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Effect of different inactivants and preservatives on the stability of 146S fraction of foot-and-mouth diseases virus

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Abstract

Foot-and-mouth disease virus (FMDV) cripples livestock by imparting devastating effects to economy. A good vaccine is the key to stopping it, but due to instability of 146S of FMDV, it is becoming difficult. This is bad because only 146S can fight against disease and its dissociation ultimately leads to decreased potency of vaccine. This study aimed to preserve the integrity of 146S in vaccine using different inactivators and preservatives. Foot-and-mouth Disease virus type 'O' was propagated on baby hamster kidney 21 cell lines and inactivated using formalin or binary ethylenimine (BEI). Size exclusion high performance liquid chromatography analysis revealed minimal 146S loss after double inactivation with formalin and BEI. This inactivated virus was further formulated into oil-based vaccine with sodium thiomersal or chloroform as a preservative. Our findings demonstrated that chloroform outperformed thiomersal in maintaining shelf life of vaccine. This claims that the combined approach of double inactivation with formalin and BEI followed by chloroform as preservative offered a promising strategy for developing efficacious FMDV.

Introduction

Foot-and-Mouth Disease (FMD) is one of the Trans-boundary and highly infectious diseases of ungulates primarily cattle, swine, sheep, goats, pigs and also of wild animals.[1] Foot-and-mouth disease virus (FMDV) can exist in three different forms based on their composition, complete infectious particle 146S, empty capsid 75S and the degraded protein fragments 12S.[2]

Due to the rapid spread of FMDV different approaches are being used to control it. Cell culture-based vaccines prepared by chemical inactivation of viruses are approved to be the best strategy by World Organization for Animal Health (Former OIE).[3]

The 146S particle is a major immunogenic component in most of the subtypes. There is a direct relationship between the dose of 146S antigen and the rate, height and persistence of antibody titer in vaccinated animals after the primary shot and booster. That is why the quantification of 146S antigen became part of the protocol in many modern FMD vaccines.[4]

To quantify the concentration of 146S particles in the vaccine several methods have been developed. Traditionally, it is determined by the sucrose density gradient (SDG) method, however, the main disadvantage of SDG is that it requires specialized expertise and equipment mainly ultracentrifuge machine. To come up with these limitations, other methods are also being used including double antibody sandwich enzyme-linked immunosorbent assay, size-exclusion high-performance liquid chromatography and lateral flow immunoassays.[5]

The 146S of FMDV is extremely unstable. It can readily dissociate into stable degradation product *i.e.*, 12S. This proteolysis is irreversible and can occur by many factors e.g. mild heat and pH variations below 6.50.[2] This instability of the virus is believed to be due to its residues cluster (pka = 6.80) that is located close to the inter-pentameric interface and at an acidic pH it becomes protonated causing the disassembly of the capsid.[6] Most widely used FMDV vaccines are inactivated whole virus vaccines that are produced from cell culture.[7]

Complete inactivation is ensured because the presence of any live particle will cause severe economic losses. That is why the most critical step in FMDV vaccine production is virus inactivation and safety test.[1] Initially, formalin was used for the inactivation of the virus. Other inactivation

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methods include binary ethylenimine (BEI), beta-propiolactone, sodium chloride (NaCl) or phosphate and physical treatments including heat and ultraviolet irradiation.[8] However, the aziridine compounds, (BEI), resulted in the innocuous vaccines followed by first-order virus inactivation.[9] Nevertheless, all of these aziridine derivatives mostly act on nucleic acid with less cross-linking and fixing properties compared to formaldehyde. Further, it has been established that the thermo stability of FMD virus (146S) is found to differ considerably between serotypes and accordingly the inactivation of FMDV with aziridine compounds can reduce thermo stability.[10]

To improve the immunogenicity of the vaccine, the use of an adjuvant is highly recommended. It enhances the time for the provision of effective protection. Adjuvants of several types have been used in vaccines.[9]

Preserving vaccine against bacterial growth and maintaining their shelf life is critical. For this, a specific concentration of thiomersal sodium and chloroform is used *i.e.*, 0.01% v/v and 0.25% v/v, respectively.[11]

The purpose of this study was to identify the best inactivation and preservation conditions and it claimed that combined approach of double inactivation with formalin and BEI followed by chloroform.

