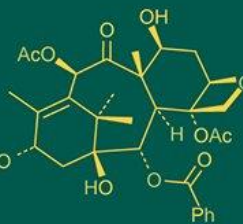
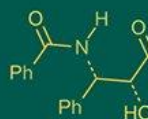


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Effect of Biochemical stabilizers on thermostability of live foot and mouth disease virus

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Abstract

Under industrial settings, bulk produced foot and mouth disease virus (FMD) is often stored before inactivation. The capsid of FMDV, which harbours immunogenic antigens, easily undergoes disassembly even with a mild increase in the storage temperature, thus decreasing the yield of 146S antigen. Thus, we hypothesized that the addition of sugar stabilizers into the viral suspension will prevent the disintegration of viral capsid. Accordingly, we screened the stabilizing effects of 0, 5, 10 and 15% (w/v) concentrations each of glycerol, sucrose, mannitol, sorbitol, and trehalose on FMDV serotype O following exposure to 37 °C, room temperature and 4 °C in terms of virus titer (Log₁₀ TCID₅₀/mL). The results indicated that addition of any of the above stabilizer at 5% prevented the loss of titer of FMDV serotype O at all the three tested temperatures as compared with stabilizer-free control group. Of the five stabilizers, glycerol and sucrose at 5% were chosen for further studies not only because of the kosmotropic properties but also due to the ease of availability. The experiment was repeated with glycerol or sucrose for the FMDV serotypes O, A, and Asia1. The results indicated that addition of either glycerol or sucrose at 5% prevented the loss of virus titer till 16 h post-exposure to 37 °C for all the three serotypes of FMDV. At 4 °C, the reduction in the virus titer was ~1.1.2 log₁₀ at 7 week post-exposure, while it was >2 log₁₀ for the no stabilizer group for all the 3 serotypes of FMDV. It was concluded that addition of 5% of either glycerol or sucrose to the FMDV serotypes O, A, and Asia1 prevented the loss of virus titer upon exposure to 37 °C and 4 °C for 16 h and 7 weeks, respectively.

Keywords: FMDV, Thermostability, stabilizers, glycerol, sucrose

Introduction

Foot-and-mouth disease (FMD) is an OIE notified list 'A' transboundary disease affecting the growth rate [1], milk production [2, 3], reproduction [4, 5] and global animal trade [6]. Considering the economic importance, India aggressively implements FMD control program through biannual vaccination of cattle with chemically inactivated FMD virus (146S) containing 10 µg of serotype O, 5 µg each of serotype A and Asia1 that is adjuvanted in oil emulsion. However, there is a vast gap between the requirement of about 800 million doses/annum [7] vis-à-vis the current production capacity of about 500 million doses. To bridge the gap, many ways and means are being explored that includes genetic engineering of the vaccine strains of the FMDV by inducing targeted mutations and addition of stabilizing agents in the vaccine. Minimizing the loss of 146S during the production and storage by the addition of stabilizing agents is a feasible approach that can be easily introduced into the commercial manufacturing units. Thermolability of the FMDV virions and 146S are the main reasons for the antigenic loss in the final vaccine formulation. For the most part, mild acidic pH below 7.0 in the surroundings and elevated temperature above 30 °C promote irreversible disintegration of intact 146S particle into a firm 12S fragments resulting in reduced protective antibody response [8]. Further, slow deterioration of 146S during the cold storage results in the reduced shelf life of an otherwise effective vaccine [9]. Maintaining the cold chain from the point of production to dispensation accounts for ~80% cost of the vaccination campaign [10] and is a major obstacle in developing countries due to

poor infrastructure facilities and logistic limitations^[11]. In the process of FMD vaccine preparation, the bulk produced virus is chemically inactivated with 3 mM binary ethyleneimine at 37 °C for 24 h. Exposure of the FMDV particles to body temperature for 24 h results in considerable loss of 146S antigen content and reduces the yield. For instance, when one liter of BHK21 suspension cell culture infected with FMDV having a titer of 7.0-7.5 log₁₀ TCID₅₀/mL is maintained for at 37 °C for 16 h, the expected antigenic yield of 146S is 5-10 µg/mL. However, in real world settings, the observed antigenic yield is 1.0-1.5 µg/mL resulting in 70% loss (personal communication), as the antigenic yield is influenced by the pH of the culture, temperature and hours post-infection. It is also reported that the yield of 146S antigen (µg/mL) was 2.87 and 2.59 for serotype O and A, respectively given the initial titer of 8.0 log₁₀ TCID₅₀/mL resulting in a loss of about 43%^[12]. Addition of stabilizers to the culture medium is a potential way to minimize the loss of live FMDV particles so that antigenic yield can be improved upon chemical inactivation^[13].

