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Comparison of different inactivation methods on the stability of Indian vaccine strains of foot and mouth disease virus

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ABSTRACT

In this study, the efficiency of binary ethyleneimine (BEI) in combination with formaldehyde (FA) and glutaraldehyde (GTA) in inactivating the Indian FMDV vaccine strains is compared. The acceptable safety of virus inactivation was faster and the inactivation rates were increased many-folds with combination of inactivants than BEI alone. FMDV A was inactivated rapidly than the other two serotypes with BEI + FA combination. Inactivation plots were linear for all the serotypes irrespective of inactivation process. Further, the integrity studies on 146S using serotype specific ELISA indicated no significant change in the antigenic mass of all the serotypes throughout the inactivation process. However, the loss of 146S antigen occurred in the subsequent steps of downstream processing. Further, the studies on intactness of viral RNA using real time PCR indicated the amplification of 1D gene sequences in all the preparations of timed samples irrespective of serotypes/inactivation process. Further, inactivated virus preparation (146S) was more stable at lower temperatures for all the serotypes/inactivation process. Among the combinations of inactivants, BEI + FA out performed compared to BEI + GTA and BEI in terms of inactivation rates, 146S yield and its storage stability, irrespective of the serotypes.
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1. Introduction

Maintaining the integrity of 146S particle of foot and mouth disease virus (FMDV) during FMD vaccine production and in final vaccine formulation is vital in inducing neutralizing antibodies and protection against the disease. One of the critical steps affecting the integrity of 146S particle during FMD vaccine production is inactivation of the virus. Historically, formaldehyde (FA) has been used to inactivate the FMDV [1]. However, the work of Sven Gard [2] demonstrated that the inactivation of polio virus with formaldehyde was non-linear or first order reaction. Similar results were obtained for the inactivation of FMDV by FA also [3–6], which may lead to improper inactivation and vaccine associated outbreaks [7,8]. Owing to its demerits, the focus has been shifted towards aziridins such as ethyl-ethylenimine (EEI), ethylenimine (EI), *N*-acetyl-ethylenimine (AEI), which were found to have high reliability of inactivation of various DNA and RNA viruses including FMD, bluetongue, rabies and others as reviewed [9]. However, AEI is highly toxic and unstable at 20 °C and above [9]. Another derivate, binary-ethylenimine (BEI), has the advantages of ease of preparation and handling and is now widely used for inactivation of FMDV and other viruses of veterinary importance. BEI inactivates FMDV more rapidly with linear inactivation kinetics [9–11]. Nevertheless, all of these aziridine derivatives mostly act on nucleic acid with less cross-linking [5] and fixing properties [12] compared to FA.

To develop a rapid and reliable inactivation, Barteling and Cassim (2004) [5] used the combined inactivation using both FA and BE, and this approach had been found to augment inactivation rates and increased yield of 146S of FMDV SAT strains. Moreover, FA cross-links the viral proteins and stabilizes the antigen [13], which makes easy accessibility of nucleic acid to BEI and faster inactivation of the virus [5]. Further, it has been established that the thermostability of FMD virus (146S) is found to differ considerably between serotypes and accordingly the inactivation of FMDV with aziridine compounds can reduce thermostability [14,15]. Glutaraldehyde, a dialdehyde, used as an amine-reactive homo-bifunctional cross-linker and reacts with amines, amides, and thiol groups in proteins [16]. Glutaraldehyde has also been used as an inactivating agent in the preparation of pertussis vaccine [17], Aujeszky's disease vaccine [18], polio vaccine and bacterial toxins [19]. The

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glutaraldehyde has shown to increase the thermostability of FMDV by increasing the diameter of the 146S particle and makes them permeable to BEI [14,19], thereby limiting the proteolytic destruction of 146S antigen and increases the antigen yield [5,6,20].

In view of these facts, the study was designed to evaluate the combination inactivation approach for rapid and safe inactivation using FA and GTA in combination with BEI for inactivation of Indian vaccine strains of FMDV.

2. Materials and methods

2.1. Cell lines and viruses

Baby hamster kidney (BHK21 clone 13) cell line and Indian vaccine strains of FMDV serotypes (P5) namely, O (O/IND/R2/75), A (A/IND/40/2000) and Asia 1 (Asia1/IND/63/1972) maintained at the FMD Research Centre, IVRI, Bengaluru was used.

2.2. Inactivation of FMDV with BEI, BEI + FA and BEI + GTA

Each FMDV serotype was inactivated with 1 mM binary ethyleneimine(BEI) at 37 °C for 24 h [21]. In brief, 1 mM BEI was prepared by dissolving 2-bromoethylamine hydrobromide (BEA) in 0.7% sodium hydroxide (NaOH). BEA gets converted in to "BEI" in the alkaline condition in 1 h at 37 °C and filter sterilized using 0.2 μm filters. Then the BEI was added to virus suspension slowly with gentle agitation at 37 °C. The inactivation was carried out at 37 °C with constant agitation for 24 h with an intermittent change of the flask at 12 h interval. Similarly, each serotype of FMDV was inactivated with the combination of formaldehyde (FA) and BEI at 30 °C for 24 h [5,20]. In short, the BEI and FA were added simultaneously at a final concentration of 1 mM and 0.04% respectively, to viral suspension. The inactivation was carried out by maintaining pH at 7.5–7.6, with constant agitation and intermittent change of the flask at 12 h interval to mimic the two vessel approach as it is carried out in large scale fermenter production. Thus allowing any virus in the air space above the liquid (less than 10% of total flask capacity) to come into contact with the inactivant. Likewise, the inactivation of each serotype of FMD virus with GTA and BEI combination was carried out at 37 °C for 24 h with constant agitation on a magnetic stirrer [22]. BEI prepared as aforementioned was added to the virus suspension at a final concentration of 1 mM followed by the addition of GTA at 0.005% and maintaining the pH at 7.2–7.4.

