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Original Article

# Comparative assessment of β-propiolactone, binary ethyleneimine and formaldehyde in inactivating Foot and Mouth Disease virus serotype O

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## Abstract

Inactivation of foot-and-mouth disease virus (FMDV) with Formalin (FA) and binary ethyleneimine (BEI) is a slow process and takes long time. The purpose of this study was to evaluate β-Propiolactone (BPL), an alkylating agent that is frequently used in vaccine development and production, as a candidate for the inactivation of FMDV serotype O. Virus was grown on confluent monolayer of BHK-21 cell line. Harvesting was performed between 18 and 20 hours after infection, when CPE was between 90 and 95%. The virus was subjected to inactivation with formalin (0.02%), BEI (2mM, 2.5mM, 3mM) and BPL (0.1%, 0.2 and 0.4%) at 4°C and 37°C. Samples (05 mL/each) were collected after 0, 2, 4, 8, 16, and 24 hours post treatment. The biological titer (TCID50/ml) of each inactivated sample was measured. An innocuity test was used to further confirm the inactivation. RT-PCR was used to detect viral genome damage by amplification of VP1 gene. Data were analyzed by one-way analysis of variance and pair wise comparison was made by post hoc Tukey's HSD test. Linear regression was used in Microsoft Excel Version 2010. The virus titers after 0.02% formalin treatment were 7.172±0.298 and 4.584±1.362 at 4°C and 37°C respectively. The virus titers were 6.036±0.513, 5.622±0.298, and 5.150±0.449 with 2mM, 2.5mM, 3mM BEI at 4°C and 1.646±1.0210, 1.050±0.644, 0.492±0.492 at 37°C respectively. Inactivation with 0.1%, 0.2% and 0.4% BPL at 4°C resulted in virus titers of 2.386±1.1104, 1.4400±0.9445 and 0.6960±0.6960 respectively. Rapid inactivation of virus with all three BPL concentrations at 37°C gave mean titer of 0.00±0.00. There was a significant difference ($P<0.05$) among all treatment groups with highest inactivation rates recorded for BPL.

**Keywords**: FMDV, BHK-21, Inactivation, BEI, BPL, Formalin

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## Introduction

Inactivation of pathogenic viruses like foot-and-mouth disease virus (FMDV) for preparation of vaccines is a critical procedure. It requires precise care and consideration as improper inactivation of virus results in emergence of outbreaks in field conditions upon vaccination (Wood et al., 2020, Karayel-Hacioglu et al., 2022). Preparation of inactivated FMD vaccines is accomplished by treating live virus with Binary ethyleneimine (BEI) alone, or in combination with formalin (Bergmann et al., 2021). Previously formalin remained as a sole principal inactivation substance for FMDV however later studies revealed that it results in incomplete inactivation and antigenicity modifications (Delrue et al., 2012; Kenubih, 2021). Inactivating agent must not alter the immunological structure, contrary to formalin which changes the conformation of virus particles. These changes thus lead to weakened immunological responses (Patil et al., 2002). Formalin fixed preparations contain live virus particles that causes disease and there is lack of 1$^{st}$ order kinetic response. Historically a number of FMD outbreaks in United Kingdom (UK) and Germany have been attributed to use of formalized vaccines. Formalin takes minimum 48 hours for inactivation of the BPL FMD virus suspensions (Kamel et al., 2019). Due to the dangers of formalin and the desire for a more effective inactivating agents, vaccine producers became interested in the aziridine group, which includes Binary ethyleneimine (BEI) and Acetyl ethyleneimine (AEI). The latter gained more traction and is now frequently used to inactivate FMDV (Willems et al., 2020). Unlike formalin that only fixes proteins, BEI penetrates into the virion and interact biochemically with the deoxyribonucleotide (DNA) or ribonucleotide (RNA) resultantly preserves the antigenic confirmations (Eladawy and El-Bagoury, 2020). BEI was more widely accepted and eventually used as the primary inactivation agent for the FMDV vaccination due to its ability to preserve the immunological qualities of the antigen and its superior deactivation kinetics over formalin. (Zheng et al., 2021). Although it is easy to formulate and deal with BEI in laboratories however safety issues are linked with it as this is irritant and a potential carcinogen that needs to be neutralized with sodium thiosulphate when used. Moreover, the recommended inactivation time for BEI is minimum 24 hours that affect the production process of industrial level biological production units (Cao et al., 2021). Prolonged duration and improper concentration of the inactivating agents consequently result in the poor stability and decrease immunogenic features of vaccine (Isakova-Sivak and Rudenko, 2021; Kim et al., 2023). Presently β-Propiolactone (BPL) is widely employed as an inactivant for both veterinary and human vaccine preparations like corona virus (Gupta et al., 2021), rabies virus (Eftekhari et al., 2016), avian influenza virus (Pawar et al., 2015), polio virus, infectious bovine rhinotracheitis virus (Kamaraj et al., 2008) and seasonal flu vaccines with the purpose of virus inactivation. Neutralizing agents are also not required for BPL as it is self-limiting agent in this perspective. Furthermore, inactivation times are remarkably shorter for BPL as compared to formaldehyde (Nunnally et al., 2015; Hotez and Bottazzi, 2022). FMD virus serotype O is found responsible for more than 60% of all outbreaks in Pakistan (Riaz et al., 2021). The present study was designed to evaluate the Beta propiolactone as another inactivating agent for Foot and mouth disease virus (FMDV) serotype O and to compare it's the inactivation kinetics with BEI and formalin.

