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In-vitro Susceptibility of FMD Virus Serotype A Endemic in KPK, Pakistan

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Abstract

Foot and Mouth Disease virus (FMDV) serotype A is considered to be antigenic ally diverse among various geographical locations leading to diverse patterns of resistance and sensitivity. This phenomenon is posing high risks to global trade. This is the first study to quantify the effects of different physical factors (temperature, pH, UV and gamma irradiation) affecting the infectivity of FMDV serotype A. The infectivity of virus was calculated in term of tissue culture infectivity dose-50 (TCID-50) and plaque forming units (PFU). Virus in suspension (6×10^6 particles/ml) was treated using Cobalt-60 source in BHK-21 cell line grown in 96 well plates. The virus showed complete inactivation on 3, 5, 9 and 11 pH and 10kGy dose of gamma irradiations.

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The results revealed that increase in temperature (both moist and dry heat) and UV light as well as increase in time of exposure with same dose of UV irradiations significantly decreased the infectivity of virus ($p<0.05$). These physical factors are a better alternate for virus inactivation than chemicals, which are toxic for the health and accumulate in the animal products. It is recommended that viral strains should be analyzed for their susceptibility to these physical methods. They could also be combined with thermal inactivation to further improve virus inactivity to obtain virus free products.

Keys words: FMD virus; temperature; pH; UV; gamma irradiation; TCID-50; plaque forming units.

1. Introduction

Foot and mouth disease (FMD) virus serotype A (FMDV-A) is antigenically diverse among various geographical locations at worldwide. This diversity is responsible for the variable sensitivity and resistance patterns among the regions. World Organization for Animal Health (OIE, L'organisation mondiale de la santé animale) has set forth the standards for FMDV inactivation. Phylogenetic analysis of variable protein (VP-I)coding sequences of FMD serotype A virus from Pakistan and Afghanistan during years 2002-2009 exhibits that two recognizably different genotypes (I and II) have four lineages that belongs to Asia topotypes, within serotype A [14, 19, 23, 24]. Although, A-Iran05 was the most frequently observed lineage of serotype A within the country but others were also found like A-Pak09. Serotype A-Iran05 lineage after analysis revealed seven distinct variants [12] and is still evolving with the rate of 1.2×10^{-2} per year [12]. A-Iran05^{AFG-07} and A-Iran05^{BAR-08} sub-lineages have been reported from Middle-East countries and still new variants of FMDV-A are expected outcomes from these high rate substitutions [12].

FMD is highly contagious disease for cattle and other cloven hoofed animals [1]. Moreover, FMDV is a threat to food security, as there might be possibility of residual virus in the food stuff prepared from animals having clinical or sub-clinical disease. For precautionary and safety measures and to meet the consumer's confidence in the foods of animal origin, there is an utmost need to evaluate *in vitro* the circulating strains within the geographical locations, before going for inactivation in bulk like industrial pasteurization of milk or meat processing. Once sensitivity and resistance pattern will be evaluated, it can be applied on industrial scale to resolve the global trade issues, for importation of livestock and their products usually banned from endemic countries.

Industrial level inactivation of virus usually involves various physical approaches including temperature treatment or exposure to ionizing irradiations (gamma rays) [8], so these methods could also be applied for viral inactivation *in vitro* [9]. FMDV could also be inactivated at acidic and basic pH [9]. However, it is quite difficult to store, handle and transport the hazardous chemicals, used in the above mentioned methods. Contrastingly, UV lamps are preferred because they have multiple benefits, since they require less space and operational cost than disinfection methods [2]. Apart from these, UV light could also be used for disinfection of contaminated ground water, feeds and foods and any contact surface as well as air [8]. However, the inactivation pattern by UV light may vary depending upon the composition of the medium treated wherein the virus is suspended e.g. serum protects the virus inactivation by UV light [7]. UV light primarily targets RNA of the

virus and leads to formation of pyrimidine dimmers that stops transcription and translation of RNA as well as replication of the virus [10]. In the same context, virus in suspension as well as in food products is irradiated by ionizing gamma radiations which completely inactivate the virus. Moreover, these radiations like UV light reduce the use of hazardous chemicals like formalin and aziridines etc which are retained as residues in the final products and cause allergic or sometimes toxic effects [3].