Stabilizers, also called as excipients, are additives that maintain the efficacy of a vaccine despite exposure to heat, light, and other adverse conditions^[14]. The choice of stabilizer is determined by the structure and properties of the viral antigen(s) in the vaccine^[15]. Carbohydrates such as lactose, sucrose, maltose, and trehalose, sugar alcohols such as mannitol and sorbitol are commonly used as stabilizers in the vaccine^[16]. Disaccharides confer more stability than the monosaccharides and non-reducing disaccharides are preferred as the reducing disaccharides compromise stability due to Maillard reactions^[16]. For instance, combination of sucrose 30% and bovine serum albumin 1% prevented the degradation of 146S antigen from dissociation into 12S pentamers for 7 months during the storage at 4°C during the storage as oil-emulsion FMD vaccine^[8]. A stabilizer comprising of pullulan 10% (w/v) and trehalose 0.5M retained the efficacy of live-attenuated HSV-2 vaccine and inactivated influenza vaccine retained its efficacy at the minimum of 2 and 3 months, respectively, when stored at 40°C^[17]. Addition of sugar stabilizers such as glycerol to the storage medium has cryoprotective effect and minimizes the loss of virus during the freeze-thaw cycles. Considering the beneficial effects of stabilizers in maintaining the structural integrity of the live virus and viral antigens, we evaluated the stabilizing effects of glycerol, mannitol, sorbitol, sucrose and trehalose on the titer of FMDV and 146S antigen content of the vaccine.

Materials and Methods

Cells and viruses

Baby Hamster Kidney (BHK-21) clone 13 cell line (passage no. 12), maintained at the FMD Research Laboratory, Indian Veterinary Research Institute (IVRI), Bengaluru was used. Glasgow's Modification of Eagle's Medium (GMEM, Himedia, India) with 10% fetal bovine serum (M P Biomedicals, South America) served as growth media for the propagation of cells incubated in 5% CO₂ incubator at 37 °C for 48 h. Foot-and-Mouth disease virus serotype strains, O/IND R2/75, A/IND 40/2000, and Asia 1/IND 63/72 of passage number 6, 5 and 7, respectively maintained at FMD Research laboratory, IVRI, Bengaluru were used in this study. All the three serotypes were further passaged three

times to produce master seed stock with a final titer of 7.67, 7.15 and 7.10 Log₁₀ TCID₅₀/mL for serotypes O, A and Asia 1, respectively. Identity of the viruses as FMDV O, A and Asia-1 serotypes were confirmed by sandwich ELISA and multiplex PCR^[18].

Stabilizers

Glycerol (Himedia, India), mannitol (S D Fine Chemicals Limited, India), D-Sorbitol (SRL, India), sucrose (SAFC, USA), and D (+) trehalose dihydrate (Himedia, India) were purchased. Stock solutions of 10%, 20% and 30% W/V were prepared for each stabilizer using double distilled water. Similarly, stock solution of 10%, 20% and 30% V/V of glycerol was prepared and autoclaved at 120°C for 30 min. For each experiment, freshly prepared and sterilized stabilizer solutions were utilized.

Virus culture

BHK-21 cells at 80-90% confluency was used to propagate FMDV serotypes O, A, and Asia1. Cells were washed once with serum free GMEM prior to infection. Cells were infected at 0.1 multiplicity of infection (MOI) by pre-adsorption method and incubated in 5% CO₂ incubator at 37 °C for 16 h after adding fresh serum free GMEM. After 16 h incubation, infected cells were harvested at 80% of cytopathic effect (CPE). The harvested cell suspension was clarified by centrifugation at 4500 rpm for 15 min at 4 °C to get neat virus that was stored at -80 °C until further use.

