Research Article

Comparative Study of the Virulency of Different Serotypes of Foot and Mouth Disease Virus by Using Baby Hamster Kidney-21 Cell Line

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Abstract | Foot and Mouth Disease (FMD) is a highly contagious and sometimes fatal viral disease affecting cloven footed animals including bovine, caprine and ovine. Globally, FMD production losses are up to 21 billion US dollars annually. Annual losses in Pakistan is 692 million dollars in terms of body weight loss, milk production, treatment cost and mortality. FMD is caused by 7 different serotypes of FMD virus having different virulence. To compare the virulence of various FMD serotypes the study was designed to propagate the virus on BHK-21 cell line. The results revealed that serotype 'A' virus virulence is more as compared to serotype 'O' and 'Asia-1'. Although less virulence difference was found between serotype 'A' and 'O'. Serotype 'Asia-1' was comparatively less virulent. Randomized Complete Block Design (RCBD) was used for determining the effect of incubation time, virus serotypes and virus titer producing CPEs on BHK-21 cells.

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Introduction

Livestock is a sub-sector of the economy of Pakistan's agriculture contributing a share of 60.6% in agriculture GDP and 11.7 % in overall GDP (Economic Survey of Pakistan 2019-2020). Almost 8 million families are directly or indirectly involved in livestock rearing and earn more than 35% of their family income. Besides this importance, this sector is facing troubles due to decreased productivity and improper marketing. One of the reason of decreased productivity is the existence of various contagious diseases of livestock. Among these contagious diseases FMD is of utmost importance. FMD presents the major hurdle in the export of livestock and its products to FMD-free countries. According to FAO, an annual loss in Pakistan is 692 million dollars in terms of body weight loss, milk production, treatment cost and mortality (Food and Agriculture Organization 2018).

Foot and mouth disease is highly infectious disease of cloven footed animals including cattle, sheep, goat and pigs etc. The disease was first defined in sixteenth century and FMD virus was the first animal pathogen isolated as virus. Foot and Mouth Disease is caused by Foot and mouth disease virus which belongs to

Family *Picornaviridae*, genus *Apthovirus*, which is a non-enveloped RNA virus, existing in seven different serotypes namely O, A, C, Asia-1, SAT1, SAT2 and SAT3 (Longjam *et al*., 2011).

FMD virus serotype 'O' can be easily transmitted to both cattle and swine regardless of donor species. In contrast serotype 'A' is only transmissible to swines and is less severe in steers. Pigs shows a severe rapid and contagious disease when infected with serotype 'A' by direct contact, direct inoculum or by infection with bovine derived virus (Pacheco *et al*., 2016).

A previous study showed that FMD virus utilizes $\alpha_v\beta_3$ integrin as primary receptors for infection and adaptation of type O1 virus to cell culture results in the ability of virus to utilize heparin sulphate (HS) as a receptor. As this integrin is absent in human cell line-K562, so serotype A_{12} cannot replicate in these cells unless transfected with cDNA encoding this integrin (K562-$\alpha_v\beta_3$). On contrast, cell surface heparin sulphate is required for type O_1 FMD virus infection and tissue culture FMDV type O_1 virus was capable of replication in non-transfected K562 cells. (Neff *et al*.,1998).

To control infectious diseases like FMD massive vaccination is required. Live cells including BHK- 21 cells, Bovine kidney cells, vero cell lines are required. For FMD virus isolation and propagation, BHK- 21 cell line is more preferable. This cell line was first introduced in 1961 from 5 Syrian Hamsters' kidney litter no. 21. Since then, this cell line is a laboratory standard for many biological processes and propagation of many viruses (Hernandez *et al*., 2010). BHK-21 cells have affinity for reovirus-3, human adeno virus and vesicular stomatitis virus (Indiana strain), but they are resistant to poliovirus-2. Furthermore, these cells are not suitable for reverse transcriptase, which is a clear indication for lacking of integral retrovirus genomes (Macpherson and Stoker, 1962). BHK-21 cell lines are susceptible to FMDV infection and can be adopted by blind passages. (Rabbani *et al*., 2007).

