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RESEARCH ARTICLE

Physical Factors Affecting *in Vitro* Replication of Foot and Mouth Disease Virus (Serotype "O")

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ABSTRACT

Effect of physical factors (temperature, pH and UV light) on replicating ability of "O" type of Foot and Mouth Disease (FMD) virus on Baby Hamster Kidney (BHK) cell line was determined. The freshly grown FMD virus containing 10^6 units of tissue culture infective dose ($TCID_{50}$) was divided into aliquots. Each of the 9 virus aliquots was exposed to 37, 57 or 77°C for 15, 30 or 45 minutes, respectively. Each of the 5 virus aliquots was mixed with MEM-199 maintenance medium having pH 3, 5, 7, 9, or 11. Similarly, each of the 3 aliquots having 1 mm depth of the medium was exposed to ultraviolet light (252.7 nm wavelength: one foot distance) for 15, 30 or 45 minutes. Each of the virus aliquot exposed to either of the temperature, pH or ultraviolet light (UV) for either of the interaction time was inoculated to 8 wells of the 96-well cell culture plate containing complete monolayer of BHK cell line. One row of 8 wells served as virus control and other row of 8 wells served as control for monolayer of the BHK-21 cell line. The plates were incubated at 37°C for 48 hours. It was observed that temperature of 57 and 77°C inactivated the virus within 15 minutes. The virus when admixed in the MEM-199 maintenance medium having pH 3, 5, 9 or 11, of the medium inactivated the virus while pH 7 did not show any detrimental effect on its survival. The ultraviolet light for 15, 30 or 45 minutes showed undetectable effect on survival of the virus as either of the virus aliquot exposed to the UV light for either of the interaction time showed cytopathogenic effects (CPE). It was concluded that the temperature of 57°C or higher for 15 minutes, acidic pH (below 5) or basic pH (more than 9) may inactivate the FMD virus.

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INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious, viral and vesicular disease affecting cloven-hoofed animals. It is caused by a picorna virus (FMD virus) with an incubation period of 3-5 days and is characterized by fever and vesicles formation, followed by erosions on the tongue and lips, in the mouth, on the teats and between the hooves. Animals show reduced feed intake and lameness while pregnant animals may abort. The disease has high morbidity while mortality is only recorded in young animals on account of severe myocarditis. The disease causes heavy economic losses to the dairy industry and enormous consternation to the livestock farmers (Geering *et al.,* 2001; Ishimaru *et al.,* 2004; Khan, 2010).

In the infected animals, the virus is excreted in the saliva, nasal discharge, milk, semen, feces, urine and fluid from ruptured vesicles. The FMDV can survive at 4°C (39.2° F) on wool for approximately two months, and for two to three months in bovine feces or slurry (Bartley *et al*., 2002). It is a potential source of virus infection to the susceptible animals in the vicinity. The virus spreads by direct and indirect contact with contaminated material/individuals, as well as through the air.

The virus may be disseminated to other countries through trade of contaminated milk, meat, animal by-products and semen. Thus, the export of the animal by-products from the country having outbreaks of the FMD to the international market is badly affected. The virus in these products may be inactivated by exposure to acidic pH or temperature without deteriorating food quality.

Several environmental factors inactivate the FMD virus in animal products (Bartley *et al*., 2002). The present study was designed to see the effects of physical factors (temperature, pH and UV light) on *in vitro* replicating ability of "O" serotype of FMD virus.

MATERIALS AND METHODS

Virus Source and Biological Titration

Live culture of foot and mouth disease virus "O" serotype (FMDV "O") and a Roux Flask containing monolayer of Baby Hamster Kidney-21 (BHK-21) cell line was obtained from Department of Microbiology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

One liter of cell culture medium was prepared by reconstituting 16.8 gm of Medium199 (M199, MP; Biomedical, USA) and was used to culture confluent monolayer of BHK-21 cell line. The inoculum of FMDV "O" virus suspension was filtered through 0.2 µm pore sized syringe filter (Millipore, USA) and transferred to a pre sterilized glass vial.

When a complete monolayer of the BHK-21 cells was formed, then virus was inoculated in it, as described by Mitev *et al*. (1976). At 24 hours post incubation, each flask was observed for any cytopathic effect (CPE). At 48 hours post incubation, the carrel flask was passed through two regular freeze and thaw processes; 2 ml inoculum was harvested, filtered through a syringe filter and then inoculated into next flasks. All the flasks including control flask were incubated. When 90% CPE was observed, the pH of the virus culture was adjusted to 7.0 and the flasks were frozen at -20°C.