Preparation of stabilizer suspension for thermo-stability studies

Suspensions of FMDV for each stabilizer were prepared by mixing FMDV and the respective stabilizer solutions at the ratio of 1:1. Similarly, a negative control was prepared by mixing FMDV and GMEM at the ratio of 1:1. The experimental groups were exposed to different temperatures for different time intervals. The unexposed virus, which was kept at -80 °C served as positive control. For the screening experiments, all the five stabilizers were taken at concentrations 10%, 20%, and 30% and equal volume of viral suspension was mixed with each concentration to get a final concentration of 5, 10 and 15% and exposed at 37 °C for 8 h along with negative control (No stabilizer group). Based on the screening experiment, glycerol and sucrose were selected for further evaluation. A suspension of FMDV in 5% glycerol was prepared by mixing equal volume of FMDV and 10% glycerol (v/v). Similarly, a suspension of FMDV in 5% sucrose was prepared by mixing equal volume of FMDV and 10% sucrose (w/v). The mixture of FMDV and the stabilizers were exposed to 37 °C, room temperature and 4 °C for 48 h, 7 days and 7 weeks, respectively along with the control group. To evaluate the combined effects of glycerol and sucrose, a suspension of FMDV in 5% glycerol and 5% sucrose was prepared by mixing one part of 20% glycerol, one part of 20% sucrose and two parts of FMDV suspension. A suspension of FMDV in 2.5% glycerol and 2.5% sucrose was prepared by mixing one part of 10% glycerol, one part of 10% sucrose and two parts of FMDV suspension. The mixture of FMDV and the stabilizers were exposed to 37 °C along with glycerol 5%, sucrose 5% control group for 24 h. All the exposed samples were collected at the pre-determined time points and were stored at 4°C transiently before virus titration.

Virus titration by TCID₅₀ assay

The samples were subjected to virus titration assay (TCID₅₀ method) as described earlier^[19] Briefly, ten-fold serial dilution of the virus samples was carried out using plain GMEM and the viruses were titrated in BHK-21 cell suspension (1×10⁶ cells/mL) in the growth medium having 10% FBS in 96-well tissue culture plates (Nunc, ThermoScientific Corporation, USA) using six replicates per dilution (50 µL/well) along with cell control. The tissue culture plates were incubated in 5% CO₂ incubator at 37 °C for 48 h. Development of CPE was observed under inverted microscope after 48 h of incubation and the readings were recorded as positive or negative.

Statistical analysis

Each experiment was repeated thrice and the mean of the triplicate for each time-point, temperature and concentration of the stabilizer was analyzed by two-way repeat measures ANOVA with Bonferroni *post-hoc* test to find the significance of pair-wise mean difference. Significance was set at 95%. Graphpad Prism 5.0 was used for data analysis and preparation of line charts.

Results and Discussion

The virus titer of the unexposed positive control was 7.68 TCID₅₀/mL in all the experiments.

Effect of stabilizers on the virus titer when FMDV serotype O was exposed to 37 °C

The effect of five different carbohydrate-based stabilizers at four different concentrations on the virus titer following 8 h exposure of the FMDV to 37 °C is presented in Fig. 1. The results indicated that there was no interaction between the type of stabilizers and the concentration ($P = 0.7456$); however, concentration had an extremely significant effect in preventing the reduction in the virus titer ($P = 0.0001$) following exposure to 37 °C for 8 h. Pair-wise comparison across the concentration revealed a significant difference between 0% (exposed control) and other concentrations as shown in the figure 1 indicating that addition of any stabilizer at a minimum concentration of 5% was beneficial. Though sorbitol stabilizes the proteins in solution as well as in the lyophilized state, it could not protect the proteins in the frozen state^[20]. Mannitol has lower thermal transition temperature than sucrose due to aliphatic side chain and gets crystallized during freezing^[21]. Despite the fact that the physico-chemical properties of the sucrose and trehalose are comparable due to structural similarity, the solubility of the sucrose at 20 °C is 203.9 g/100 mL which is higher than that of trehalose^[22]. Glycerol is readily soluble in the water due to the presence of 3 hydroxyl groups, and is an excellent cryoprotectant^[23], viscous liquid at room temperature, decomposes at 290 °C and is non-toxic to cells at lower concentrations^[24]. Sucrose dissociates at temperature above 186°C^[25], glycerol and sucrose are osmolytes and prevent unfolding and denaturation of the proteins at high temperature^[26]. Further, they are cost-effective for use at industrial scale and are readily available. Because of the better stabilization of the proteins, glycerol and sucrose were chosen for further investigation.