Materials and Methods

Cells revival. Preserved baby hamster kidney cells obtained from Quality Operation Laboratory (QOL), University of Veterinary and Animal Sciences Lahore, Pakistan were revived. For this purpose, the preserved cell vials were thawed in a water bath at 37.00 ˚C for 2 min and decontaminated by spraying or dipping in 70.00% ethanol. After this, cells were centrifuged at 2,000 rpm for 4 - 5 min (Thermo Scientific, Waltham, USA) followed by the addition of chilled sterile media (Caisson, Smithfield, USA). The supernatant was discarded and the pellet was suspended in culture media (Caisson). Cells were counted by the hemocytometer and those suspended cells were shifted into a cell culture flask (Nest, Jiangsu, China). Growth media was added to the flask and incubated for 24 to 48 hr in an incubator (Memmert, Heidenheim, Germany) with 5.00% carbon dioxide at 37.00 ˚C. After incubation, the cells were observed for establishment of proper monolayer (80.00 - 100%) under an inverted microscope (BioBase, Shandong, China).[12]

Virus propagation. A BHK-21 flask with 80.00% mono-layer was taken and washed 2 - 3 times with phosphate buffered saline (PBS) and cells were inoculated with pure FMD type O virus taken from QOL (10.00 mL). Serum-free Glasgow Minimum Essential Media (30.00 mL; Caisson) was added to the flask. It was then wrapped in an aluminum foil and incubated at 37.00 ˚C for 24 hr. After incubation, morphological changes in cells also known as cytopathic effects (CPEs) were observed under microscope.[13]

Biological titration of live virus. A 96 well tissue culture plate seeded with BHK-21 cells (2.50×10^4 cells per well) was taken and 100 µL of maintenance media was added in each well. In a separate V-shaped plate, 2-fold virus dilution with media was prepared *i.e.*, 10^{-1} to 10^{-8}. Then 100 µL of each dilution was inoculated vertically up to the 10th well of each column. In the 11th column, 100 µL pure virus was added (virus control- from QOL, University of Veterinary and Animal Sciences) while in the 12th column, there was only media (cells control). The plate was then incubated with 5.00% CO_2 at 37.00 ˚C for 24 hr.[14]

PEGylation of virus. Polyethylene glycol (Daejung, Siheung, South Korea) solution (50.00 % w/v in 10.00 X TRIS buffer) was added to a virus (8.00 %v/v), stirred for 2 hr at room temperature and centrifuged at 3,000 – 3,500 rpm for 30 min at 4.00 - 6.00 ˚C. After discarding the supernatant, pellet was suspended with 3.30 mL PBS 0.011 M (pH 7.40). It was again centrifuged at 3,000 - 3,500 rpm for 30 min at 4.00 - 6.00 ˚C. This time supernatant was collected and stored at 4.00 ˚C. The PBS was added again in the pellet 2 - 3 times and all the supernatants were mixed (≈ 10.00 mL).[15]

Quantification of 146S. The 6.00 mL of the PEGylated virus sample was injected into the size exclusion high-performance liquid chromatography (SE-HPLC; Biologic LP chromatographic system 731-8350; BioRad, Hercules, USA) column by rotating the knob of the pump anti-clock-wise. The knob was then rested at its clockwise position with a buffer running at the speed of 0.375 mL per min. A sample was collected. The absorbance of the sample was then taken at 254 nm by multiplying it with 131.55 (extinction coefficient) and the concentration of 146S in µg per mL was obtained.[16,17]

Inactivation of virus. Chemical inactivation of FMDV with 0.04% formalin (Thermo Fisher Scientific) and 1.00 mM BEI (BEI, MP Biomedicals, Santa Ana, USA) was performed in cell culture flasks at different time and temperature intervals. Those flasks were incubated under specific given conditions without shaking. The BEI was prepared by dissolving 2.00% of BEA solution (0.10 M) in pre-warmed 0.20 N NaOH solution (Thermo Fisher Scientific). The BEA was converted into BEI in the alkaline condition for 1 hr at 37.00 ˚C and filtered using 0.20 µm filters.[18] A 20.00% stock solution of sodium bisulfite and sodium thiosulphate (Thermo Fisher Scientific)was prepared in double distilled water to neutralize formalin and BEI (2.00% final concentration), respectively.[19] After each inactivation period, a small sample was taken and inoculated on cells up to seven passages to confirm the inactivation of the virus. During inactivation, the optimum pH was maintained at 7.20 - 7.40. Formalin is relatively a slower inactivator, about 0.20 to 0.30 $\log_{10}$ per hr. This mainly happens due to its unfocused inactivation kinetics. It not only reacts with virus but also with other substances including proteins and amino acids. On the other hand, BEI

tend to inactivate at 0.50 to 1.00 log_{10} per hr. However, formalin and BEI together produce a synergistic effect with 2.50 to 3.50 logs per hr.[20]

Experimental strategy. Eleven groups were formed for inactivation with different factors *i.e.*, inactivates, time and temperature (Table 1). A virus without inactivation was run along the process at the same experimental conditions.