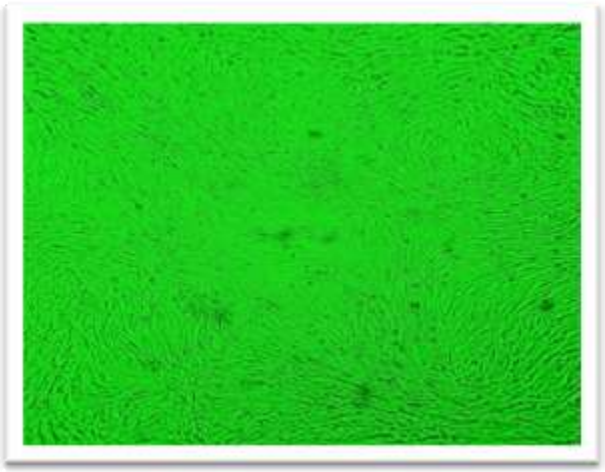
## Material and Methods

### Propagation and inactivation of virus

Baby Hamster Kidney cell line (BHK-21 clone-13, ATCC®-CCL-10™) was grown in Dulbecco`s Modified Eagle Medium (Caisson Labs, USA) with 10% fetal bovine serum (FBS, Gibco®, USA) in 175cm$^2$ cell culture flasks. Confluent monolayers (85-90%) of BHK-21 were infected with FMDV serotype O/ME-SA/Ind-2001e. The virus inoculated flasks were observed critically and harvesting was performed when cytopathic effects (CPE) were 90-95% that appeared within 18-22 hours post infection (Figure 1). The harvest was freeze thawed twice for the maximum recovery of virus and centrifuged at 3000 RPM for 30 minutes to separate the cell debris. Aliquots of supernatant were made and stored at -70°C till further use (Hernandez and Brown, 2010; Yi et al., 2023).

FMDV serotype O/ME-SA/Ind-2001e was inactivated using BPL (0.1%, 0.2% and 0.4%), BEI (2.0 mM, 2.5 mM and 3.0 mM) and 0.02% formalin for 24 h at 4°C and 37 °C (Kamaraj et al., 2008). 5ml samples were collected at interval of 0, 2, 4, 8, 16, and 24 h. In case of BEI, each collected sample was

neutralized by addition of 0.5ml sodium thiosulfate from 20% stock solution to reach final concentration of 2%. The samples were stored at −70 °C until further analysis.