In the current study virulence of different serotypes were compared to know about the most virulent among the present FMD virus serotype.

Materials and Methods

Growth media preparation for BHK-21 cells

Glasgow Minimum Essential Media of Biowest was dissolved in distilled water as per manufacturer's instruction. Sodium bicarbonate was added with a concentration of 0.4 g per liter. Media was filtered using syringe filter of 0.22 µm. Fetal bovine serum with a concentration of 10% was added in media. Antibiotic and antimycotic solution were added as per manufacturer's preparation. All the procedures were performed in Class II-A2 Biological Biosafety cabinet.

Propagation of cell culture flask

Revival of cells was done as per ATCC Animal Cell Culture guide. A 25 ml of growth media was taken 75 square cm cell culture flask. Cryovails containing frozen cells were thawed and poured in 15 ml falcon tube containing 9 ml media. Centrifugation was done at 2000 rpm for 10 minutes. Supernatant was poured off and 3 ml growth media was added in falcon tube and vortexed for few seconds. Growth media containing cells were poured in 75 square cm cell culture flask and incubated for 24 hours. All the procedures were performed in Class II-A2 Biosafety Cabinet.

FMD virus

FMD virus was recovered from Foot and Mouth Disease Vaccine Research Center, Veterinary Research Institute Peshawar. Strain serotype A was AT06, serotype O is Pan Asia-2 and serotype Asia-1 is sin 08. Virus sample was kept at -80℃. Each sample was confirmed by sandwich ELISA through their specific OD values. TCID-50 was performed according to the visual method of TCID-50 according to Spearman-Kerber method described by Ramakrishnan (2016).

Enzyme linked immunosorbent assay (ELISA) for virus confirmation

A 250µl of virus sample was taken and 250µl diluent buffer was added and mixed properly. A 50µl of diluted sample was distributed from well 1 to well 10 in all respective columns. A 50µl of diluent buffer was distributed in all wells of 11th and 12th column. (control positive and control negative). Plates were incubated for 1 hour at room temperature. (18-30 ℃). After incubation all wells were emptied and tapped hard to remove all remaining residual fluid. Entire wells were filled with 200 µl of washing solution. Plates were incubated for 3 min at room temperature. Wells were emptied and procedure was repeated thrice. A diluted conjugate A was added from rows A to row F and diluted conjugate B into rows G and H. Plates were covered and incubated for 1 hour at room tempera-

ture. Four washing cycles were repeated as above leaving last one for 5 min. A 50 µl of substrate/ chromogen solution was added to each well in a dark room. Plates were covered and incubated for 20 min at room temperature in a dark room. A 50 µl of stop solution was added to each well. Plate was read and OD values were recorded at 450 nm wavelength. OD values of confirmed FMD virus was greater than 0.1.

Tissue culture infective dose-50

TCID-50 was performed according to the visual method of TCID-50 according to Spearman-Kerber method described by Ramakrishnan (2016).

Preparation of 96-well cell cultured plate: A cell cultured plate of 75 square cm was taken. Cell counting was done with the help of automatic cell counter (Machine and made). Cell count of 1 X 10^5 cells/ ml was adjusted. Growth media having 10% Fetal bovine serum was added to make a total volume of 60 ml growth media for two cell culture 96 well plates. Growth media of 100µl was added in each well. Cell cultured plate was kept in incubator at 37℃ for 24 hours.

Determining virus titer: A 10-fold dilution of each virus serotype was made in Eppendorf tubes. For this purpose, 900 µl of GMEM was taken. Virus of 100 µl was taken and added to first Eppendorf tube. Virus was properly mixed in GMEM with help of micropipette. A series of dilutions were made up to 8^{th} Eppendorf tube. A 100µl of volume was discarded from last Eppendorf tube. Cell cultured plate was marked up to 8^{th} dilution. A 100µl of diluted virus was added in plate. Each dilution was repeated 5 times. Plate was kept in incubator. Methylene blue of 100 µl was added in each well after 48 hours. Cell cultured plate was kept in incubator. Results were interpreted after 1 hour. A virus titer of $10^{6.2}$/ml was maintained.