The current *in vitro* studies were designed to assess the growth and replication of FMDV-A in response to application of different variables i.e. temperature, pH, UV and gamma radiations. Due to possibility of appearance of new serotype A variants within its subtype as previously reported [12], this study was designed to investigate the susceptibility of FMDV-A virus endemic in Khyber Pakhtunkhwa (KPK) province of Pakistan. The study elaborates the procedures that are in commercial use or set by OIE, for inactivation of serotype A viruses circulating in KPK, Pakistan. Although inactivation data are available for serotype O viruses but inactivation pattern may vary among various serotypes, topotypes or lineages. Serotype A of FMDV used in this study is assumed to be representative of its field population and the inactivation data will be applied to commodities or biological products to combat the risk associated to FMDV in terms of food safety.

2. Materials and methods

The work was performed in BSL-II, Laboratory of Foot and Mouth Disease Research Center, Veterinary Research Institute, Peshawar, Pakistan

2.1 Source and culture of FMDV-A

The samples were collected from field out breaks. Samples were brought to the serology laboratory of FMD Research Center, Peshawar for serotyping by FMDV Antigen Detection ELISA (Serotyping of FMDV O, A, C, Asia-1, (Exotic Disease Research Station, National Institute of Animal Health, National Agriculture and Food Research Organization, Josuihoncho Kodaira, Tokyo, Japan) as previously described [15]. Briefly, the tissue samples were put in tissue grinder and after trituration, the lysates were collected in falcon tubes and passed through nitrocellulose membranes (0.2µm diameter) using syringe filters (Sartorius®, Gottingen, Germany. The filtrates were then collected in eppendorff tubes (Eppendorff, Hauppauge, NY) and serotyping was performed by using ELISA [15].

2.2 Propagation of BHK-21 cell line

A Roux flask (Corning®, USA) containing a monolayer of Baby Hamester-21 kidney cell line) was obtained from the cell culture laboratory of our research center. Cells were grown in GMEM medium (Glasgow MEM BHK 21, Biowest®, Riverside, MO, USA) supplemented with 5% Fetal Calf Serum (FCS), 100 U/ml of penicillin and 10 mg/ml of streptomycin (Invitrogen, Cergy-Pontoise, France) and maintained at 37ºC in an atmosphere of 5% CO_2 and 95% humidity, as previously described (Ali et al 2014). The virus was adapted to BHK-21 cells by serial passages (5th passage) on monolayer formed after 72 hours and was harvested and stored at -80^{o}C for further use. Virus adapted to cell culture at 4th passage on propagation with BHK-21 cell line. All the procedures were performed in Biosafety Cabinet-II/Class A2 according to safety rules and regulations of the

research center.

2.3 Cultivation of virus

For the growth of virus, maintenance media used was GMEM with 5% FCS with 1% antobiotics and 1% antimycotics. Virus suspension was filtered through 0.2µm filter (Millipore®, Billerica Massachusetts, USA) and poured into the roux flask containing monolayer and incubated at 37°C for 48h. Cytopathic Effects (CPEs) were observed at 48h post inoculation. The virus was harvested and filtered through 0.2µm syringe membrane filter. The resultant virus was poured into 50ml falcon tubes and aliquots were stored in eppendorff tubes of 2ml capacity for further use in the designed study. All the procedures were performed in Biosafety Cabinet-II/Class A2**.**

2.4 Tissue Culture Infectivity Dose-50

TCID-50 of the virus was calculated by Spearman and Karber method and it was 6×10^{6}. The resultant virus was stored at -80°C for further study.