A

B

**Figure-1. (A) Confluent monolayer of BHK-21 Cells (B) Cytopathic Effects (CPE) of FMDV serotype O after 20 hours of incubation at 37°C.**

**Virus titration**

Biological titration of each sample was performed in 96-well flat bottom micro titration plates (Nest® Scientific USA) using BHK-21 cells. Counting of the cells was performed by using Neubauer chamber by adding 0.4% trypan blue and adjusted at a concentration of $2 \times 10^5$ cells/ml by mixing cell culture medium (Louis and Siegel, 2011). 180µl of cell suspension was dispensed in each well of the 96-well plate and was placed in incubator at 37°C. Upon completion of monolayer after 24 hours, the previous growth media was discarded and 180µl maintenance media (2%FBS) was added in each well. 20ul of the sample virus suspension was added to each well of column 1. After gentle pipetting, the 20ul virus suspension was transferred to respective wells of the column 2 to carry out 10-fold serial dilution. The dilution process was carried up to column 10 while wells of column 11 and 12 were kept as control. The plate was again incubated at 37°C and observed at regular intervals for CPE. The final readings were made after 72 hours incubation and virus titer was calculated as tissue culture median infectious dose (TCID50) using Reed and Muench, 1938 method.

**Innocuity test**

Samples that manifested no readable titer in 96well microtitration plates were subjected to innocuity test for further confirmation. The virus samples were inoculated in $25cm^2$ tissue culture flasks and allowed to adsorb on confluent monolayer of BHK-21 cells by keeping the flasks in incubator at 37°C for 1hour. After incubation, cell culture media with 2%FBS was added in the flasks and again placed in incubator for 24-48 hours and examined regularly under inverted microscope for CPE. Flasks were freeze thawed thrice to liberate the maximum virus from cells and next passage was carried out in same way. This protocol was continuously repeated for 03 consecutive passages. Samples that showed no CPE at the end of 3rd passage were considered as completely inactivated having no live virus. While the samples that showed CPE at the end of 3rd passage were considered as viable.

**Identification of virus post inactivation**

**Extraction of RNA**

Extraction of viral RNA from collected samples was performed using TRI-reagent method. Briefly 100µl sample was taken in 1.5ml microcentrifuge tube (Eppendorf® Germany) and 300µl of TRI-reagent (Cat No.15596026 Invitrogen®) was added. The mixture was vortexed for 5minutes. Afterward 80µl chloroform (Cat No.22711.324 AnalaR NORMAPUR®) was added and vigorously mixed. After an incubation of 15 minutes at room temperature, the tube was centrifuged at 14000RPM for 15 minutes at 4°C. The supernatant having aqueous phase was collected in a new sterile microcentrifuge tube and 500 µl isopropanol (Cat No.20842.330 AnalaR NORMAPUR®) was added and mixed properly. For precipitation of RNA the

tube was kept for 10 min at room temperature. Centrifugation was performed again at 14000 RPM for at 10 minutes at 4°C. Supernatant was discarded and 70% ethanol was used for washing of RNA pellet. Centrifugation was done at 8000 RPM for 5minutes and pellet was allowed to be air dried. 20 µl of nuclease free water (NFW) was added and heat shock was provided by incubation at 55-60°C for 10 minutes. The extracted RNA was kept at -80 °C before cDNA was prepared.

**Preparation of complementary DNA (cDNA)**

Extracted RNA was transcribed into cDNA using kit method (Cat No. K1622 Thermo scientific). In the RNA template (5µl), 1µl random hexamer (0.2µg/ µl), 6 µl of nuclease free water, 4µl of 5X RT buffer, 1µl RiboLock RNase inhibitor (20 units/µl), 1 µl 10 mM dNTP mix and 1 µl RevertAid M M-MuLV reverse transcriptase (200 units/µl) enzyme were added. The reaction mixture was mixed properly and incubated in thermocycler (Bio-Rad USA) using the condition 25°C for 10 minutes, 42°C for 1 hour and 99°C for 5 minutes in a single cycle. cDNA was stored at -20°C for further use.

**PCR amplification**

Polymerase Chain Reaction (PCR) was performed using serotype 'O' specific VP1 gene primers SA-F (5´-ACCACCTCCACAGGTGA-3´) and SA-R (5´-CAAAAGCTGTTTCACAGGTGC-3') procured from ABI Sciences (Kanwal et al., 2014). A total volume of 25 µl reaction mixture was prepared containing cDNA (5µl), 12.5 µl of 2X Dream Taq Green PCR master mix (Cat no.K1081 Thermo scientific), 1 µl of each forward primer (10 pmol/µl) and reverse primer (10 pmol/ µl), and 5.5 µl nuclease free water to make final volume of 25 µl. The PCR was performed with the thermal cycling condition as follows: 95°C, for 5 minutes, 1 cycle; 95°C for 40 seconds, 56°C for 40 seconds, 72°C for 40 seconds, 30 cycles followed by 72 °C for 3 minutes, 1 cycle (Das et al., 2022).