Effect of 5% glycerol and sucrose on the virus titer when FMDV serotype O was exposed to 22° and 37 °C

The protective effect of 5% each of glycerol (v/v) and sucrose (w/v) on the virus titer following exposure of

FMDV serotype O to room temperature and 37 °C is presented in Fig. 2. In the absence of any stabilizer, the virus titer was undetectable as compared with either glycerol or sucrose at 37 °C ($p < 0.0001$). However, the mean virus titer was comparable between glycerol and sucrose either at room temperature or 37 °C ($p > 0.05$). Further, at room temperature, the mean virus titer was significantly high by about 1 log₁₀ for both glycerol and sucrose compared with the no stabilizer group ($p < 0.05$). The results indicated that at 37 °C, addition of either glycerol or sucrose at 5% concentration reduced the loss of virus titer by about 42% (titer ~4.4 log₁₀ TCID₅₀/mL) when exposed to 37 °C for 24 h, compared with the unexposed control group.

Combined effect of glycerol and sucrose on the virus titer when FMDV serotype O was exposed to 37 °C for 24 h

The interaction between different combination of glycerol and sucrose either at 2.5% or 5% did not have beneficial effect on the titer of FMDV serotype O as compared with the solitary effects of 5% glycerol or 5% sucrose at 37 °C, as evidenced by < 1 log₁₀ variation in the titer at any time point. However, when no stabilizer was added, the virus titer showed a steep reduction between 16 and 24 h post-exposure of the FMDV to 37 °C (Fig. 3). As the results of the first three experiments collectively suggested that addition of either glycerol or sucrose at 5% concentration significantly minimized the loss of virus titer when FMDV serotype O was exposed to 37 °C for 24 h (Fig. 1, 2 and 3), we repeated the experiments with FMDV serotypes (A, and Asia1) that are used in vaccine formulation in India.

Effect of glycerol or sucrose on the virus titer upon exposure of the FMDV serotypes to different temperatures

In the absence of stabilizer, minimum of one log₁₀ reduction in the virus titer was evident at 8 h after exposing the three FMDV serotypes to 37 °C and the magnitude of difference was wider for the serotype O, possibly because of its thermolability. Further, no-stabilizer group showed a drastic reduction in the virus titer at 20 h for FMDV serotype O. The protective effect of glycerol and sucrose in preventing the time-dependent reduction of the virus titer was evident till 16 h post-exposure to 37 °C; after 20 h, a steep reduction in the virus titer was recorded for all the three FMDV serotypes (Fig. 4A, B, and C). A significant interaction ($p < 0.001$) between the stabilizer treatment and time post-exposure to room temperature indicated that, in the absence of stabilizer, FMDV serotype O virus showed a steep reduction in the titer by day 4 as compared with glycerol or sucrose groups, whereas it was day 5 for serotype Asia 1 (Fig. 5). When the three vaccine strains of FMDV serotypes were exposed to 4 °C for 7 weeks, the main effect of stabilizer indicated that the addition of either glycerol or sucrose at 5% significantly prevented the loss of virus titer as compared with the no stabilizer group (Fig. 6). However, lack of simple main effect indicated that there was no interaction between the stabilizer treatment and week post-infection ($p = 0.497$).

The indigent stability nature of the inactivated FMD virus is one of the challenge for the vaccine production. Symmetrical arrangement of intact capsid and dynamics of viral particles plays a vital part in infectious life cycle^[27]. The interaction of interpentameric residues are the key

factor responsible for infectivity and also stability of the virion [28]. Capsid assembly is highly prone for disintegration with slight increase in temperature [29] results in 12S pentamers. 12S pentamer is less immunogenic and will not maintain antibody response in host animals, and for vaccine production disassembly of 146S is disadvantageous. Denaturation of the antigen results in less potent vaccine, high cost of production and finally vaccination failure. Stabilization of live virus by retaining the infectivity consequent immunogenicity is highly challenging task since nucleic acid assembly and protein integrity should not be altered.