The BHK-21 cells in one cell culture plate were used for determining the biological titer (Tissue Culture Infective Dose-50: TCID-50) of the virus as described by Reed and Muench, (1938), which was 10^6. After calculating $TCID_{50}$, the virus was stored at 4°C and was later subjected to different physical factors.

Physical Factors

The FMD "O" virus (10^6 units of $TCID_{50}$) was subjected various temperature, pH and UV light for varying time intervals. The virus suspension of 10^6 units of $TCID_{50}$ was collected in three Eppendorf tubes of 2.5 ml capacity, each containing 2 ml of virus suspension. The tubes were exposed to 37, 57 and 77°C, respectively. The sample from each of the tubes was collected after 15, 30 and 45 minutes intervals.

The virus suspension of 10^6 units of $TCID_{50}$ was collected in five Eppendorf tubes of 2.5 ml capacity, each containing 2 ml of virus suspension. The pH of the tubes was adjusted as 3, 5, 7, 9 and 11, respectively by adding hydrochloric acid and sodium hydroxide. From each tube one half of the virus suspension was stored at 4°C and the second half at 37°C for 24 hrs. After the exposure time, pH of the each aliquot was brought to 7.2.

The virus suspension of 10^6 units of $TCID_{50}$ was dispersed in a sterilized petri-dish. This petri-dish was placed in a chamber having ultraviolet light source (252.7 nm). The distance between light source and petri-dish was 12 inches and the depth of the virus suspension was 1mm at the ambient temperature. From the petri-dish, virus inoculum was collected at 15, 30 and 45 minutes intervals.

Sample of 50 µl from each of the virus aliquot treated with either of the factor was then transferred to monolayer of BHK-21 cell line in 96 well cell culture plates to see its viability. Each well of the plate was having monolayer of the BHK-21 cell line with 200 µl of the growth medium. One column of 8 wells of the cell culture plate was kept as virus positive control in which the virus without exposure to any physical agent was inoculated. One column of the cell culture plate was kept as virus negative control in which no virus was inoculated. The plates were then incubated at 37°C for 48 hrs to observe the cytopathogenic effect (CPE).

RESULTS

Physical agents, including temperature, pH and ultraviolet light showed variable effects on the survival of FMD "O" virus. Effect of temperature on *in vitro* replication of FMD "O" virus is represented in Table-1. The virus suspension when exposed to heat treatment at 37°C was not inactivated after 15, 30 and 45 minutes of exposure, and showed CPE on to BHK cell line. However the virus suspension exposed to heat treatment at 57 and 77°C got inactivated after 15, 30 and 45 minutes of the interaction time. These results showed that temperature exposure of ≥ 57°C for 15 minutes can inactivate the virus.

Effects of pH treatment on survival of virus samples are shown in the same Table. The virus suspension when exposed to pH 3, 5, 9 and 11 got inactivated after 24 hours interaction time. Each of the pH treated virus suspension did not show any CPE. However, the virus exposed to pH 7 for 24 hours remained viable and showed CPE on the BHK cell line.

Effects of UV treatment (in darkness and light) on survival of virus have been shown in Table 1. The virus exposed to UV light either in light or darkness was not inactivated after interaction time of 15, 30 and 45 minutes and each of the virus sample showed CPE on the BHK-21 cell line. No CPE were observed in the virus negative control well as well as virus inactivated samples (Fig. 1), while the significant CPE were noted in virus positive as well as viable virus samples after treatment to physical factors (Fig. 2).

DISCUSSION

Temperature treatment of 37°C for 15, 30 and 45 minutes of interaction time had undetectable effect on survival of "O" serotype of FMD virus. The serotype of FMD virus grows more efficiently on cell culture at 29°C than at 37°C (Auti, 1980). However, infectivity titers of FMD "O" virus suspension in maintenance cell culture medium is reduced by 2 log units on storage at 37°C for 12 hours. It indicates that virus temperature interaction time at 37°C has inverse effect on the virus infectivity (Razdan *et al.,* 1996). Heat treatments at 57°C and 77°C for period of 15 min or longer time inactivated the virus. Heat treatment at 60°C inactivates FMD virus (Turner *et al.,* 2000; Kamolsiripichaiporn *et al*., 2007). Heating the virus suspension at 56°C for 60 minutes reduces its

Table 1: Effect of physical factors on survival of Foot and Mouth Disease Virus serotype "O"

	Temperature (°C)	Temperature exposure time (minutes)				
		15		30		45
Temperature	37	+++		+++		+++
	57	---		---		---
	77	---		---		---
	Positive control	+++		+++		+++
	Negative control	---		---		---
	Storage temperature	pH of the virus suspension				
		3	5	7	9	11
pH	4	---	---	+++	---	---
	37	---	---	+++	---	---
	Positive control	+++	+++	+++	+++	+++
	Negative control	---	---	---	---	---
	Ultrviolet light	UV Exposure				
	exposure time (min)	Light			Darkness	
Ultraviolet Light	15	+++			+++	
	30	+++			+++	
	45	+++			+++	
	Positive control	+++			+++	
	Negative control	---			---	

+++ means that the virus was not inactivated by the physical factors, and it induced cytopathogenic effect (CPE) in the cell line, while --- means that the virus did not produce CPE.