The PCR product was analyzed by agarose gel electrophoresis. Samples (5µl) were loaded along with 100bp ladder (5µl) (GeneRuler) ®. 100volts electric current was supplied for 45minutes (Zheng et al., 2015). The gel was visualized on UV transilluminator (JUNYI China) ®.

**Statistical analysis**

The results of inactivation kinetics were analyzed by using one-way analysis of variance (ANOVA) in SPSS software (SPSS Inc., Chicago, USA, Version 16.0). The differences were considered significant at $p < 0.05$. Pair wise comparison was made by Tukey's Honest Significant Difference Test (Tukey's HSD). Inactivation rates were analyzed by simple linear regression in Microsoft Excel Version 2010.

## Results

The FMDV suspension contained biological titer ($TCID_{50}$) 9.88 $log_{10}$TCID50/mL before starting the inactivation process. By measuring the decrease in virus titers (log10TCID50/ml), the inactivation time of FMD virus serotype O with BEI, Formalin, and BPL at 4°C and 37°C was determined (Table:1).

**Table-1. Effect of Temperature on Virus Titer in response to different concentrations of BPL, BEI and Formalin**

| Inactivant | Temperature | | Significance |
|---|---|---|---|
| | 4°C | 37°C | p-value |
| **2mM BEI** | 6.130±0.414 | 1.640±1.021 | 0.004 |
| **2.5mM BEI** | 5.520±0.372 | 1.050±0.644 | 0.000 |
| **3mM BEI** | 5.150±0.449 | 0.490±0.492 | 0.000 |
| **0.1% BPL** | 2.386±1.110 | 0.000 | 0.000 |
| **0.2% BPL** | 1.440±0.9445 | 0.000 | 0.000 |
| **0.4% BPL** | 0.696±0.69 | 0.000 | 0.000 |
| **0.02% Formalin** | 7.170±0.298 | 4.580±1.360 | 0.100 |

**Note:** Data are represented as Mean ± SE p values less than 0.05 are considered significant.

Inactivation of FMDV with BEI when used 2mM, 2.5mM and 3mM BEI concentrations at 4°C and 37°C resulted significantly different inactivation kinetics (Figure 2). Although the inactivation rates were slightly higher for 3mM (0.1769 log/hour) as compared to 2.5mM (0.1575 log/hour) and 2mM BEI (0.1476 log/hour) however this difference was non- significant (p=0.273) to have considerable variation in virus titer. Based on a linear regression model, the anticipated time needed to completely inactivate FMDV serotype O was determined to be 55, 49, and 43 hours at 4°C with 2 mM, 2.5 mM, and 3 mM BEI, respectively. The predicted durations at 37°C for the same BEI doses were 11, 08, and 06 hours, respectively.

After inactivation with 0.02% formalin at two different temperatures (4°C and 37°C) the titration was performed for each time course sample .The virus titer declined from 9.88 to 8.02, 7.6, 7.22, 6.54, 6.48 ($log_{10}TCID_{50}$/ml) with mean titer of 7.17±0.29 after 2, 4, 8, 16 and 24 hours respectively at 4°C. inactivation was performed at 37°C and were recorded as 7.75, 6.72, 5, 3.45, 0 ($log_{10}TCID_{50}$/ml) with mean titer of 4.58±1.36 after 2, 4, 8, 16 and 24 hours respectively. Based on regression model and results formalin requires an estimated time of minimum 78 and 25 hours for complete inactivation of FMDV serotype O at 4°C and 37°C respectively (Figure 3).

