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Determination of TCID₅₀ of O, A, Asia-1 serotypes foot-and-mouth disease virus to the production of trivalent vaccine in bioreactor

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Abstract: This research work was designed to ensure the production of quality Foot and Mouth Disease (FMD) vaccine. For ensuring the standard quality of vaccine, it is important to propagate the FMD Virus (FMDV) into the cell line. Tissue Culture Infective Dose₅₀ (TCID₅₀) assay was used to determine the biological titer of FMD virus serotypes. Baby Hamster Kidney-21 (BHK-21) Monolayer cell and BHK-21 Clone 13 Suspension cell were used to determine the TCID₅₀ of FMDV. The result for calculating TCID₅₀ under Monolayer cell showed that the highest biological titer of O, A, Asia-1 serotype were $10^{-9.83/ml}$, $10^{-9.36/ml}$ and $10^{-9.70/ml}$ respectively. On the other hand, the result for calculating TCID₅₀ under BHK-21 Clone 13 suspension cell of O, A, Asia-1 serotypes were $10^{-6.66/ml}$, $10^{-6.50/ml}$ and $10^{-6.50/ml}$ respectively. BHK-21 cell line, either the monolayer or the suspension cell culture system were used in FMD vaccine production to ensure the potency and the quality of the vaccine to be produced. BHK-21 monolayer cell sub-cultured by applying roller cell culture cultivation system and BHK-21 clone 13 suspension cell sub-cultured by using suspension cell culture cultivation system. Suspension culture of BHK-21 cells allows large-scale virus propagation and cost-efficient vaccine production by using Bioreactor with capacity of 50 liters maintaining biosafety and biosecurity.

Keywords: foot-and-mouth disease; BHK-21; TCID₅₀; bioreactor; vaccine

1. Introduction

FMD is a highly contagious and trans-boundary viral disease of domesticated and wild cloven-hoofed animals showing symptoms of fever, erosions and blister like lesions on the hooves, lips, mouth, teats and tongue; associated with huge economic loss to the livestock farming and industry worldwide and is considered as a major threat to cattle, buffalo (both milk and meat) and pig production in endemic countries and therefore, considered to cause food insecurity, both locally and globally (Giasuddin *et al.*, 2016). Domesticated species such as cattle, pigs, sheep, goats and buffaloes are susceptible to FMD, in addition, many species of cloven-hoofed wildlife, such as deer, antelope and wild pigs may become infected (FAO, 1984; Barnett *et al.*, 1999; Grubman *et al.*, 2004; Chang *et al.*, 2007; Rahman *et al.*, 2022). There is 100% morbidity in susceptible animal population and negligible mortality in adults (Hassan, 2016). However, high mortality in a young animal is due to the virus-induced necrotic lesions and myocardial degeneration (Khawaja *et al.*, 2009).

FMD virus, a positive sense single-stranded RNA virus, is belongs to the genus Aphtho virus of family *Picornaviridae* and occurring in seven serotypes that is O, A, C, Asia-1, SAT1, SAT2 and SAT3 with a wide diversity (Domingo *et al.*, 2002). FMDV serotype O, A and Asia-1 are the currently circulating FMDV in Bangladesh as revealed by RT-PCR (Chowdhury *et al.*, 2015). Annual loss due to FMD in Bangladesh has been estimated at about US\$ 62 million (FAO/OIE, 2012; Sarker *et al.*, 2015).