Virus cultivation

A total of nine 75 square cm flasks were cultured having equal BHK-21 cells confluency. Maintenance infectious media having 02% FCS was prepared. Virus suspension was filtered through 0.2 µm filter (Millipore®, Billerica Massachusetts, USA). A 2 ml volume of virus was poured in respective 75 square cm flasks and incubated at 37°C for 48 hours. Cytopathic effects (CPEs) was observed. Cell counting was performed with hemocytometer to analyze live cells. All the procedures were performed in Biosafety Cabinet-II/Class A2.

Statistical analysis

Randomized complete block was applied as the experimental units are homogeneous. Virus titer i.e $10^{6.2}$/ ml, incubation temperature (36-37 °C) and incubation time (6, 12, 24 and 48 hours) are kept constant for recording cytopathic effects at different intervals.

Results and Discussion

Equal amount of FMD virus (0.5 ml), having tissue culture infected dose (TCID-50, $10^{6.2}$ / ml) was inoculated over 75 square cm BHK-21 cells culture confluent flasks in 3 replicates. Cytopathic effects were recorded on the basis of changes in shape, size and living status. A normal living cell was identified as elongated attached with the culture flask visualized at 4X and 10X under inverted microscope. Cytopathic effect was detected via round shape with small size and floating non-adherent cells. The culture was examined at intervals of 6, 12, 24 and 48 hours after the onset of incubation.

Table 1: *Changes in BHK-21 cells morphology infected with FMD virus.*

Virus type	Incubation time (hours)	Cytopathic Effects (%)			
		Round cells	Clusters	Dead cells	Total
Serotype "O"	6	10 ±0.3	0 ± 0	0 ± 0	10 ± 0.3
	12	50 ±1	50 ± 10	50 ± 1	50 ± 0.1
	24	90 ± 0.5	90 ± 0.5	90 ± 0.0.5	90 ± 0.5
Serotype "A"	6	20 ± 0.6	0 ± 0	0 ± 0	20 ± 0.6
	12	60 ± 0.5	60 ± 0.5	60 ± 0.5	60 ± 0.5
	24	100 ±0	100 ± 0	100 ± 0	100 ± 0
Serotype "Asia-1"	6	5 ± 0.1	0 ± 0	0 ± 0	5 ± 0.1
	12	30 ± 0	30 ± 0	30 ± 0	30 ± 0
	24	70 ± 0.5	70 ± 0.5	70 ± 0.5	70 ± 0.5

According to Table 1, Cytopathic effects were minimum after 6 hours as slight decrease in cell count was observed. Cell count of serotype 'O' came to 3X10^5 cells/ml from an initial cell count of 3.3X10^5 cells/ml. In 12 hours, cell count was 1.6X10^5 cells/ml and in 24 hours no live cell was detected. Cell count of serotype 'A' after 6 and 12 hours were 2.8X10^5 cells/ml, 1.4X10^5 cells/ml and no live cells were detected after 24 hours. Live cell count of serotype 'Asia-1' in 6, 12 and 24 hours was 3.2X10^5 cells/ml, 2.7X10^5 cells/ml and 1.7X10^5 cells/ml respectively.

Different types of cell lines can be used for growth purpose of FMD virus in-vitro. Among these LFBK, BHK-21, bovine kidney cells, calf thyroid cells are included. According to previous research, LFBK cells are most sensitive cell line because of expression of appropriate $\alpha_V\beta_6$ integrin receptors. (Larocco *et al.*, 2013). Another study reveals that Bovine kidney cell line is 2.25 times more suitable than BHK- 21 cell line (Kamal *et al.*, 2014). Hence in this study we prefer BHK-21 cells because they were easily available at the center.