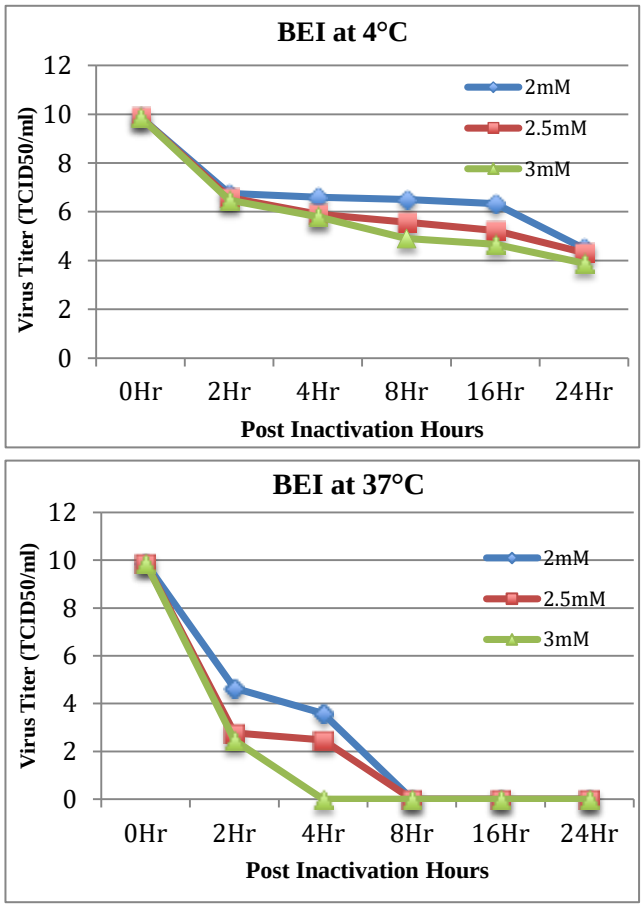



**Figure-2. Inactivation of FMD virus serotype O with BEI at 4°C and 37°C. Virus titers are presented as $log_{10}TCID_{50}$/ml.**

As per the results of linear regression model the estimated time required for the complete inactivation of FMDV serotype O is 15,8 and 4 hours with 0.1%,0.2% and 0.4%BPL respectively at 4°C.Rate of inactivation for was found greater at 37°C than 4°C for BPL

BPL induced complete inactivation of FMDV at 37°C in the shortest amount of time just 15 minutes for all three concentrations of BPL (0.1%, 0.2%, and 0.4%) (Figure 4). However, at 4°C the rate of inactivation was comparatively slower and the virus titer was recorded as 5.70, 3.90, 2.33, 0, 0 ($log_{10}TCID_{50}$/ml) after 2, 4, 8, 16 and 24 hours respectively with mean titer of 2.386±1.11 with 0.1%BPL.The virus titer was 4.67, 2.53, 0, 0, 0 ($log_{10}TCID_{50}$/ml) after 2, 4, 8, 16 and 24 hours respectively with mean titer of 1.44±0.94 with 0.2%BPL. The virus titer was 3.48, 0, 0, 0, 0 ($log_{10}TCID_{50}$/ml) after 2, 4, 8, 16 and 24 hours respectively with mean titer of 0.696±0.696 with 0.4%BPL. The anticipated duration needed for the complete inactivation of FMDV serotype O is 15, 8 and 4 hours at 4°C with 0.1%, 0.2%, and 0.4% BPL, respectively, based on the results of the linear regression model.

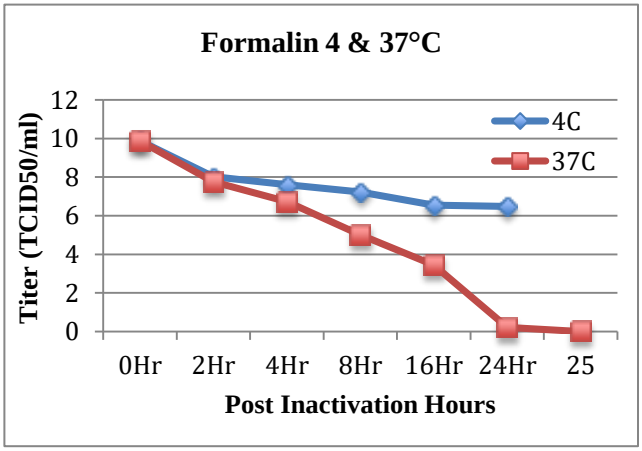



**Figure-3. Inactivation of FMD virus serotype O with Formalin at 4°C & 37°C. Virus titers are presented as $log_{10}TCID_{50}$/ml.**