In addition, because of the emergence of new FMD viruses, vaccination with one serotype cannot confer protection against strains of different serotypes or heterologous strains of the same serotype based on antigenic variation and also cannot respond promptly (Rodriguez *et al.*, 2009). BHK-21 cell line either the monolayer or the suspension systems are used in FMD vaccine production and to ensure the potency and the quality of the vaccine produced (Hassan, 2016). Suspension culture has certain advantages, such as larger production volumes, continuous medium perfusion and certain parameters including pH, oxygen and carbon dioxide levels which can be monitored and controlled continuously (Current Laboratory Techniques, Research and Prevention, 2014). For Suspension cell culture, Bioreactors with capacity of 50L were used maintaining biosafety & biosecurity for production of vaccine in the Livestock Research Institute (LRI), Dhaka. Bioreactors are the vessels/containers which provide biological, biochemical, and biomechanical requirements for the optimal growth of the fermenting microorganisms and/or biochemical reactions on the industrial scale for the synthesis of desired products. Determination of high biological titer of the FMD virus by using Tissue Culture Infective Dose₅₀ (TCID₅₀) assay is the basic requirement for vaccine production (Reed and Meunch, 1938). However, its infectivity titer is enhanced by repeated passage on BHK-21 cell lines either anchorage dependent (adherent) or anchorage independent (suspension). Therefore, the objective of this study was to prepare a quality full FMD vaccine against O, A, Asia-1 serotype of FMDV on BHK-21 cell lines, to know the effect of different cell culture systems including roller cell culture system for monolayer, suspension cell culture system by Bioreactor and to know the efficacy, potency of FMD trivalent vaccine by TCID₅₀.

2. Materials and Methods

This research work was conducted at FMD Vaccine Section, Livestock Research Institute, Mohakhali, Dhaka from February 2017 to March 2019. Vaccine seed used for FMD Vaccine containing three serotypes namely O, A, Asia-1 strains. Accession numbers of Gen Bank Serotypes A, O, Asia-1 were KP119763, KP119762 and KP119756 respectively.

2.1. Procedure for the preparation of FMD vaccine used BHK-21 monolayer cell by using roller cell culture cultivation system

BHK-21 cells were grown in tissue culture flasks. All the media and reagents were brought to room temperature before use. Cryovial containing BHK-21 monolayer cell (1 milliliter) brought from liquid nitrogen & poured into 25cm² flask containing growth media and then incubated at 37°C with 5% CO₂ Incubator for 24 hours. Cultures were viewed using an inverted Microscope (Nikon ECLIPSE, Tokyo, Japan) to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants. Spent medium was removed. The monolayer cell was washed with PBS without Ca²⁺/Mg²⁺ using a volume equivalent to half of the volume of cultured medium. Repeat this step of wash, if the cells were known to adhere strongly. Trypsin was poured into the washed cell monolayer using 1ml per 25cm² of surface area. After that flask was rotated to cover the monolayer with trypsin. Excess trypsin was decanted. Then, the flask was returned to the incubator and left for 2-3 minutes. The cells were examined using an inverted Microscope to ensure that all the cells were detached and floating. Approximately 35 ml of cell culture maintenance media was added in a 75 cm² flask. Afterward, transferred the required amount of cells to a new labeled flask containing pre-warmed medium. Incubated at 37°C with 5% CO₂ incubator for 24 hours for confluent growth. Thus, monolayer cell was harvested and transferred to 175 cm² cell culture flask with 35 ml of the growth medium (Khawaja *et al.*, 2009), the roller bottle (Duran, GmbH, Germany) containing 100 ml of the growth medium in order to observe the effect of culture system on the production of FMD virus. Thereafter, the same procedure was repeated as previous.

2.1.1. Infection and adaption of FMD in BHK-21 cell culture

The virus was readily adapted to BHK-21 cell line within first 3-5 passages. The BHK-21 cells those formed complete and confluent monolayer in the roller bottle within 24 hours of subculture were selected for infection with viruses. Necessary number of monolayer cell culture bottle was taken out from the roller hot room. The growth media from the roller bottle containing BHK-21 cell was removed and then inoculated by 5ml prepared virus seed separately for each type of virus (O, A, Asia-1) in separate bottle. Rolled the bottle gently in order to roll the virus over the whole cell layer. Then, incubated at 37°C for 5-10 minutes in the hot room. After

incubation, added 300 ml virus media in each bottle. Again incubated for 18-24 hours at 37°C. After incubation periods, the cells were examined twice under inverted Microscope until the inoculated FMD virus exhibited characteristic Cytopathic Effect (CPE). Propagation of FMDV in BHK-21 cell was confirmed by appearance of CPE comprising rounding and flattening of the cells, breaking down of the intracellular bridges and finally, cell death. Subsequently, the virus fluid was collected and stored at -20°C to -80°C for further production of vaccine.