2.4.1 Fixation of plates

After at least 48h CPEs started to appear. The wells were marked. The plate was fixed by adding 100µl of fixative i.e. Histo choice containing crystal violet or Methyl blue in each well of plate. The plate were kept overnight in refrigerator, washed gently under tape water and tapped on adsorbent paper and observed under microscope. TCID-50 was calculated using Spearman and Karber method by using the formula: Log of 50% end point = log of most concentrated dilution – (total no of cells with CPE – 0.5/ no. of wells per dilution) × of dilution factor

2. 5 FMDV inactivation by different physical factors

2. 5.1 Temperature (moist and dry heat)

The virus was treated at 37°C, 57°C and 77°C with dry heat (heat provided in hot air oven) and moist heat (heat provided in water bath) for 15, 30 and 45 min each.

2.5.2 UV irradiations

The samples were left in Biosafety Cabinet under UV rays of 258nm for 15, 30 and 45 min.

2.5.3 pH treatment

The pH of the media containing virus was adjusted to 3, 5, 7, 9, 11 by using 0.1N HCL and 0.1N NaOH solutions and incubated at 37°C for overnight.

2.5.4 Gamma irradiations

Virus was exposed to Co60 source with dose rate of 6.3146 kGy/hr. Samples in triplicate were given doses of gamma irradiation as 10kGy, 20kGy, 25kGy, 35kGy, 40kGy, 45kGy and 50kGy [20].

And then TCID-50 of the treated virus samples were calculated as discussed above.

2.6 Statistical Analysis

The data obtained were analyzed using SPSS software (version 16.0) by using one-way ANOVA multiple comparisons by Post-Hoc tests. $p<0.05$ was considered as statistically significant difference.

3. Results

1. Serotyping of FMDV-A virus:

Serotyping of FMDV was carried out by ELISA as describes previously [15] and the serotype of virus was identified as A with OD value 2.34.

2. Cell culture adaptation of the virus:

Virus was adapted to cell culture on BHK-21 cell line at 4^{th} passage level. This virus was grown in infectious media using Roux flasks in BHK-21 cell line.

3. Biological titrations of FMDV-A virus:

Virus was titrated biologically please mention this method sentence in materials and methods and the calculated TCID-50 value was 6×10^{6}.

4. Effect of heat on susceptibility of FMDV-A:

Significantly results were obtained when FMDV-A was exposed to various temperature intensities during different intervals of time. Significantly higher values of TCID-50 and PFU of virus were obtained when FMDV-A treatment temperature was increased from 57°C to 77°C and when time of exposure was increased from 30 to 45 min (Table 1, 2 and 3). Greater the temperature higher was the inactivation of the virus (Figure 2.1 and 2.2). Similarly, when exposure time was increased at a specified temperature, inactivation was also increased (Figure 3.1 and 3.2).

Moist heat was found to be more efficient at all temperatures in comparison to dry heat (Figure 4.1 and 4.2). Thus either by increasing the exposure time or temperature significantly affects the rate of inactivation of FMDV serotype A.

At elevated temperature lesser time of exposure may be required or by increasing the exposure time for a specified temperature, lesser amount of heat will be required.