However virus titers were comparatively less when Samples without a readable titer underwent three successive cycles of the innocuity test to establish full inactivation. No CPE was observed for any of the three continuous passages in the 16- and 24-hour samples that were treated with any of the three BEI concentrations at 37°C. The inactivant concentration did not significantly change between the 08-hour samples, despite the 2 mM sample exhibiting CPE in the second and third passage while the 2.5 mM and 3 mM samples remained negative for all three passages. Similar to the first passage, no live virus was found; however, the second and third passages

showed CPE in 4 hours when 3mM BEI samples were obtained. Since the viral titration showed that the inactivation with BEI at 4°C was not completed within the 24 hours, passage of cells in flasks was not required to analyze this group's sample further.

A 24-hour sample maintained at 37°C was the only one tested for innocuity in the formalin group. The findings revealed CPE in the second and third passages along with partial inactivation of the active virus in less than 24 hours.

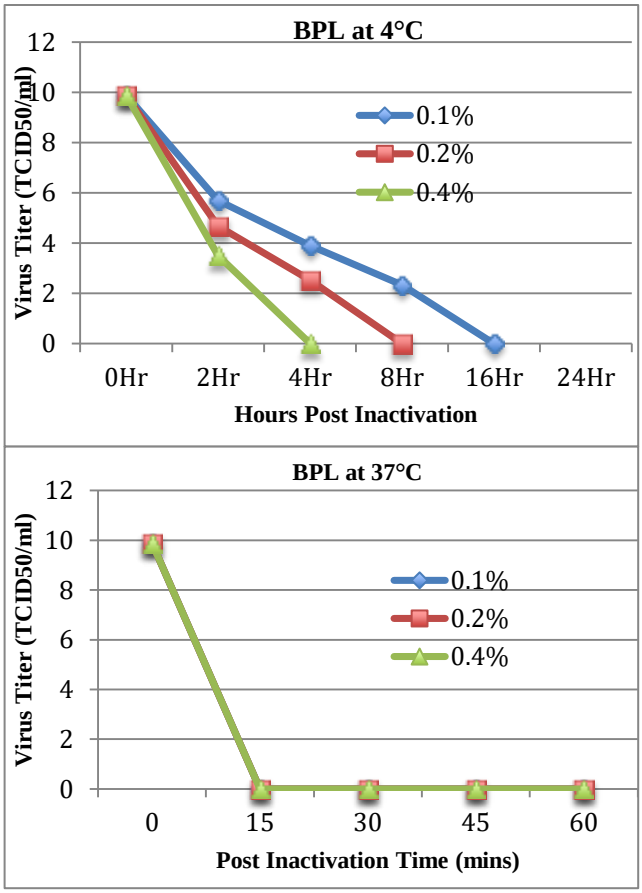



**Figure-4. Inactivation plot of FMD virus serotype O at 4°C and 37°C with BPL. Virus titers are presented as $log_{10}TCID_{50}$/ml.**

**(1)** Ladder 100bp **(2)** Positive Control **(3)** 0.4% BPL 2hour sample 4°C **(4)** 0.4% BPL 4hour sample 4°C **(5)** 0.4% BPL 8hour sample 4°C **(6)** 0.4% BPL 16hour sample 4°C **(7)** 0.4% BPL 24hour sample 4°C **(8)** 0.4% BPL 15minutes sample 37°C **(9)** 0.2% BPL 15minutes sample 37°C **(10)** 0.1%BPL 15minutes sample 37°C **(11)** 0.02% FA 24hour sample 4°C **(12)** 0.02% FA 24hour sample 37°C **(13)** Negative Control

None of the BPL samples tested at 4°C or 37°C in the innocuity test showed any signs of active virus. Using FMDV serotype O-specific primers, reverse transcriptase PCR was performed on each treated sample. A band size of 639 bp was observed by gel electrophoresis (Figure 5). The presence of detectable viral nucleic acid indicated that none of the inactivants had significantly weakened the viral RNA.

