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Original article

# Preparation of purified vaccine from local isolate of foot and mouth disease virus and its immune response in bovine calves

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ABSTRACT

Foot and Mouth Disease (FMD) is globally pandemic which badly affect the economics of livestock based countries like Pakistan. There are different types of Foot and Mouth Disease Virus (FMDV) among these types O is most prevalent in Pakistan. Recently Pakistan is producing approximately fifteen million doses of non-purified FMD vaccine against the demand of 160 million doses annually. More over the Pakistan is still striving for the development and optimization of concentration as well as purification of FMDV. The present project was designed to develop the technology for the purification of FMDV indigenously. The locally isolated and adapted FMDV type O virus was propagated on adherent culture of BHK-21cells to get final volume of virus one liter. This virus suspension was concentrated by peggylation as well as ultra-filtration method. The purification and quantification of concentrated virus was done by size exclusion chromatography. The results showed that peggylation is better method of concentration up to 603.75 μg/ml with 82.80 % recovery rate than ultra-filtration with 43.90 % followed by chromatography for purification. The $PD_{50}$ was calculated in bovines at 24, 12, 6, 3 and 1.5 μg of FMDV Ag/dose and it revealed that antigen load of 1.98 μg is the dose, where the 50 % of inoculated animals showed the protective antibody level based upon percent inhibition through antibody detecting ELISA. According to the British pharmacopeia, the vaccine should contain $3PD_{50}$ which found equivalent to our findings about 6 μg/dose. The group of animal injected with 6/dose (3.23PD50) showed protective titer up to 20th week post priming.



## 1. Introduction

Foot and mouth disease (FMD) is a virally originated, highly contagious and economically damaging disease of cattle, buffalo, goat, sheep and pigs (Jamal and Belsham 2013). The infected animal shows fever, ptyalism, lameness and development of vesicles in mouth, feet, teats (Arzt et al., 2011). The direct economic losses caused by the disease includes reduced in production, mortality in young animals (Ferrari et al., 2014), while indirect losses are reduced draught capacity, still birth, abortion in pregnant animals and infertility in recovered animals (Ranjan et al., 2016). Many developing countries of the Asia, Middle East and Africa are endemically infected with the disease and experience economic hindrance to the international trade in livestock and their byproducts (Barasa et al., 2008).

Foot and Mouth Disease Virus (FMDV) is the causative agent of the disease belongs to Aphthovirus genera of family Picornaviridea. The FMDV is non-enveloped, +SS RNA exist in seven immunologically distinct serotypes i.e A, O, C, Asia-1, SAT-1, SAT-2 and SAT-3 lacking cross immunity (Metwally et al., 2016). During infection, the FMDV produces four types of structural proteins i.e $VP_1$, $VP_2$, $VP_3$, $VP_4$ and ten types of non-structural proteins (NSPs) i.e $L^{pro}$, 2A, 2B, 2C, 3A, $3B_1$, $3B_2$, $3B_3$, $3C^{pro}$, $3D^{pol}$ including some cleavage intermediates (Cao et al., 2016).The structural proteins each having sixty copies forms the capsid of FMDV, while NSPs are regulatory proteins mostly exist in combinations like 3ABC participating in replicatory and other functions within host cell (Liu et al., 2017). $VP_1$ is highly variable polypeptides and involved in inducing neutralizing antibodies in host against the disease (Belsham 2005). The new subtypes evolved within serotype in a particular region

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make the control of disease difficult (Knowles and Samuel 2003). In Pakistan, serotype O is more prevailing (70 %) than Asia-1 (25 %) and A (4.67 %) causing economic losses more than PKR. 6.00billion annually (Nawaz et al., 2014).

Regular vaccination of animals with high quality and more potent efficacious vaccine is the major cornerstone for the elimination of disease in endemic regions (Waters et al., 2018). In Pakistan, vaccine production units are producing whole cell culture vaccine through amplification of FMDV on BHK-21 cells and then inactivated with BEI. Following inoculation of whole cell culture vaccine, antibodies against both types of proteins i.e structural and NSPs are produced. Neutralizing antibodies induced against structural proteins protect the animals from infection (Kitching et al., 2007), while anti-NSP are considered as hallmark of natural infection (Uttenthal et al., 2010). Purification of intact FMDV from its NSPs is a crucial step for the production of high quality vaccines because naturally infected and vaccinated animals are distinguishable based upon the presence of anti-NSP antibodies during serological surveillance (Park et al., 2022).