2.2. Procedure for the preparation of FMD vaccine used BHK-21 clone 13 suspension cell culture cultivation system by using bioreactor machine

2.2.1. Preparation of O, A and Asia-1 virus fluid

In FMD laboratory, three types of virus fluid were producing i.e. A, O and Asia-1. The following procedure was maintained to produce those viral fluids separately. First of all BHK21 suspension cell was kept in a 25 cm² flask with media and broth. Then, the flask was placed in a shaking incubator at 37°C temperature and 40 rpm for 24 hours. Next day, the live cells were counted by using Haemocytometer Auto Cell Counter. The media and broth were placed in new flasks to increase the number of cells. After that, all the flasks were placed in a shaking incubator following same procedure i.e. at 37°C temperature and 40 rpm for 24 hours. After 24 hours, the cells were centrifuged using falcon tube at 2000 rpm in a centrifuge machine for 10 minutes. After centrifuging, the supernatant fluids from the falcon tube were removed. Then 10 ml media were added with the cell pellet at the base of the falcon tube using pipette. After mixing the media, cells sediments were collected using a pipette and placed in a new 25 cm² flask. The flask was kept in a shaking incubator at 37°C temperature and 40 rpm for 72 hours. After 72 hours, the same procedure was repeated. In this phase, to increase the amount of live cells, partial amounts of cells were kept from 25 cm² flasks to 75 cm² flasks. Then media and broth were added in both flasks. By following the same steps the amounts of cells were increased by taking partial amount of liquid from 75 cm² flasks. After 72 hours, the liquid was kept in a 175 cm² flasks. In this case, media and broth were added in the both flasks again. Then, partial amounts of liquid were collected, after 72 hours from 175 cm² flask and kept in the roller bottle. Then, same way, media and broth were added both in the flask and the roller bottle. The procedure was continued to increase the cell amount. For 20 liters of media added 2 liter cells, Fetal Bovine Serum, L- Glutamine and Antibiotic and Antimycotic. All the reagents were poured into the bioreactor. Then, Carbon Di Oxide (CO₂) was given in the Bioreactor at 5-liter flow rate for 5 seconds at 3-minute interval for 24 hours. The fluid was collected and centrifuged at 2000 rpm for 10 minutes. After that, the sedimentation fluid was collected and the same procedure was repeated. Afterwards, the centrifuged cells were kept in another container and virus seed was added at 1:1 ratio i.e. the amount of centrifuged cell and virus seed were same or equal for each container. Then, the mixture was kept in the hot room at 37°C for 1 hour. After that 20-liter Virus Media (VM) was prepared. Then cell and seed virus mixture from the hot room (after 1 hour) were added with 20-liter Virus Media and antibiotic was also added. The liquid mixture was kept in Bioreactor for 6-18 hours at 37°C temperature (Because seed virus produces CPE within 6-18 hours). After 6-18 hours, CPE observed under inverted Microscope. Thereafter, the virus fluid was collected from Bioreactor and kept into a container. The container was kept into the deep freeze (at -20°C temperature) for further use.

2.3. Biological titration of the virus (TCID₅₀)

The harvested culture fluid was processed for biological titration (TCID₅₀) before using for vaccine preparation. Virus titration was conducted in 48-well tissue culture plates. The BHK-21 cell line was grown in 48-well tissue culture plate and infected with tenfold dilutions of the virus. After 6-18 hours, the plate was examined for CPE and TCID₅₀ was calculated by the Reed and Meunch (1938) method.

3. Results and Discussion

The main scope in vaccine production procedures is usually constructed in the production of a large amount of vaccine doses so as to fulfill the huge need of vaccine doses for different animals and also in addition to the potency of the produced vaccine regarding its high infectivity and antigenicity of the serotypes included in the vaccine.