In a previous study, BHK-21 cells were cultured in tissue cell culture flasks and incubated at 37℃. After 48 hours' monolayers were observed. Confluent monolayers were infected with FMD virus serotype 'O' having TCID-50 $10^{4.5}$/ ml. Cells were showing cytopathic effects in the form of clumping and detachment after 48 hours. Nine calves of 6-8 months were vaccinated with titer having TCID-50 10^6/ ml and challenged with FMD virus having TCID-50 $10^{4.5}$/ ml. All animals survived the challenge showing no clinical disease. On the other side, unvaccinated animals show systemic reactions after challenge. Mouth lesions from 10 suspected animals were collected and adopted on BHK-21 cell line. No cytopathic effects were observed after 3 passages. This could be due to very low or no antigen present (Kitching *et al.*, 2007). In one another study, systemic reactions were observed after injecting cattle, pigs and mice with FMD virus having TCID-50 10^6/ ml (Fayyaz *et al.*, 2011). FMD virus titer was kept 10^6 /ml in this study. The biological titer of the virus in this study was evaluated by making monolayer in 96-well flat bottom plate. Cell count kept for monolayer was $1x10^5$ cells/ml. (Rahman *et al.*, 2006). Ten-fold dilution of virus was used in their study (Muhammad *et al.*, 2011).

A previous study was done for detection and serotyping of FMD virus present in a district of Bangladesh during 2013. Twelve samples of tongue epithelium (n=8) and interdigital tissue (n=4) were collected from suspected cattle. Inoculum was prepared later on, and inoculation was done in confluent BHK-21 cells for further propagation. Cytopathic effects were observed after 3 subsequent passages including breaking down of intracellular bridges, rounding and flattening of cells and finally cell death (almost 100%). These were indication of successful virus growth in the cells (Alam *et al.*, 2015).

In a previous study, three different serotypes of FMD virus (A, O and Asia-1) was adopted in BHK-21 cells for FMD trivalent master seed preparation. Samples were collected during 2011-2014 from different districts of Bangladesh. Parts of samples included tongue epithelium, milk, saliva and inter digital spaces of suspected animals. RNA extraction and RT-PCR was performed for detection and serotyping of FMD virus. Prepared inoculum of serotype A, O and Asia-1 were inoculated in BHK-21 cells and cytopathic effects were observed. CPE were characterized by rounding and flattening of cells, breakage of intracellular bridges, multinucleated giant cells and finally cell death. These all changes were confirmation of FMD virus propagation (Mahmud *et al.*, 2017).

FMD virus has very short infectious life cycle. Cytopathic effects begin to appear 4 to 6 hours post infection. In this study maximum time for recording CPEs were 48 hours. In a previous study maximum time period for recording CPE were 72 hours (Rahman *et al.*, 2006).

FMD virus can be easily grow on BHK-21 cells. Normal BHK-21 cells are somewhat elongated and shiny. Cytopathic effects in BHK-21 cell line is a proof of propagation of virus. Cytopathic effects included rounding and floating of cells, multinucleated giant cells visualizing at 4X and 10X under inverted microscope.

Conclusions and Recommendations

This study concludes that FMD virus serotype 'A' is more virulent as compare to serotype 'O' and 'Asia-1'. Although less virulence difference was found between serotype 'A' and 'O'.

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Novelty Statement

FMD Virus serotype 'A' causes more cytopathic effects as compare to serotype 'O' and 'Asia-1'. Although less virulence difference was found between serotype 'A' and 'O'.

Author's Contribution

Sania Subhan Qureshi: Designed and conducted re-

search.

Baitullah Khan: Helped in research methodology

Shahid Khan: Helped in samples

Hanif Ur Rahman: Results write-up

Muhammad Subhan Qureshi: Proofreading and final write-up of final manuscript.

Conflict of Interest

The author has declared no conflict of interest.

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