The efficacy of vaccine used in FMD eradication programs depends upon the structural stability, infectivity, antigenicity and quantity of FMDV particles in vaccine (Yang et al., 2015). Potency rigorously meant for the vaccine's ability to elicit the protective immunity required for $PD_{50}$ analysis is carried out as per WOAH Manual section 5.3 and European Pharmacopeia monograph 01/2017:0063 (Anonymous 2006). Potency testing on vaccine is performed either in vitro or in vivo for determining the vaccine's capability for conferring the protective immunity (Taffs 2001). Estimation of vaccine potency in vivo is highly expensive experiment because of maintaining high bio-security of animal facilities to safeguard the premises from challenging infection, unprotected animals will face painful clinical manifestation of the disease and even protected animals will show lesions at live virus inoculation (Goris et al., 2008a). Instead of in vivo challenge phase, serological assays such as ELISA and virus neutralization test (VNT) can be used to assess the expected ex vivo in vitro protection percentage against virulent pathogen reducing the number of required animals and level of suffering (Alkan et al., 2008; Ferrari et al., 2016).

In current project the local isolate of FMDV type O was cultivated on BHK-21 cells and concentrated the cell culture harvest through precipitation with polyethylene glycol and ultrafiltration through membrane. The concentrated FMDV was purified from NSPs through size exclusion chromatography and five different types of purified monovalent FMD vaccines containing different concentration of FMDV Ag were prepared. The $PD_{50}$ and efficiency of locally produced purified FMD vaccine was determined in bovine calves through solid phase competitive ELISA, VNT and 3-ABC NSP ELISA.

## 2. Materials and methods

### 2.1. Collection and serotyping of FMDV

Locally isolated FMDV type O collected from Quality Operation Laboratory (QOL); University of Veterinary and Animal Sciences (UVAS) Lahore was characterized through reverse transcriptase polymerase chain reaction (RT-PCR). Genome of FMDV (RNA) was extracted by TRIzol reagent (Thermo scientific) and used as template for synthesis of cDNA through commercially available Revert Aid first strand cDNA kit (Thermo scientific). Quality of RNA and cDNA was ensured by determining the ratio of OD at 260/280 through scientific Nano-drop 2000/2000c spectrophotometer (USA). The cDNA was amplified through RT-PCR by using commercially available Dream Taq green PCR master mix (Thermo scientific) and FMDV type O specific primers (Macrogen 639 bp) at pre-optimized conditions. The size of amplicon was confirmed followed by gel electrophoresis (Stear 2005).

### 2.2. Establishment of BHK-21 cell monolayer

Cryopreserved Adherent Baby Hamster Kidney cell culture (BHK-21) collected from QOL were revived and propagated in Glasgow's Minimum Essential Medium (GMEM) (Caisson, USA) with 10 % bovine calf serum. The cell culture flasks of 75 $cm^2$ capacity were seeded at density of $10^5$ cells /ml and incubated in $CO_2$ incubator at 37 °C for 36, 48 and 72 h. Following incubation, the cell monolayers were disaggregated with trypsin (GIBCO, 0.25 %), stained with trypan blue dye (0.4 %) and viability was determined through haemocytometer as followed.

$$\%\ viability = \frac{No.\ of\ viable\ cells \times 100}{Total\ No.\ of\ cells}$$

Cell suspensions with maximum viability were transferred into roller bottles of 1900 $cm^2$ capacity at density as said above for establishing monolayer in bulk quantity (Freshney 2015; Park et al., 2021).