Sucrose and trehalose at a concentration of 250 mM each were reported to stabilize etanercept in solution by showing increase in thermal transition temperature. Higher the T_m higher the stability from the thermodynamic perspective [30]. Sucrose has been reported to stabilize membranes and proteins [31]. The other advantage is that sucrose is safe for parenteral administration to animals. It is used as the stabilizer of viral vaccine for human diseases, such as influenza, zoster and Japanese encephalitis, owing to its safety. One of the most consistent and reproducible results of the study was that addition of glycerol or sucrose at 5% prevented the loss of virus titer when the three Indian vaccine strains of FMDV were exposed to 37 °C, room temperature and 4 °C for different periods. However, there was no beneficial effect in preventing the loss of virus titer

when the concentration of the stabilizers was increased beyond 5%. Similarly, the combination of glycerol and sucrose did not have any additive or synergistic effect in maintaining the virus titer upon exposure to 37 °C. The results of the study is consistent with the theory of preferential exclusion by which the hydroxyl moieties in the carbohydrates stabilize the protein [30]. For instance, the three hydroxyl groups of glycerol or seven hydroxyl groups of sucrose form multiple hydrogen bonds with the surrounding water molecules in the solution thereby stabilizing the structural integrity of FMDV particles [30]. The results suggest that glycerol or sucrose can be added to the virus culture during the bulk production of FMDV as practiced by the vaccine manufacturers. Though the time-dependent decrease of virus titer showed a similar trend for the three FMDV serotypes, the loss of virus titer was quick for serotype O for the different temperatures tested. This is due to the fact the FMDV serotype O is highly thermolabile as compared with serotype A [29, 32].

It is concluded that addition of polyols such as glycerol and non-reducing disaccharides such as sucrose at 5% concentration to the FMDV suspension culture confer thermostability to the FMDV particles by minimizing the loss of virus titer. The effect of addition of 5% glycerol or sucrose to FMDV 146S antigen in preventing the disintegration of capsid into less immunogenic subunits needs to be investigated.

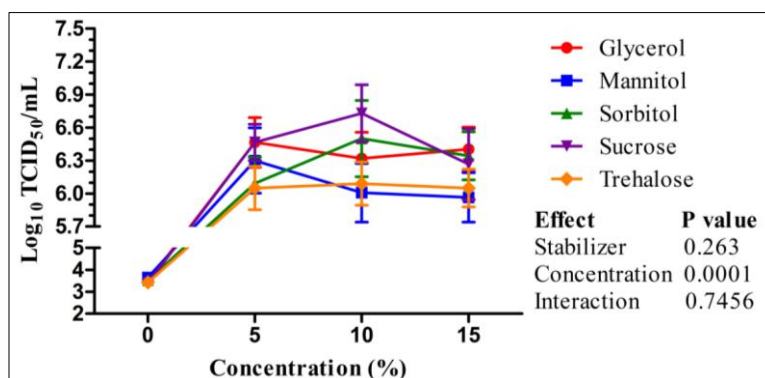


Fig 1: Effect of different stabilizers from 0 to 15% concentration on the titer ($\log_{10}$ TCID₅₀/mL) of foot-and-mouth disease virus (FMDV) serotype O exposed to 37 °C for 8 h.