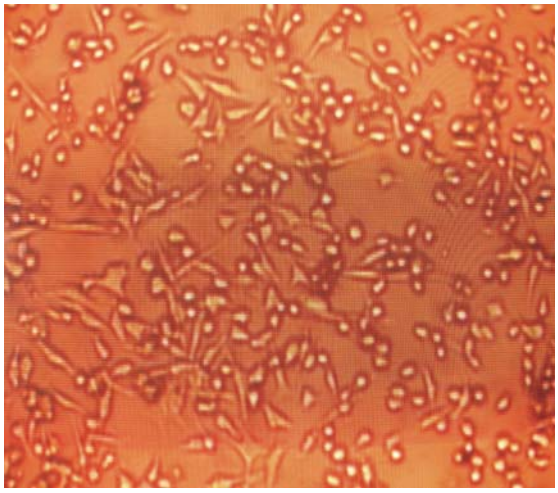

Fig. 1: Photomicrograph of BHK cells showing no CPE from virus negative control samples

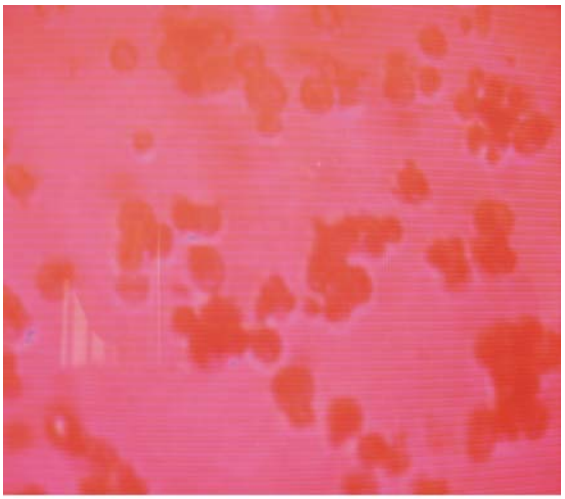

Fig. 2: Photomicrograph of BHK cells showing CPE from live FMDV

infectivity (Kariwa *et al.,* 2006). At high temperature, there might be destruction of virus receptors, which ultimately declined its infectivity. However, the virus suspension in cream or milk may survive to heat treatment at 72-95^{0}C for 18.6 to 36 seconds (Tomasula *et al*., 2007), while in the pig tissues it got inactivated at 50-70^{0}C for 15 days (Guan *et al*., 2010). Milk cream may protect virus from heat treatment. The "O", "C" and "Asia-1" serotypes of FMD virus may endure 54^{o}C for 1 hour (Nettleton *et al.,* 1982).

The FMD "O" virus suspension was inactivated when exposed to maintenance medium of cell culture having pH 3, 5, 9 or 11 while it retained its infectivity in the medium having pH 7.0. The picorna virus is pH labile and is inactivated in acidic pH or pH below 6 (Cavanagh *et al.,* 1978; Armstrong *et al.,* 2000; MacLachlan *et al.,* 2004). Presumably, viral capsid is hydrolyzed or dissociated at low pH (Newman and Brown, 1997). It means that spray of weak acids may mitigate the virus load on the FMD contaminated farms.

Third physical factor studied was the ultraviolet light, which had no effect on the virus and the virus produced cytopathic effect on BHK cell line. Our findings are in aligned with Hazem (2002) where Bacillus phages survived the UV exposure for 13 and 20 minutes. He concluded that the virus was resistant to UV light. Our results are in partial accordance with Darnell and Taylor (2006) where the presence of bovine serum albumen helped the corona virus to survive the UV treatment. These findings are not in aligned with Rabia *et al.* (1990) and Nuanualsuwan *et al.* (2008) who observed that vesicular stomatitis and FMDV were inactivated when exposed to UV light source, respectively.

It is concluded that the temperature of 57°C or higher for 15 minutes, acidic pH (below 5) or basic pH (more than 9) may inactivate the "O" type of FMD virus. The results of the study may help bio containment of the virus and to promulgate effective bio-security measures.

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