan VA. 1992. Screening of Indian elephants, cattle, and sheep for antibodies to Foot and Mouth Disease virus infection associated antigen. Ind. Vet. J. 69: 390-392.

Piatti P, Hassard S, Newman JFE and Brown F. 1995. Antigenic variants in a plaque-isolate of foot-and-mouth disease virus: implications for vaccine production. Vaccine. 13: 781-784.

Patil PK, Bayry J, Ramakrishna C, Hugar B, Misra LD, Prabhudas K and Natarajan C. 2002-a. Immune responses of goats against foot-and-mouth disease quadrivalent vaccine: comparison of double oil emulsion and aluminium hydroxide gel vaccine in eliciting immunity. Vaccine. 21: 2781-2789.

Paranjape S. 2004. Goat serum: an alternative to fetal bovine serum in biomedical research. Ind. J. Exp. Biol. 42: 26-35.

Paton DJ, Valarcher JF, Bergmann I, Matlho OG, Zakharov VM, Palma EL and Thomson GR. 2005. Selection of foot and mouth disease vaccine strains--a review. Rev Sci Tech. 24: 981-993.

Perez AM, Thurmond MC, Grant PW and Carpenter TE. 2006. Spatial distribution of foot-and-mouth disease in Pakistan estimated using imperfect data. Prev Vet Med. 76: 280-289.

Reed LJ and Muench H. 1938. A simple method for estimating fifty percent end point. Am. J. Hyg. 27: 493-497.

Rubino MC. 1946. La Fiebre Aftosa, Estado Actual de Los Conocimientos sobre su profilaxis biologica in: Compilacion de Trabajos Cientificos del Dr. Miguel C. Rubino Uruguay. 297-353.

Radlett PJ, Telling RC, Stone CJ and Whiteside JP. 1971. Improvements in the Growth of BHK-21bCells in Submerged Culture. Appl. Microbiology. 4: 534-537.

Rauf AM, Khan AN and Ahmed M. 1981. Typographical study of foot and mouth disease virus in Pakistan. Pakistan V. J. 1 (1): 13-14.

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

Ryan JM. 1997. Effect of different fetal bovine serum concentrations on the replicative life span of cultured chick cells. *In vitro.* 15: 895-89.

Reid SM, Grierson SSN, Ferris P Hutchings GH and Alexandersen S. 2003. Evalution of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease irus. J Virol Methods. 107(2):129-39.

Racaniello VR. 2006. Picornaviridae: the viruses and their replication. In Fields Virology, 5th edn, pp. 795–838. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Rzeczinski S, Victorov IV, Lyjin AA, Aleksandrova OP, Harms C, Kronenberg G, Endres M and Dirnag U. 2006. Roller Culture of Free- Floatin Retinal Slices: A New System of Organotypic Cultures of Adult Rat Retina. j. Ophthalmic. 38: 263-269.

Ryan J, Deng M, Wang H, Ward G, Gidlewski T, McCollum M, Metwally S, McKenna T, Wainwright S, Ramirez A, Mebus C and Salman M. 2008. Foot-and-mouth disease in North American bison (bison bison) and elk (cervus elaphus nelsoni): susceptibility, intra- and interspecies transmission, clinical signs, and lesions. J Wild Dis. 44: 269-79.

Rweyemamu M, Roeder P, MacKay D, Sumption K, Brownlie J and Leforban Y. 2008. Planning for the progressive control of foot-and-mouth disease worldwide. Transbound and Emerg Dis. 55: 73-87.

Rashid A, Rasheed K and Akhtar M. 2009. Factors influencing vaccine efficacy A general review. J Anim and Plant Sci. 19: 22-25.

Rodrigue LL and Grubman MJ. 2009. Foot and mouth disease virus vaccines. Vaccine. 27: 90-94.

Skinner HH. 1951. Propagation of stains of Foot and Mouth disease virus in unweaned mice. Proc. Roy. Soc. Med. 44: 1041-1044.

Sadettin SO and Bernhard IP. 1990. Effect of initial cell density on hybridoma growth, metabolism, and monoclonal antibody production. J. of Biot. 16: 259-278.

Sharma SN and Adlakha SC. 1995. Veterinary Virology. Vikas Publishing House, New Delhi. p 212-223.

Snowdon WA. 1966. Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. Nature. 210: 1079-1080.

Swayne DE, Jhon RGM, Jackwood EJW and Pearson RMW. 1998. Cell culture methods In: A Laboratory manual for isolation and identification of Avian pathogens, 4th Edi, pp: 223-234. American Association Avian pathologists, Washington State University Press, Washington D.C

Shen F, Chen PD, Walfield AM, Ye J, Brown F and Wang CY. 1999. Differentiation of convalescent animals fromthose vaccinated against foot-and-mouth disease by a peptide ELISA. Vaccine. 17: 3039–3049.

Sakamoto K, Kanno T, Yamakawa M, Yoshida K, Yamazoe R and Murakami Y. 2002. Isolation of Foot-and-Mouth disease virus from Japanese black cattle in Miyazaki Perfecture, Japan. J. Vet. Med. Sci. 64(1): 91-94.