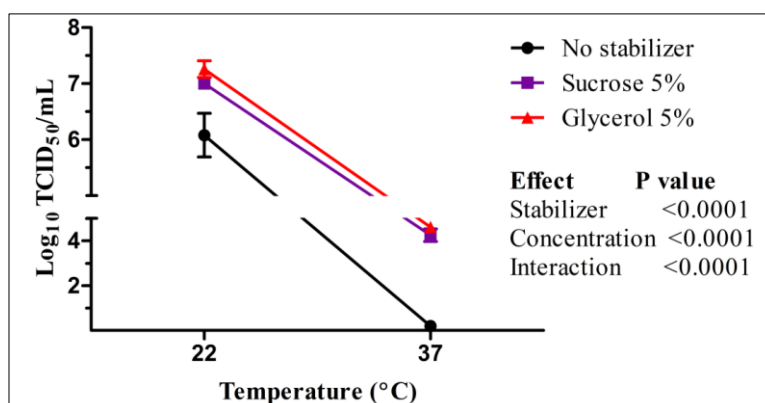


Fig 2: Effect of glycerol 5% and sucrose 5% on the titer of FMDV serotype O exposed to two different temperatures for 24 h.

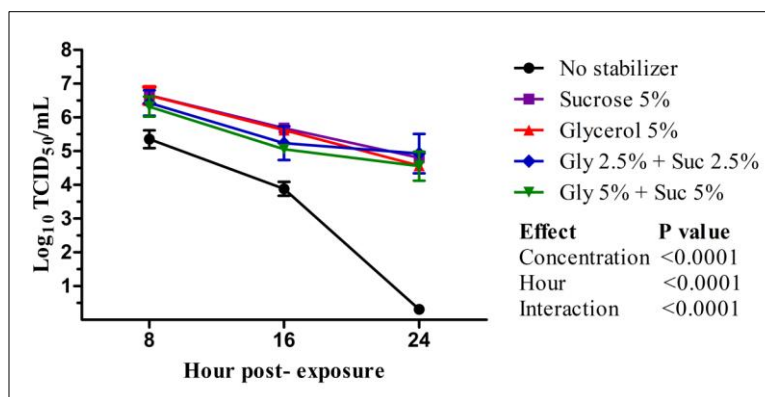


Fig 3: Addition of glycerol and/or sucrose each at 2.5% or 5% concentration to FMDV serotype O on the virus titer upon exposure to 37°C for 24 h.