### 2.3. Propagation of FMDV type O on BHK-21 monolayer

Following establishment of confluent monolayer, the exhausted media from roller bottles was discarded and cells were washed with PBS three times. The cells were inoculated with FMDV type O and incubated at 37 °C/30 min for better interaction. The infection media (GMEM with 1 % serum) was poured into FMDV infected bottles and incubated in $CO_2$ incubator for 24 h. The bottles were observed under inverted microscope for development of cytopathic effects (CPEs) as a result of FMDV replication. Following obtaining optimal CPEs, the bottles were freeze and thaw repeatedly 3–4 times for the release of FMDV. The FMDV infected cell culture suspension was collected, centrifuged at 1000 rpm/10 min and stored the supernatant at −40 °C till further use (Huang et al., 2011).

### 2.4. Biological titration

The harvested FMDV was serially diluted 10-fold in infection media. Following the development of confluent monolayer in 96-well plate, the exhausted media was replaced with 100 µl serially diluted FMDV and incubated in $CO_2$ incubator 24 h. The results were recorded by observing CPEs in 50 % of infected wells as an end point. Biological titer of harvested pool of FMDV was determined in 10Log of tissue culture infective dose ($TCID_{50}$) as described by (Reed and Muench 1938).

$$Proportionate\ distance\ (PD) = \frac{\%positive\ at\ or\ above\ 50\% - 50}{\%\ positive\ at\ or\ above\ 50\% - \%\ positive\ below\ 50\%}$$

50 % endpoint titer ($TCID_{50}$/0.1 ml) = 10Log of dilution above 50 % + $PD^{*log\ (dilution\ factor)}$.

### 2.5. Inactivation and concentration of FMDV suspension

The harvested FMDV suspension was subjected to standard chemically inactivation process with binary ethyleneimine (BEI, MP BioMedicals)) and formaldehyde (MP BioMedicals) to final concentration of 3 mM & 0.04 % respectively (Harvey et al., 2022). The residues of inactivating reagents were neutralized with 2 % w/v of each of sodium bisulfate & sodium thiosulfate respectively (Barteling and Cassim 2004).

The half of inactivated FMD vaccine antigen was concentrated up to 1/10th of original volume through precipitation with polyethylene glycol (Daejung, PEG-6000) (7.5 % w/v). The mixture was stirred at 4 °C, centrifuged and pellet was collected. The FMDV was eluted by re-suspending the pellet in phosphate buffer saline (PBS) to a volume 1/10th of original volume (Jackson et al., 2021). The remaining half of inactivated FMDV was filtered through ultra-filtration (UF) membrane having 300 kDa (BIOMAX) molecular weight cutoff. The retainate of UF membrane left 1/10th of original volume was collected (Kim et al., 2019). Both PEG treated and UF retenate were run through size exclusion chromatography for estimation of FMDV yield.

### 2.6. Purification and estimation of FMDV

The BioRad Biologic LP chromatograph system 358-BR3506 with UV monitor (2 mm path length) at 254 nm fitted with BioRad Econo-column 15/50 was used for the purification and estimation FMDV in PEG treated, UF retenate and compared with non-concentrated inactivated FMDV samples as control. The column was filled with sepharcrlye S-300 (GE Healthcare) resin and equilibrated with mobile phase (phosphate buffer 0.01 M). The chromatographic run of above said samples were performed at already optimized conditions (1st component of project).The absorbance of FMDV with extinction coefficient $E_{1cm}{}^{1}$ % 72 in each sample recorded at 254 nm was appeared in the form of chromatogram. The chromatogram of each sample obtained at chart speed of 12 cm/hour was analyzed for the estimation of FMDV (μg/ml) (Doel et al., 1981) by following formula as described by (Rweyemamu et al., 1989; Spitteler et al., 2011).

$$Concentration\ of\ FMDV\ (\mu g/ml) = \frac{FR \times PA \times FSD \times 1000}{S \times PL \times E \times W}$$

Whereas FR is flow rate of mobile phase, PA area under peak, FSD full scale absorbance unit setting, S chart recorder speed, PL path length of the flow cell, E extinction point for FMDV, W sample volume. Purity of eluted FMDV was ensured through SDS PAGE and 3ABC-NSP ELISA following raising hyper immune serum in goats (Results not shown here).