Shin JH, Sohn HJ, Choi KS, Kwon BJ, Choi CU, Kim JH, Hwang EK, Parj JH, Kim JK, Choi SH and Kim OK. 2003. Identification and isolation of foot-and mouth disease virus from primary suspect cases in Korea. J. Vet. Med Sci. 65: 1-7.

Sutmoller P, Barteling SS, Olascoaga RC and Sumption KJ. 2003. Control and eradication of foot and mouth disease virus research. 91: 101-144.

Singh SN. 2003. Foot and mouth disease: Present status and future strategy for control. In: Proceedings of the 4th Asian Buffalo Congress on buffalo for food security and Rural Employment. Lead papers. 1: 267-271.

Shi XJ, Wang B, Zhang C and Wang M. 2005. Expressions of bovine IFN-gamma and foot-and-mouth disease VP1 antigen in P.*pastoris* and their effects on mouse immune response to FMD antigens Vaccine, 24: 82–89.

Slivac I, Srček VG, Radošević K, Kmetič I and Kniewald Z. 2006. Aujeszky's disease virus production in disposable bioreactor. J. Biosci. 31: 363–368.

Rehman S, Rabbani M, Sahidullah, Muhammad M and Iqbal Z. 2007. Studies on *In vitro* Culture Characteristics of Adherent Baby Hamster Kidney-21 (BHK-21) Cell Line. Int. J. Agri. Biol. 6: 821–826.

Sathya R, Mathialagan A, Flora FB and Ramalingum K. 2008. Determinent factors for hybridoma culture (H9r9) in the yield of anyi-D- impact of medium (IMDM and RPMI), serum (FCS) and cell density. j.Biochem tech. 1: 53-56.

Trautwein K. 1927. Die pluralitat des maul-und- Klauenseuche virus. Arch. Wiss. Prakt. Tierheilk. 56: 505.

Tewari SC, Kant R, Prasad S, Ahuja KL and Sharma VK. 1980. A note on the use of bovine kidney cell culture for recovery of foot and mouth disease virus from field samples. Bull.Off. Intl. des Epiz. 92: 167-169.

Thomas AA, Woortmeijer RJ, Puijk W and Barteling SJ. 1988. Antigenic sites on foot-and-mouth disease virus type A1 0. J. Virol. 62: 2782-2789.

Torres A, David MJ and Bowman QP. 2002. Risk management of international trade: emergency preparedness. Rev Sci Tech Off Int Epiz. 21: 493-498.

Tosh C, Hemadri D and Sanyal A. 2002. Evidence of recombination in the capsid-coding region of type A foot-and-mouth disease virus**.** J Gen Virol. 83**:** 2455-2460.

Tariq MA. 2006. Immunoprophylaxis of foot and mouth disease in bovine Ph.D. Thesis Microbiology, University of Veterinary and Animal Sciences, Lahore, Pak.

Tizzard IR. 1998. Veterinery immulogy, Saunders, Philadelphia, USA, 8th edn, 2008, ISBN 1416049894.

Urushihara H, Ueda MJ, Okada TS and Takeichi M. 1977. Calcium-dependent and –independent adhesion of normal and trans-formed BHK cells. Cell Struct. Funct. 2: 289-296.

Villegas P and Purchase HG. 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, 248-253.

Vallee H and Carre H. 1922. Sur la pluralite' due virus aphteux. As quoted by Sutmoller, P., S.S. Barteling, R.C. Olascoaga and K.J. Sumption. 2003. Control and eradication of foot and mouth disease. Virus Research. 91: 101-144.

Velthuis AG, Mourits MC. 2007. Effectiveness of movement-prevention regulations to reduce the spread of foot-and-mouth disease in the Netherlands. Prev Vet Med. 82: 262-281.

Westbury HA, Doughty WJ, Forman AJ, Tangchaitrong S and Kongthon A. 1988. A comparison of enzyme-linked immunosorbent assay, complement fixation test and virus isolation for foot and mouth disease diagnosis. Vet. Microbiol. 17(1):21-28.

Wani SA, Ahuja KL, Prasad S and Arvind K. 1997. Differentiation of foot and mouth disease virus type "O" isolates by micro-serum neutralization and in vivo cross protection methods. Intl. J. of Anim. Sci.12 (1): 73-75.

Wei Wei D, Zwang D, Ran, Huang J and Meng Y. 1998. Cloning and probe preparation of pseudorabies glycoprotein gp 50. Chinese J. Vet. *Sci*. 18: 457–59.

Yadav MP. 2003. Health barrier to buffalo productivity and their management. In: Proceedings of the 4th Asian Buffalo Congress on "Buffalo for Food Security and Rural Employment, held at New delhi, during February 25-28.

Zulfiqar M. 2003. Draft report for development of national disease control policy for foot-and-mouth disease in Pakistan under the FAO project "support for emergency prevention and control of main trans-boundary animal diseases in Pakistan Rinderpest, FMD and PPR

Zahur AB, Irshad H, Hussain M, Anjum R and Khan MQ. 2006**.** Transboundary animal diseases in Pakistan. J Vet Med. Series B**.** 53: 19-22.

Zhang C, Bin W and Ming W. 2011. GM-CSF and IL-2 as adjuvant enhance the immune effect of protein vaccine against foot-and-mouth disease. Virology J. 8:7.