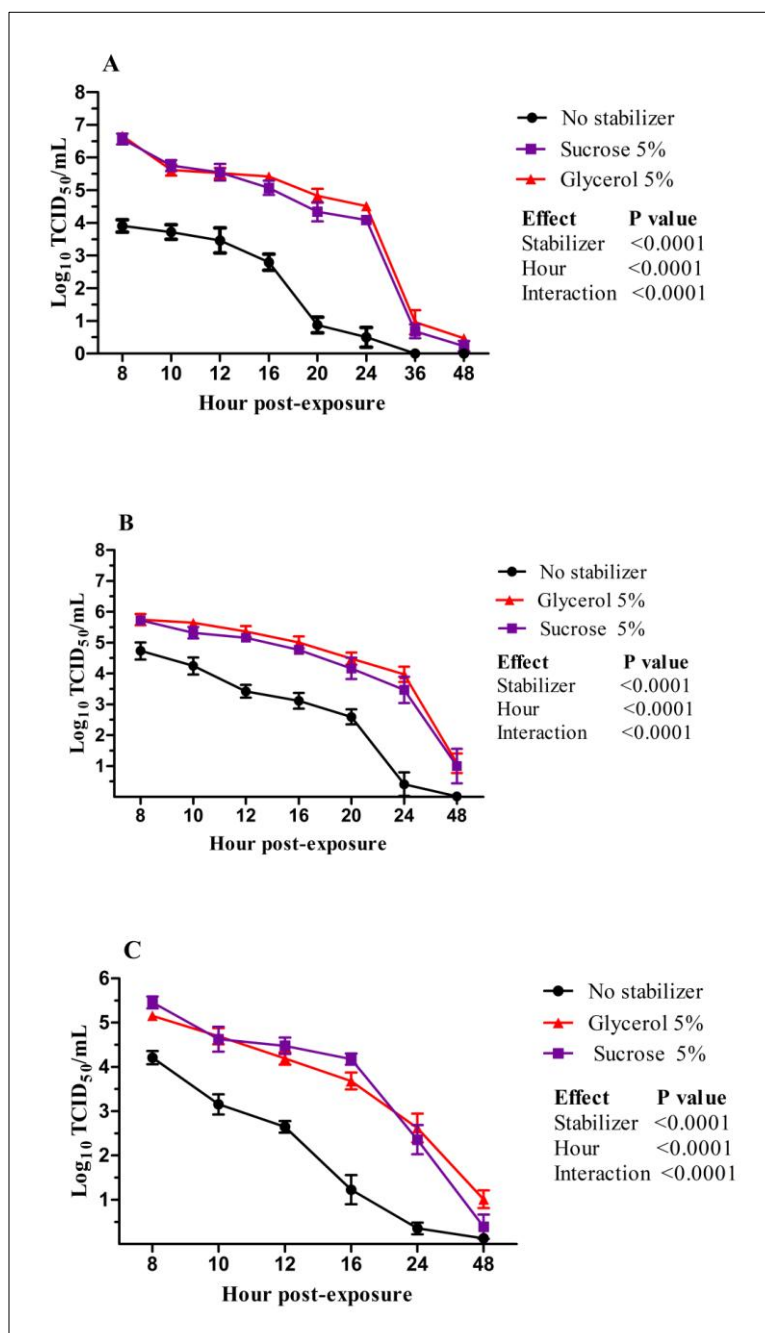


Fig 4: Addition of glycerol 5% or sucrose 5% to FMDV serotype O, A, and Asia1 on the virus titer upon exposure to 37°C for 48h.