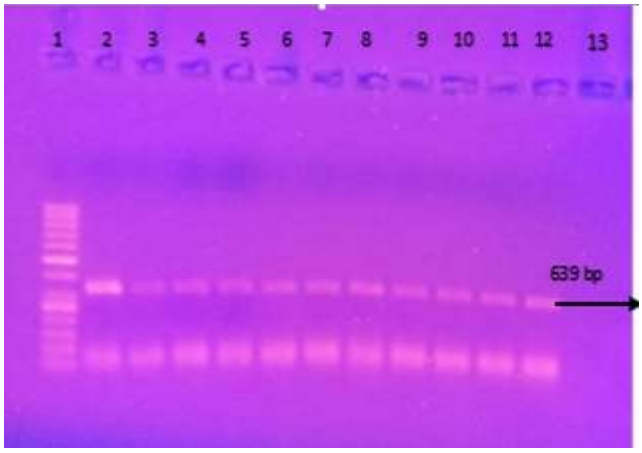



**Figure-5. PCR products of inactivated samples on agarose gel electrophoresis**

## Discussion

Immunizing susceptible animals in endemic areas is the most effective way to control foot-and-mouth disease for which safer and more effective vaccines are required (Sseguya et al. 2022). Inactivation time is of prime importance to gain the maximum production within shortest possible time without deteriorating the vaccine quality (Romey et al. 2018). BEI and formalin have been used as virucides to inactivate a variety of viruses, including FMDV. BPL was evaluated in this study as a potential means of inactivating the local FMDV serotype O vaccine strain.

Significant differences in inactivation kinetics were observed between 4°C and 37°C with BEI inactivation. Even after a full day at 4°C, inactivation remained incomplete, and no concentration attained total inactivation Regression equation of y= -0.1476x+8.088, y= -0.1575x+7.666 and y= -0.1769x+7.5329 with coefficient of determination ($R^2$ = 0.929, 0.8763, 0.8665) were obtained with 2mM, 2.5mM and 3mM BEI at 4°C respectively. While the inactivation rate for 3mM BEI was somewhat greater (0.1769 log/hour) than for 2.5mM (0.1575 log/hour)

and 2mM (0.1476 log/hour), this difference was not statistically significant (p=0.273).

With improved inactivation kinetics, BEI's performance was noticeably enhanced at 37°C. At 4°C, applying different BEI concentrations did not significantly affect the virus titer (p=0.571). However, it did increase the variation in the rates at which different BEI molarities inactivated the virus. This could be because BEI is an agent that depends on temperature, and as temperature rises, so does its inactivation efficiency. (Ismail et al., 2013) have reported on the same, looking into the BEI's ability to inactivate FMDV serotype SAT-2 at 25°C and 37°C. In comparison to inactivation at room temperature, his studies showed that at 37°C, the inactivation rate of 1.6 mM BEI was five times higher and complete inactivation was reached 24 hours earlier. With 3 mM, 2.5 mM, and 2 mm BEI, respectively, inactivation of FMDV serotype O with no residual virus was accomplished in 06, 08, and 11 hours. According to a recent study (El din Mahdy et al., 2015), FMDV serotype O, A, and SAT-2 were inactivated by 1 mM BEI within 20, 24, and 16 hours, respectively. These results of the inactivation rate and total time required for complete titer loss with BEI inactivation at 37°C are consistent with those findings. The time needed for total viral inactivation decreased when the concentration was raised from 2 mM to 3 mM, indicating a direct correlation between inactivation rate and BEI concentration and an inverse relationship between BEI concentration and inactivation time. Thus, according to Rueda et al., 2000 inactivation with BEI is a first order kinetic response. Significant differences were seen between the BEI group at 2 mM (p=0.004), 2.5 mM (p=0.000), and 3 mM BEI (p=0.000) when the influence of temperature (4°C and 37°C) was examined. This demonstrates that BEI works best at 37°C, and that lowering the temperature will result in much longer inactivation times and slower inactivation rates. These findings are consistent with a study that found that FMDV serotype O could be successfully inactivated at 37°C for five and eight hours, respectively, using 02 mM and 1.6 mM BEI (Aarthi et al., 2004). According to Sarkar et al. (2017), a different investigation found that treatment to 1 mM BEI for 8 hours at 37°C completely inactivated FMDV serotype O. A PPR viral inactivation investigation using 4mM BEI at 37°C showed total virus inactivation in 5 hours (Akbarian et al., 2021).