Table 1: Effect of temperature on TCID-50 and PFU/ml when time remains constant

Dependent Variable	Effect of Temperature	Effect of Temperature	Sig.
TCID-50	37°C Moist heat	57°C Moist heat	.000
		77 °C Moist Heat	.000
	57°C Moist heat	37°C Moist heat	.000
		77 °C Moist Heat	.935
	77 °C Moist Heat	37°C Moist heat	.000
		57°C Moist heat	.935
PFU	37°C Moist heat	57°C Moist heat	.000
		77 °C Moist Heat	.000
	57°C Moist heat	37°C Moist heat	.000
		77 °C Moist Heat	.935
	77 °C Moist Heat	37°C Moist heat	.000
		57°C Moist heat	.935
TCID-50	37°C Dry heat	57°C Dry heat	.000
		77 °C Dry Heat	.000
	57°C Dry heat	37°C Dry heat	.000
		77 °C Dry Heat	.999
	77 °C Dry Heat	37°C Dry heat	.000
		57°C Dry heat	.999
PFU	37°C Dry heat	57°C Dry heat	.001
		77°C Dry Heat	.001
	57°C Dry heat	37°C Dry heat	.001
		77°C Dry Heat	1.000
	77°C Dry Heat	37°C Dry heat	.001
		57°C Dry heat	1.000

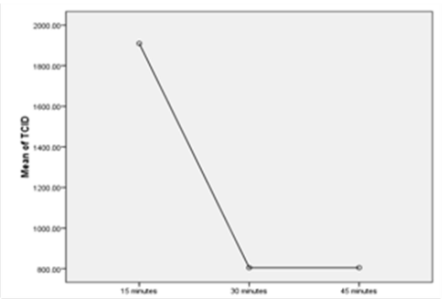

Figure 2.1 Effect of time with moist heat on TCID-50 of FMDV serotype and susceptibility pattern at different times (15 minutes, 30 minutes and 45 minutes)

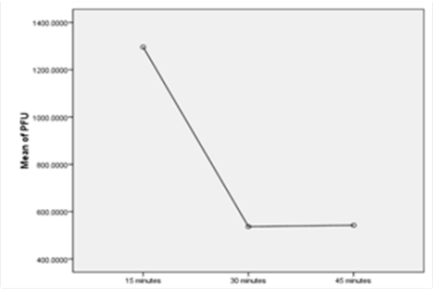

Figure 2.2 Effect of time with moist heat on PFU of FMDV serotype and susceptibility pattern at different times (15 minutes, 30 minutes and 45 minutes)

Table 2: Effect of exposure time on TCID-50 and PFU/ml of FMDV-A virus at constant temperature (moist heat) (37^{o}C, 57 ^{o}C, 77 ^{o}C)

Effect of Time of exposure on TCID-50 and PFU/ml of the virus Vs Moist Heat			
Dependent Variable	**Effect of Time**	**Effect of Time**	**Sig.**
TCID-50	15 minutes	30 minutes	.223
		45 minutes	.223
	30 minutes	15 minutes	.223
		45 minutes	1.000
	45 minutes	15 minutes	.223
		30 minutes	1.000
PFU	15 minutes	30 minutes	.216
		45 minutes	.219
	30 minutes	15 minutes	.216
		45 minutes	.993
	45 minutes	15 minutes	.219
		30 minutes	.993

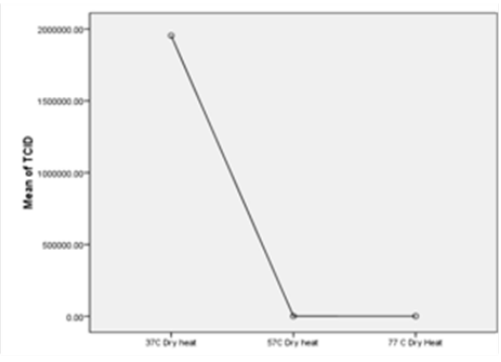

Figure 3.1 Effect of Dry Heat on TCID-50 of FMDV serotype and susceptibility pattern at different times (15 minutes, 30 minutes and 45 minutes).

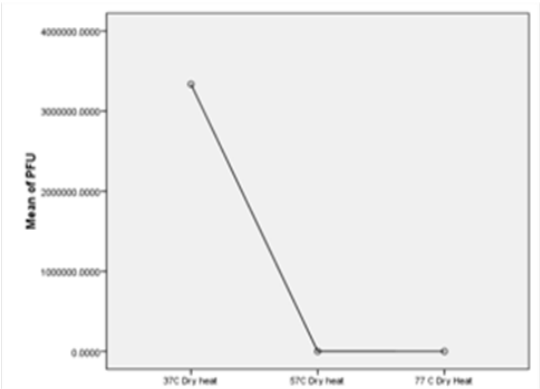

Figure 3.2 Effect of Dry Heat on PFU of FMDV serotype A prevalent in KPK and susceptibility pattern at (37°C 57°C and 77°C).