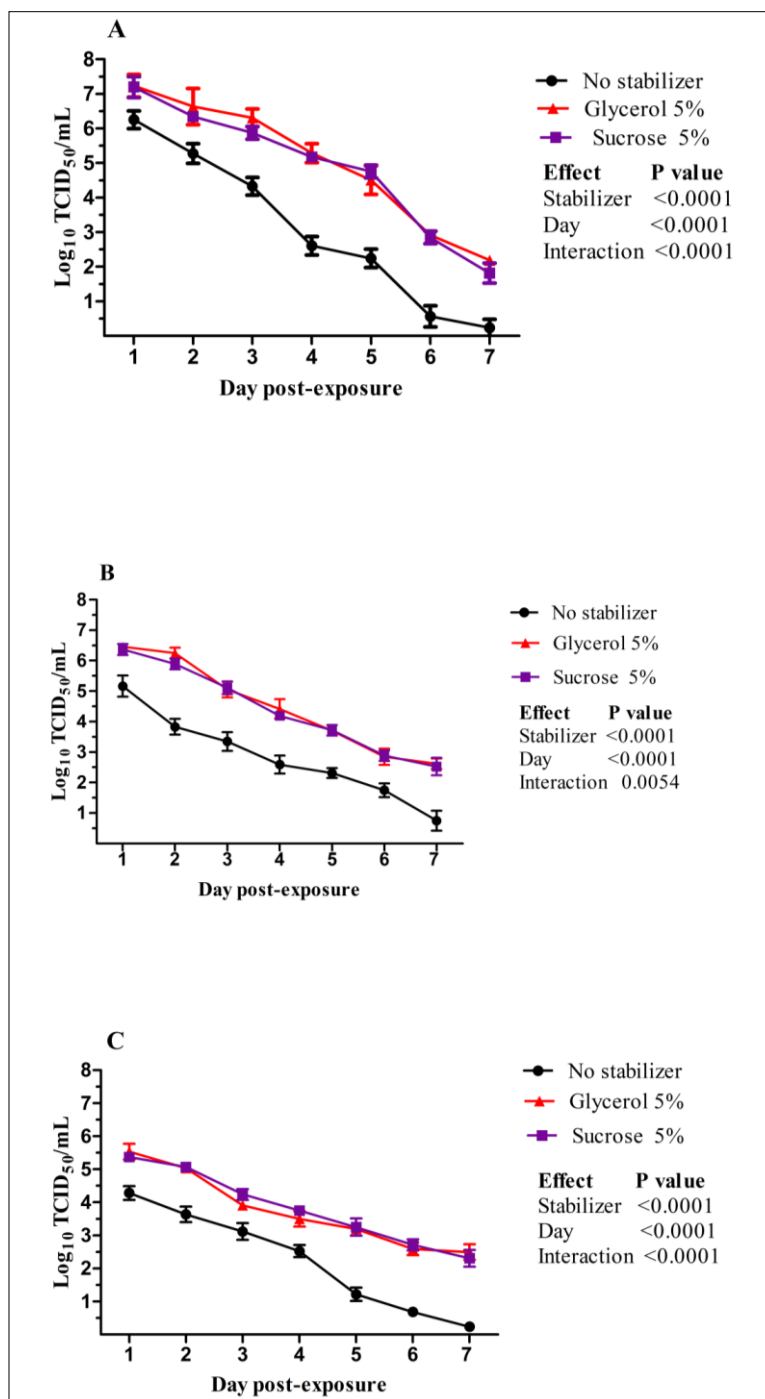


Fig 5: Addition of glycerol 5% or sucrose 5% to FMDV serotype O, A, and Asia1 on the virus titer upon exposure to room temperature for 7 days.

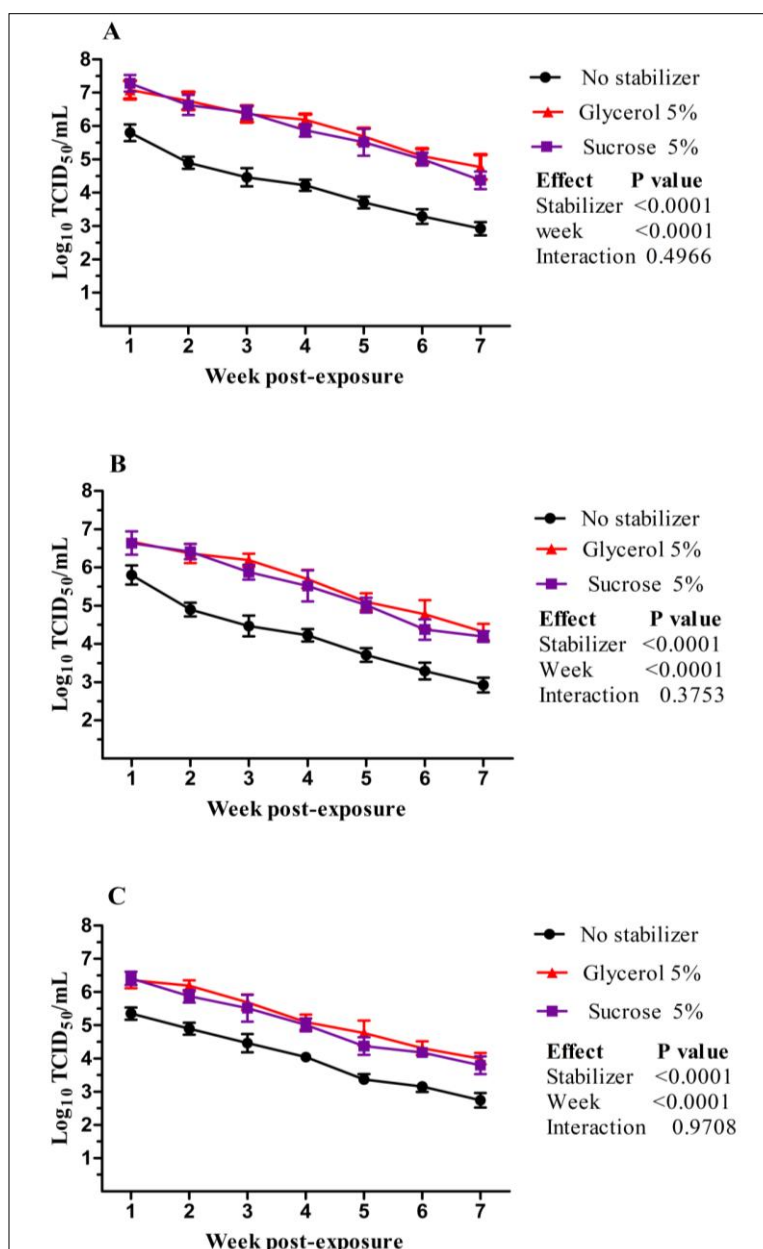


Fig 6: Addition of glycerol 5% or sucrose 5% to FMDV serotype O, A, and Asia1 on the virus titer upon exposure to 4°C for 7 weeks.

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