As the BHK-21 cell line either the monolayer or the suspension systems are used in FMD vaccine production and to ensure the potency and the quality of the vaccine produced, It was of interest to follow-up the infectivity, complement fixing activity of FMDV type A, O and Asia-1 on BHK cell culture in roller and suspension culture systems.

In this experiment the BHK-21 monolayer cell and cytopathic effects were observed by a inverted microscope (Figure 1 and Figure 2). BHK-21 clone 13 Suspension Cell and cytopathic effects also observed under Contrast Microscope (Figure 3 and Figure 4).

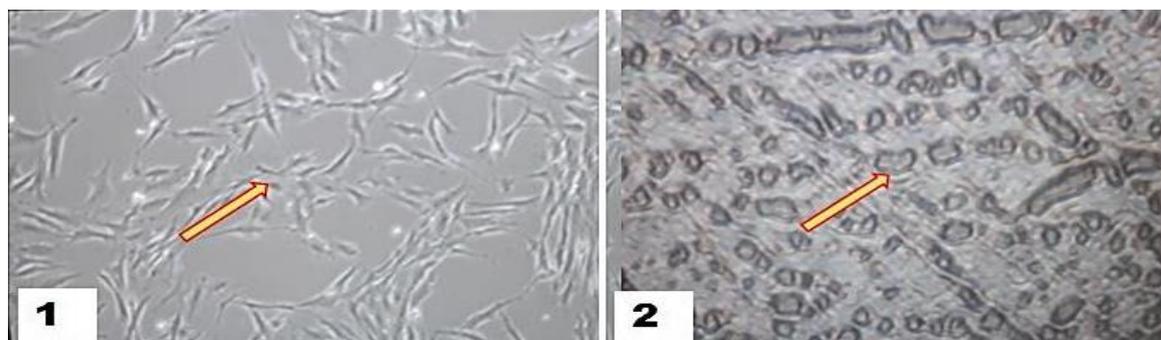


Figure 1. Elongated shape of BHK-21 Monolayer Cell which was observed under 10X objective is marked with arrow shape; Figure 2. The arrow in the picture is showing Cytopathic Effect (CPE) i.e. structural changes in BHK-21 cell line resulting from viral infection which was observed under 40X objective Phase Contrast Microscopy (Nikon ECLIPSE, Tokyo, Japan).

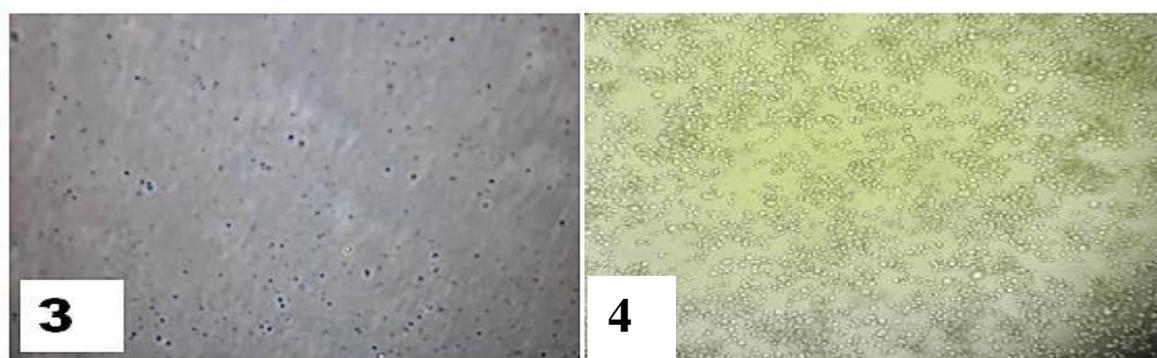


Figure 3. BHK-21 clone 13 Suspension Cell was observed under 10X objective, Phase Contrast Microscopy (Nikon ECLIPSE, Tokyo, Japan); Figure 4. Cytopathic Effect (CPE) was observed under 10X objective, Phase Contrast Microscopy (Nikon ECLIPSE, Tokyo, Japan).