Table 1. Inactivation of foot-and-mouth disease virus with formalin and binary ethylenimine (BEI) at different incubation temperatures and times. After incubation neutralizer was added and a sample was taken at 30 min (for all the groups).

Groups	Subgroups	Inactivants	Temperature (°C)	Time (hr)
1	1A	Formalin	37.00	24
	1B	BEI	4.00	24
2	2A	Formalin	37.00	24
	2B	BEI	37.00	24
3	3A	BEI	37.00	24
	3B	Formalin	37.00	24
4	4A	BEI	4.00	24
	4B	Formalin	37.00	24
5	5A	Formalin	37.00	12
	4B	Formalin	37.00	24
6	6A	Formalin	37.00	12
	6B	BEI	4.00	12
7	7A	BEI	37.00	12
	7B	Formalin	37.00	12
8	8A	BEI	4.00	12
	8B	Formalin	37.00	12
9	-	Formalin	37.00	12 + 12
10	-	BEI	37.00	12 + 12
11	-	BEI*	26.00	24 + 24

* OIE Standard.

Confirmation of inactivation. Each sample was passaged seven times in a 25.00 cm^2 tissue culture flask with the full monolayer. In all flasks, 20.00 mL virus growth media and 0.50 mL of respective sample were added.[21]

146S estimation. The 146S concentration of each sample was measured again with SE-HPLC and spectrophotometer at 254 nm.

Vaccine preparation. The sample that maintained a maximum of 146S stability was used for vaccine production. A whole culture oil-based vaccine (60:40) was prepared using different preservatives *i.e.*, chloroform (0.05%) and thiomersal sodium (0.01%). Montanide ISO 206 (Seppic, Fairfield, USA) oil and antigen were mixed in 60:40 ratios. 60.00% was oil phase containing montanide oil and 40.00% of aqueous phase containing FMDV antigen in a beaker. The mixture was stirred at high speed and then vortexed to ensure that there was no phase separation.[22] That vaccine was then divided into four aliquots (1st day, 10th day, 20th day, 30th day) and 146S was calculated thought out that time period to check ultimate effect of preservatives on integrity of 146S.

Quality control testing of vaccine (antigen elution and 146S estimation). Antigen elution was carried out using benzyl alcohol (Jiangling, Wuhan, China). About 13.50 mL of vaccine sample was collected in a 50.00 mL centrifuge tube followed by the addition of 1:10th volume of benzyl alcohol slowly along the wall. To break the oil-in-water emulsion, it was vortexed for 5 min followed by centrifugation at 12,000 *g* for 5 min. Viral antigen containing the aqueous phase was collected.[7] Antigen was eluted at 1st, 10th, 20th, and 30th day. The 146S of eluted antigen was measured with SE-HPLC and spectrophotometer at 254 nm.

Statistical analysis. To statistically evaluate the results, we performed one way ANOVA test and calculated *p*-value. The concentration of 146S in different groups was compared by analysis of variance (one-way ANOVA) using Minitab (version 17.1; Minitab Inc., Boston, USA) to evaluate the significant level and *p*-value was calculated. If ANOVA showing $p \leq 0.05$, then the concentration of ANOVA in that group was significant. Group 6 and 9 showed *p*-value 0.001 so these were most stable group with maximum preservation of 146S, while 1, 2, 4 and 7 with *p*-value greater than 0.05 were non-significant groups.

Results

To maintain the stability of 146S content of FMDV type O, different inactivation and preservation strategies were implemented. The 146S was measured before and after the inactivation of the virus to check the effect of inactivation on virus stability. The oil-based vaccine was prepared. Then, again 146S of eluted antigen was quantified in different timelines. The following summarizes the results of tissue culture infective dose 50 obtained (Table 2).

Table 2. Biological titer of virus in terms of tissue culture infective dose 50 of virus.