Table 3: Effect of exposure time on TCID-50 and PFU/ml of FMDV-A virus at constant temperature (dry heat) (37^{o}C, 57 ^{o}C, 77 ^{o}C)

Effect of Time of exposure on TCID-50 and PFU/ml of the virus Vs Dry Heat			
Dependent Variable	**Effect of Time**	**Effect of Time**	**Sig.**
TCID-50	15 minutes	30 minutes	.359
		45 minutes	.043
	30 minutes	15 minutes	.359
		45 minutes	.242
	45 minutes	15 minutes	.043
		30 minutes	.242
PFU	15 minutes	30 minutes	.145
		45 minutes	.030
	30 minutes	15 minutes	.145
		45 minutes	.431
	45 minutes	15 minutes	.030
		30 minutes	.431

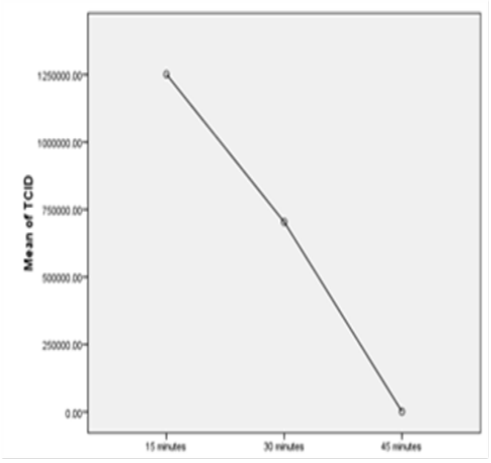

Figure.4.1 Effect of time with Dry Heat on TCID of FMDV serotype and susceptibility pattern at different times (15 minutes, 30 minutes and 45 minutes).

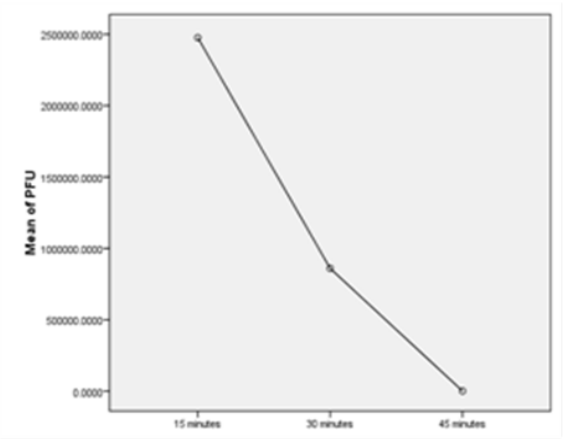

Figure 4.2 Effect of time with Dry heat on PFU of FMDV serotype and susceptibility pattern at different times (15 minutes, 30 minutes and 45 minutes)

5. Effect of UV on inactivation of FMDV-A:

FMDV-A was exposed to UV-light with 252nm wavelength in a biosafety cabinet for 15, 30 and 45 min. The treated virus revealed decrease in the infectious titer; however, virus remained viable and CPEs were observed. Post HOC tests revealed that UV-based inactivation of the virus is the function of time and intensity of UV-light source (Table 4, Figure 5.1).