2.3. Inactivation kinetics and innocuity testing

To study the inactivation kinetics of FMDV serotype O, A and Asia 1, timed series samples (1mL/serotype/inactivation process) with different combinations of the inactivation process were collected at 20' (minutes), 40′, 60′, 80′, 100′, 120′, 140′, 160′, 180′ followed by every hr up to 8hr and 16 and 24hr post inactivation (hpi). At the end, BEI was neutralized with 10% 1 M sodium thiosulfate (STS) to a final concentration of 2%. The tissue culture supernatant of timed samples were neutralized with 10% 1 M sodium thiosulfate, serially 10-fold diluted and subjected to virus titration in 96 x well tissue culture plates to deduce the inactivation kinetics. Then, first 3 samples belonging to time period, where no CPE was observed in virus titration plates was inoculated onto duplicate tissue culture flask containing BHK21 cell and examined for cytopathic effect (CPE), if any, for three serial passages. The timed sample which was not showing CPE even after three blind passages was taken as the cut-off for deciding the inactivation point against each serotype with different combinations of inactivation process.

2.4. Stability studies and quantification of146S of FMDV

Inactivated virus samples of three serotypes of FMDV were evaluated for their storage stability at 4 °C, −20 °C and −80 °C after one month period. After PEG precipitation each sample was assayed for its 146S content [23] in the beginning and at the end of the storage period and expressed in μg/mL to evaluate the loss due to inactivation/concentration procedure and after storage. Briefly, quantification of 146S particles of FMDV was carried out by adding the aliquots of virus in separate tubes having 4 mL each of 1.42 and 1.38 g/mL CsCl gradient, slowly to obviate admixing. Ultracentrifugation was carried out in a swinging bucket rotor (AH629) OTD 75B Sorvall WX ultra series centrifuge (M/s Thermo scientific, USA) at 27,000 rpm/4 °C for 4 h. The gradient was fractionated from the bottom of the tube using a long siphon tube. Six fractions were collected and absorbance of the fractions was measured at 259 and 239 nm wavelengths. The fraction with a ratio of 1.3–1.4 at 259/239 was selected and 146S concentrations were calculated at an extinction coefficient (E1% 259) of 76.

2.5. Quantification of antigen in timed samples by sandwich ELISA

The antigen concentration of timed samples (FMDV type O, A and Asia 1) collected during different inactivation processes were checked by sandwich ELISA [11] using standard concentrations (1, 0.1, 0.01 and 0.001 μg/mL) of 146S of each FMDV serotype. The standard curves were established for each serotype/inactivation method using standard concentrations against respective OD values and the unknown antigen concentrations in timed samples were extrapolated. Live virus, inactivated positive antigen controls for FMDV O, A and Asia1, healthy BHK-21 cell suspension and blank were also maintained throughout the test.

2.6. Detection of FMDV nucleic acid sequences in inactivated preparations

2.6.1. Oligonucleotide primers

The primers specific for the conserved region (1D) of VP1 gene of FMDV (DHP9, DHP13, DHP15) [24] and common reverse primer NK61 [25] were commercially synthesized (M/s Eurofins, Bengaluru). The primers were reconstituted at a stock concentration of 100 pmol/μL in nuclease free water (NFW). Primers at a working concentration of 10 pmol/μL in sterile NFW were used for SYBR-Green® based RT-PCR. The details of the primers used are depicted in Table 1.

2.6.2. RNA isolation

RNA isolation from inactivated timed samples was done [26] using "TRI-Reagent" (Cat #T9424, M/s Sigma Aldrich, St. Louis, USA). Briefly, 250 μL of sample was taken in 2.0 mL microcentrifuge tube (M/s Eppendorf) and 750 μL of TRI-Reagent was added. The suspension was mixed thoroughly and kept at room temperature for 15 min. Thereafter, the suspension was centrifuged at 12,000x*g* for 15 min at 4 °C. The clear 0.5 mL supernatant was collected in a 1.5 mL centrifuge tube and mixed with 0.5 mL isopropanol to precipitate the RNA. The suspension was incubated at room temperature for 10 min, centrifuged at 12,000x*g* for 10 min at 4 °C and then supernatant was discarded. The RNA pellet was washed with 700 μL of 70% ethanol, air-dried for 15 min, dissolved in 25μLNFW, labelled and stored at −20 °C till use.

2.6.3. Reverse transcription and PCR amplification

Reverse transcription was carried out in a 500 μL PCR tube by adding 4 μL of the extracted RNA and 1 μL of oligo-dT primer. The RNA-primer mix was incubated at 70 °C for 10 min and snap-chilled

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