Dilutions	Wells with CPEs (%)	Wells with no CPEs (%)
10^{-1}	8.00 (100)	0.00 (0.00)
10^{-2}	8.00 (100)	0.00 (0.00)
10^{-3}	8.00 (100)	0.00 (0.00)
10^{-4}	7.00 (87.50)	1.00 (12.50)
10^{-5}	7.00 (87.50)	1.00 (12.50)
10^{-6}	6.00 (75.00)	2.00 (25.00)
10^{-7}	5.00 (62.50)	3.00 (37.50)
10^{-8}	0.00 (0.00)	8.00 (100)
10^{-9}	0.00 (0.00)	8.00 (100)
10^{-10}	0.00 (0.00)	8.00 (100)

CPEs: Cytopathic effects.
The titer of the experimental foot-and-mouth disease virus type O was $10^{8.2}$ $TCID_{50}$ mL^{-1}.

146S of experimental virus. The concentration of 146S in the experimental virus was *i.e.*, 30.78 µg mL^{-1} measured by SE-HPLC and spectrophotometer.

Inactivation and inequity testing of virus. The inactivation test showed that there were no live viruses in BEI, however, formalin after some passages reverted to an infectious form.

146S estimation. Following different methods of inactivation, a concentration of 146S was calculated through a spectrophotometer (Table 3). The concentration of 146S before inactivation treatment was 30.78 µg mL^{-1}. Experimental results indicated that there was a minimum loss of 146S when formalin and BEI were used in combination for 24 hr at 37 and 4.00 ˚C, respectively (group 6). The concentration of 146S in group 6 was 20.20 µg mL^{-1}. The next more stable groups were formalin and BEI (groups 9 and 10) with 45.00 and 59.00% loss of 146S, respectively. The 1st four groups showed relatively minimum stability of immunogenic particles (Table 3).

The 146S of control viruses. The 146S of control virus (kept under the same conditions as the experimental virus but not treated with inactivates) were also measured. Without any inactivates treatment at a relative time and temperature *i.e.*, (24 hr, 37.00 ˚C), (24 hr, 4.00 ˚C), (12 hr, 37.00 ˚C), FMDV type O indicated the stability of 146S at one specific point *i.e.*, about 15.00 µg mL^{-1}. The rate of 146S dissociation was up to 50.00%. However, a sample (12 hr, 4.00 ˚C) showed an odd behavior with a 70.00% loss of 146S.

Vaccine preparation. Group 6, which maintained maximum 146S stability *i.e.*, 20.20 µg mL^{-1}, was used to prepare oil-based vaccine. Antigenic mass per dose was 15.00 µg mL^{-1}. 146S of eluted antigen was measured again. The result of the storage stability of vaccines with different treatments is summarized in Table 4. It is observed that upon storage of the vaccine for 1 month, 146S was shown to be more stable with chloroform *i.e.*, 6.00 to 38.00% of 146S was dissociated throughout the process. Instead, the loss of 146S following thiomersal was significantly higher. The dissociation rate of 146S content in the control group was found to be similar to that of chloroform. However, on day 30 a sudden drop in stability was observed in the control group *i.e.*, 52.00% loss of 146S.

Discussion

In the current study, FMDV was titrated according to the Reed and Munch method.[23] These results were also supported by others[24] who used the same method for virus titration to prepare the FMD vaccine.

In the previous study to purify the inactivated FMDV from crude supernatant, SE-HPLC was used. The entire process took 3.50 hr to complete with 75.00% final FMD recovery. The purity of the final product was above 98.00%.[25] In this study, after a purified 146S fraction of the sample was obtained through SE-HPLC, the concentration of 146S was calculated by spectrophotometer method.[17]

Inactivation is a crucial step in the preparation of an inactivated viral vaccine. Before addressing the question of potency, the innocence of the vaccine must be ensured. As part of this study, double inactivation was used because formalin alone can result in improper inactivation ultimately leading to vaccine-associated outbreaks. Therefore, to develop a quick and reliable virus inactivation, formalin and BEI were used together. This approach not only enhanced inactivation efficiency but also increased the stability of 146S content of FMDV.