Table 4: Effect of Time of Exposure vs UV-light on TCID-50 and PFU/ml

Effect of Time of exposure on TCID-50 and PFU/ml of the virus Vs UV-light			
Dependent Variable	**Effect of Time**	**Effect of Time**	**Sig.**
TCID-50	15 minutes	30 minutes	.031
		45 minutes	.000
	30 minutes	15 minutes	.031
		45 minutes	.006
	45 minutes	15 minutes	.000
		30 minutes	.006
PFU	15 minutes	30 minutes	.000
		45 minutes	.000
	30 minutes	15 minutes	.000
		45 minutes	.022
	45 minutes	15 minutes	.000
		30 minutes	.022

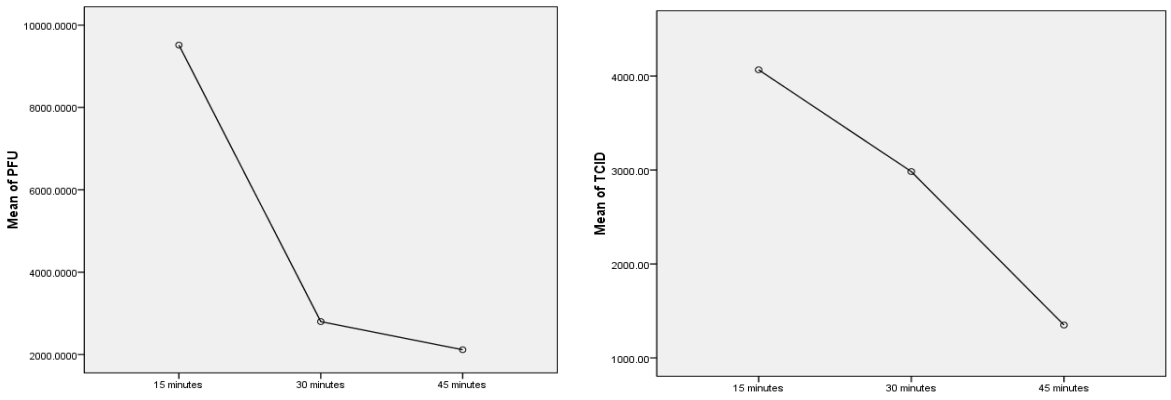

Figure 5.1: Effect of UV light vs exposure time on TCID-50 and PFU/ml of FMDV serotype A virus

6. Effect of acidic and basic pH on inactivation of FMDV-A:

Virus was exposed to acidic and basic pH as described in materials and methods section. Complete inactivation of the virus was observed at acidic and basic pH of 3, 5, 9 and 11 as confirmed by monolayer of BHK-21 cell line after staining with Histochoice. Zero TCID-50 and zero PFU were observed per ml; interestingly, virus at pH 7 remained viable, as indicated by CPEs and plaques in cell culture plates.

7. Effect of gamma irradiation on inactivation of FMDV-A:

FMDV-A virus susceptibility was checked after gamma irradiation with Co60 with dose rate of 6.3146kGy/hr.

Samples in triplicate were given doses of 10kGy, 20kGy, 25kGy, 35kGy, 40kGy, 45kGy and 50kGy. Results revealed complete inactivation of FMDV-A at all the tested doses; wherein, zero TCID-50 and zero PFU/ml were observed for the virus.

4. Discussion

FMDV-A is evolving continuously and there is a need to analyze newly emergent strains *in vitro* in order to ensure complete inactivation as well as FMDV free animal products. Inactivation of FMDV is not dependent on the physical factors, rather it depends on the serotype and strain of the virus and subtypes of a specific serotype [16]. Previous workers while working on HTST pasteurization of naturally infected milk, found alive FMDV in milk after HTST pasteurization and post pasteurization evaporation at 65°C for 1h, once inoculation was made into experimental animals [11, 4]. While, FMDV infectious titer dropped in milk when observed after Ultra High Pasteurization at 148°C for 2-5 sec in a continuous flow apparatus [6]. The difference in the results might be due to the diversity of strains as serotype A viruses are genetically diverse among all the FMDV. Other factors are also involved in the discrepancy of the results including variation in protocols or the form of heat employed as well as the dilution factor of the virus. During *in vivo* studies, variation among the results might be due to presence of proteins or fat contents in the treated milk e.g. milk protein may shield FMDV [22].