At 4°C and 37°C, the inactivation of FMDV serotype O with 0.02% FA did not differ statistically (p=0.1). Even after being exposed to FA for a full day, the viral particles persisted; however, at 37°C, the inactivation rate improved. Similar results have been reported by Möller et al. (2015), who found that the inactivation effectiveness of FA was low at 4°C and required longer than that of the human adenovirus, the vaccinia virus, and the murine norovirus at 25°C and 37°C. it is estimated that 0.02% FA will need a minimum of 78 and 25 hours to fully inactivate the FMDV serotype O at 4°C and 37°C.

Pairwise comparison revealed FA at 4°C have non-significant differences (p=0.99, p=0.94, p=0.73) in inactivation kinetics with 2mM, 2.5mM and 3mM BEI (4°C). While at 37°C FA have insignificant differences (p=0.967, p=0.998, p=1.00, p=0.611, p=0.177, p=0.111) with 2mM, 2.5mM ,3mM BEI ,0.1%BPL of 4°C and 2mM BEI ,0.1%BPL groups of 37°C respectively. When compared to BEI and BPL, FA had poorer inactivation kinetics, though improving at a high temperature of 37°C in terms of inactivation rate.

BPL is a member of lactone group comprising a ring of carbon atoms. The mechanism of action is similar to BEI and alkylation or acylation reaction occurs when BPL interacts with nucleic acid of the virus. The reactions of BPL are fast and rapid due to alkylation reactions with nucleophiles present in nucleic acid. Owing to interactions with viral genome only, it is anticipated that BPL does not alter the epitopic part of the virus (Sabbaghi et al., 2019). BPL when hydrolyzed by incubating at 37°C for two hours separates into betahydroxypropionic acid and lactate neither of which is poisonous to cells.

In the current study, BPL at 0.1%, 0.2%, and 0.4% concentrations at 37°C quickly inactivated FMDV in under 15 minutes. It just takes 30 minutes at 37°C for 0.4% BPL to inactivate the IBR virus, according to (Kamaraj et al., 2008). At 4°C, BPL's behavior completely changed with a slow inactivation rate, in contrast to 37°C. Our data show a higher inactivation rate of 2.47 log/hour compared to 1.153 log/hour and 0.536 log/hour for 0.4%, 0.2%, and 0.1% BPL, respectively, which indicate that the virus will completely inactivate in 04, 08, and 15 hours.

Kamaraj et al. (2008) showed similar results, showing that BPL needs just 04, 05, and 12 hours at 4°C to inactivate the IBR virus at 0.4, 0.2, and 0.1% concentrations, respectively. There must be an indirect correlation between inactivation time and

concentration and a direct relationship between inactivation rate and BPL concentration for FMDV to be completely inactivated. It takes around 16 hours for 0.1% BPL to fully deactivate Avian Influenza Viruses, according to earlier research. According to Zhao et al. (2020), at 4°C, TGEV was inactivated in 6 and 12 hours with 0.03 and 0.01% BPL, respectively. Our findings also agree with those of (Gupta et al., 2021), who used 0.05% BPL at 4°C for 16 hours to inactivate SARS-COV-2. For FMD vaccine quality testing to confirm the presence of FMDV, PCR-based nucleic acid identification is a requirement. The results of our investigation demonstrated that BPL can be a candidate to inactivate FMD serotype O successfully as it showed better kinetics as compared to Formalin and BEI.

## Conclusion

BPL showed the highest inactivation rate, followed by BEI and FA. Statistically significant differences (p=0.000) were observed among the inactivation kinetics of all treatment groups used in this study. Inactivation rates were notably higher for BPL (BPL 37°C > BPL 4°C >BEI 37°C > BEI 4°C >FA37°C >FA4°C) and temperature plays a more critical role than concentration of inactivating agent especially when treatment was done at 37°C. Further studies should be conducted to determine the effects of BPL on post immunization antibody titers in animals.

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### Contribution of Authors

Iqbal MA: Executed the research work and wrote the manuscript, provided technical guidance and revised the manuscript before final submission.

Sarwar N: Conceived the idea and supervised the work, edited and approved final draft.

Raza S, Firyal S & Akram AM: Literature review and data interpretation.

Riaz R, Khan AR, Sarwar M & Arshad MI: Analyzed the data.

Munir R: Provided technical guidance and revised the manuscript before final submission.

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