The result of this study showed that when the virus was inactivated with formalin for 24 hr (group 9), then, the concentration of 146S was higher than that of BEI (group 10) for the same time *i.e.*, 16.80 and 12.50 µg mL^{-1}, respectively (Table 3). Group 5 and group 6 showed maximum stability of 146S *i.e.*, 13.61 and 20.20 µg mL^{-1}, respectively. These results were supported by Rowlands *et al.*[26] according to which prior inactivation with formalin stabilized the antigenic mass of the virus due to its cross-linking ability. In the latter group, BEI was processed at 37.00 ˚C which caused more dissociation of 146S, because BEI worked much better below 20.00 ˚C and boiled above 20.00 ˚C.[27] Values of groups 7 and 8 were also

Table 3. Concentrations of 146S in samples in different groups.

Groups	Inactivants	Time (hr)	Temperatures (˚C)	OD 254	Dilution factor	146S (µg mL^{-1})	Loss of 146S (%)	*p*-value
1	Form + BEI	24 + 24	37.00, 4.00	0.061	7.50	9.99 ± 0.21	68.00	0.22
2	Form + BEI	24 + 24	37.00, 37.00	0.073	7.50	11.9 ± 0.20	61.00	0.48
3	BEI + Form	24 + 24	37.00, 37.00	0.065	7.80	9.16 ± 0.12	63.00	0.07
4	BEI + Form	24 + 24	4.00, 37.00	0.069	7.50	8.74 ± 0.12	91.00	0.33
5	Form + BEI	12 + 12	37.00, 37.00	0.083	7.50	13.61 ± 0.99	56.00	0.0007
6	Form + BEI	12 + 12	37.00, 4.00	0.112	8.10	20.20 ± 1.48	34.00	0.001
7	BEI + Form	12 + 12	37.00, 37.00	0.052	7.50	8.55 ± 0.65	72.00	0.008
8	BEI + Form	12 + 12	4.00, 37.00	0.072	7.60	11.99 ± 1.40	61.00	0.131
9	Form	12 + 12	37.00	0.098	7.80	16.80 ± 0.90	45.00	0.001
10	BEI	12 + 12	4.00	0.085	7.90	12.50 ± 0.80	59.00	0.04
11	BEI (OIE)	24 + 24	26.00	0.071	7.50	11.60 ± 0.70	62.00	-

Form: formalin, BEI: binary ethylenimine.
A *p*-value of ≤ 0.05 is significant and *p*-value of > 0.05 is non-significant.

Table 4. The 146S concentrations (µg mL^{-1}; %) in vaccines at different time and aliquots.

Vaccine Aliquots	Preservative	Day 1	Day 10	Day 20	Day 30
Aliquot 1	Chloroform (0.05%)	14.10 (6.00)	12.21 (19.00)	10.58 (29.00)	9.20 (38.00)
Aliquot 2	Thiomersal sodium (0.01%)	12.70 (15.00)	9.77 (34.00)	9.00 (40.00)	7.04 (53.00)
Aliquot 3	No preservative	14.50 (3.30)	12.00 (20.00)	10.68 (28.00)	7.08 (52.00)

in accordance to the previous studies. In both groups prior treatment was done with BEI causing dissociation of 146S. Rapid dissociation of 146S took place were stored at 37.00 ˚C for a long time. The 1st 4 groups being stored at 37.00 ˚C for 24 hr fell under this theory.[28]

Additions of preservatives in a vaccine, to ensure its sterility, also affect the immunogenicity of the vaccine. According to a study, the presence of thiomersal sodium in the vaccine resulted in a significant loss of 146S throughout the process.[29] In another study, it was indicated that chloroform was the best substitution for thiomersal sodium. It maintained 146S content of virus and also had antifungal and antimicrobial properties.[11]

In the present study, an experiment was conducted using 2 different preservatives i.e., thiomersal and chloroform (OIE). Gradual 146S loss occurred starting from 15.00% at zero-day up to 53.00% at 30 days, in the case of sodium thiomersal. The 146S dissociation rate of the control group was quite similar to the chloroform group, however, on day 30 a sudden decrease in 146S concentration was observed. This loss in concentration could be due to chemical modification and proteolysis of virus.[7] On the other hand, preservation with chloroform maintained maximum 146S stability with 38.00% loss.

This research project aimed to explore and find out the inactivants as well as preservatives that could help minimize the loss of 146S fraction of the FMD virus. It was concluded from this research that a double inactivation procedure with formalin at 37.00 ˚C for 12 hr followed by inactivation of virus stock with BEI at 4.00˚C gave a better yield with minimum loss.

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Conflict of interest

The author declares that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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