In our study, complete inactivation of the virus was observed at 57°C and 77°C for all the interaction temperatures ($p>0.005$) for both moist and dry heat, as well as inactivation *vs* exposure time (as depicted in Tables 1, 2 and 3). However, it was observed that at elevated temperature lesser exposure time is required for inactivation; while decreasing the temperature at increased exposure time might have the same effects on the virus inactivation. According to OIE Report (2007), meat should reach internal core temperature of 70°C for 30 min or longer to inactivate FMDV and 37°C for 8 days to inactivate FMDV in wool and hair. For the inactivation of FMDV in milk and cream for human consumption, ultra-high pasteurization is required at 132°C for one sec or double pasteurization of milk at 70°C for at least 15 sec is required [21].

Virus when exposed to UV light source at 252nm wavelength, decrease in the titer of virus was observed with longer exposure time however, the virus was found viable and CPEs were observed after inoculation in cell culture plates. Conversely, inactivation of FMDV was observed by UV light exposure by others [16,18]. Presence of serum in the media might protect the virus from UV inactivation as reported earlier [7]; thus both might have same UV sensitivity pattern. There is a possibility that higher radiation doses might be used for complete inactivation due to the presence of fats and proteins and varying pH ranges in animal products. Sensitivity pattern of FMDV-A strains were reported higher than serotype O viruses [13]. The variation among the results might due to UV source or exposure time that link to serum contents; possibly protect the virus from UV light inactivation. Gamma irradiations also reduce the use of chemicals for virus inactivation for vaccine production or for sterilization of food items and it is a preferred method for FMDV inactivation as virus could be completely inactivated with this method. However complete inactivation of virus was observed when irradiations dose attains to 40-44kGy [20]. Difference in the results might be due to the dose rate of the source of gamma irradiations and the possibility of production of small amount of heat while irradiating the sample. Irrespective of complete inactivation, irradiation treatment could be a method of choice, in order to prevent the

safety hazards associated with the storage of corrosive chemicals as well as their use. Moreover, it could be used for the disinfection of contaminated water, food, contact surfaces and air [2,8].

It was found from the results that FMDV-A virus included in our study completely inactivated at both acidic and basic pH except at at pH 7.0. Thus, animal skins or trophies should be soaked in acidic pH 3.0 or basic pH 11.0 for complete inactivation of FMDV. As, wool and hides are soaked in sodium hydroxide for virus inactivation [17]. However, still some acid resistant mutants have been reported [5]. But FMDV-A circulating in KPK province is susceptible to both acidic and basic pH. Thus, standards set by OIE could be effectively applied to the serotype A virus in KPK at industrial level. Furthermore, acid spray might also be used for sterilization of laboratory work surfaces contaminated with FMDV, as well as the vehicles used for the transport of animals, to prevent the spread of virus through transportation. This will also be a method of choice wherein UV lamps could not be applied.

5. Conclusions and recommendations

The use of chemicals is toxic for the health as they might accumulate in the animal products and thus could be transferred to humans consuming these products. Moreover, virus strain could be chemical resistant and might escape the inactivation method by these means. In these cases, instead of providing virus free food or animal products, they will act as reservoir of the virus. It is recommended that newly emergent strains should be analyzed for their susceptibility pattern to all of these physical variables set forth by the OIE. Gamma irradiation pattern of sensitivity should be checked for all the serotypes and strains of FMDV. It can be used in combination with other physical means of inactivation like thermal inactivation and may be a better alternative for the laborious processes already practiced in our industry for the preparation of FMDV free products. All these physical means of virus inactivation open new ways for in-depth studies of FMDV-A circulating in Pakistan, regarding industrial food processing and vaccine production.

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Conflict of interest

The authors confirm that there is no conflict of interest.

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