The Median Tissue Culture Infectious Dose (TCID₅₀) under Monolayer cell showed biological titer of O, A, Asia-1 serotype were 10^{-9.83/ml}, 10^{-9.36/ml} and 10^{-9.70/ml} respectively. On the other hand, the result for calculating TCID₅₀ under BHK-21 Clone 13 suspension cell of O, A, Asia-1 serotypes were 10^{-6.66/ml}, 10^{-6.50/ml} and 10^{-6.50/ml} respectively. In this experiment, BHK-21 Monolayer cell shown better TCID₅₀ in comparison to BHK-21 Cl 13 Suspension Cell. Details are shown in Table 1.

Table 1. TCID₅₀ value of BHK-21 (Monolayer Cell and Suspension Cell) ml⁻¹ viral titer

SL	Type of virus fluid (serotype)	No. of experiments	Viral titer in BHK-21 Monolayer Cell	Viral titer in BHK-21 Cl 13 Suspension Cell
1	O serotype	20	10 ^{-9.83}	10 ^{-6.66}
2	A serotype	20	10 ^{-9.36}	10 ^{-6.50}
3	Asia-1 serotype	20	10 ^{-9.70}	10 ^{-6.50}

This finding is contributed to the result obtained by Ali *et al.* (2013) who found that the highest infectivity for FMDV serotype A virus was achieved monolayer cell. This difference in infectivity between the roller and suspension cultivation system may be attributed to the surface area difference between the suspension and roller cultivation systems as the surface area and subsequently the exposed area for growing cells is greater in the roller cultivation system and in agreement with Akram *et al.* (2013) who found that the virus infectivity titer in the stationary monolayer of BHK- 21 cells in roller flask is higher than that in the suspension flasks and also agreed with Altaf *et al.* (2012), Khawaja *et al.* (2009) and Salivac *et al.* (2006) who stated that the infectivity titer of the virus is directly proportional to number of BHK-21 cells in the culture system.

There is a comparative result of BHK-21 Monolayer Cell in roller bottle and BHK-21 Cl13 Suspension Cell in Bioreactor for FMD vaccine production shown in Table 2.

Table 2. Comparative results of BHK-21 Monolayer Cell in roller bottle and BHK-21 CI 13 Suspension Cell in Bioreactor for FMD vaccine production

BHK-21 Monolayer Cell	BHK-21 CI 13 Suspension Cell
The exposed area for growing cells was greater in the roller cultivation system and the result was similar with Akram <i>et al.</i> , 2013.	Not like Monolayer cell
The longevities of suspension cell lines used for FMD vaccine production is Lower	The longevities of suspension cell lines used for FMD vaccine production is Higher
The Cytopathic Effect (CPE) was clearly understood because the shape of Healthy BHK-21 Monolayer cells appeared elongated, spindle shaped and the infected BHK 21 monolayer cell (infection by FMDV)	BHK-21 Suspension cell was suspended within the media, that's why it was easily used in Bioreactor machine and used to produce huge
The highest value of TCID ₅₀	Comparative lower TCID ₅₀

Depending on the fact reported by Ali *et al.* (2013) that the protective capacity of FMD vaccine is the FMDV antigenicity; the FMDV yield from different cultivation systems must be tested and compared on their antigenicity by CFT and quantifying the 146S intact virion content.

4. Conclusions

It is clear that the best cultivation system on Baby Hamster Kidney BHK-21 cell line used for production of FMD vaccine regarding its infectivity and antigenicity is the roller cultivation system as it induce the highest titer and antigenicity either in the complement fixing titer and that is reflected on the antibody titer and the protection period in vaccinated cattle.

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Data availability

All relevant data are within the paper.

Conflict of interest

None to declare.

Authors' contribution

Safeth Arju, Samsun Nahar and Most. Rawshan Ara, Mohammad Zakir Hossain: conceptualization, methodology, interpretation of data, manuscript writing; Md. Farhad Hossain, Md. Safiul Ahad Sardar: supervision, reviewing and editing; Parinita Basak and Md. Enamul Haque: reviewing and editing. All authors have read and approved the final manuscript.

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