### 2.7. Formulation of purified inactivated monovalent FMD vaccines and quality control

The chromatographic elute of PEG treated sample was diluted to obtain purified FMDV stock (24 μg/ml). Five types of purified monovalent FMD vaccines viz $B_1$, $B_2$, $B_3$, $B_4$ & $B_5$ containing FMDV to be 24 μg, 12 μg, 6 μg, 3 μg and 1.5 μg per 2 ml dose respectively were prepared. Fixed quantity of Montanide ISA 206 was added with each batch of vaccine at 1:1 ratio and final volume adjusted with sterile PBS (El-Sayed et al., 2012a). The in-house quality of vaccine *i.e* sterility, safety and innocuity was ensured through standard protocols (WOAH 2018).

### 2.8. Animal inoculation and collection of serum samples

The calves (above 5 months age) of cholistani cattle breed were used for the estimation of potency testing of purified monovalent FMD vaccine. Prior inoculations, animals under experiment were screened for the presences of antibodies against FMDV type O through solid phase competitive ELISA (SPCE) and anti-NSPs antibodies through 3-ABC NSP ELIA (Roeder and Knowles 2009). The animals found non protective were selected for potency testing and divided into five groups viz $G_1$, $G_2$, $G_3$, $G_4$ & $G_5$ each containing five animals. The animals of specific groups were inoculated at day 1 and booster at 4th week with 2 ml vaccines containing 24 μg, 12 μg, 6 μg, 3 μg and 1.5 μg FMDV antigen respectively.

### 2.9. Estimation of protective dose ($PD_{50}$)

Sera samples from animals of all groups were collected at 4th weeks post booster inoculation and analyzed for neutralizing antibodies through SPCE by using commercially available ELISA kit (PrioCHECK™ FMDV Type O Ab Strip Kit, Cat # 7610420). The sera samples with percent inhibition (PI) < 50 % and ≥ 50 % were considered as negative and positive respectively as per manufacturer instructions. Based upon PI, type of vaccine containing minimum concentration of FMDV eliciting 100 % protection was found and $PD_{50}$ of that vaccine type was estimated as described by Spearman-Karber's method (Doel et al., 1997; Goris et al., 2008a; Alkan et al., 2008; Ferrari et al., 2016).

$$PD_{50}\ (Log10) = -[Xo - D/2 + D\ (\Sigma R/N)].$$

Where, $X_0$ is Log10 of reciprocal of lowest concentration of antigen at 100 % protection: D is the Log 10 of dilution factor; N is total animals in each group: R is protected out of total animals.

### 2.10. Protective potential of purified FMD vaccine

The animals of specific group inoculated with lowest concentration of FMDV but eliciting 100 % protection were maintained for determining the protective potential. Serum samples of this group were collected at zero day, 4th weeks, 8th weeks, 12th weeks, 16th weeks and 20th weeks post primary inoculation were analyzed through SPCE and virus neutralization test (VNT) for the determination of protection against FMDV type O (Bazid et al., 2016; Gamil 2010). Anti-NSP antibodies in sera samples were determined by using commercially available test kit (IDEXX FMD 3ABC Bo-Ov). The sera samples with NSP % < 20 % and ≥ 30 % were considered as negative and positive respectively as per manufacturer instructions.

### 2.11. Virus neutralization test

Two fold dilutions of pre-heated sera samples started from 1:10 were prepared in 96-well plate by using infection media. The fixed quantity of 100 $TCID_{50}$ FMDV was added in each sera dilution for preparation of infection mixture. The infection mixture was inoculated in BHK-21 cell monolayer grown in flat bottom cell culture plate and incubated at 37 °C for 24 h. Following incubation, the cells were stained with 0.05 % methylene blue prepared in 10 % formalin solution. The reciprocal of highest test serum dilution neutralizing 100$TCID_{50}$ FMDV in 50 % of infected wells was considered as end point titer. The titer determined in<1.2 Log10 of GMT was considered as non-protected and more than 1.65 as protected (WOAH 2018).

## 3. Results

### 3.1. Serotyping of FMDV

The RNA from FMDV was successfully extracted and used as template for cDNA synthesis. The 260/280 ratios of extracted RNA and respective cDNA were 1.92 & 1.74 respectively as shown in Fig. 1a and 1b. The 260/280 ratios were within acceptable limits. The cDNA was amplified through PCR by using serotype O specific primer and yielded similar amplicon size of 639 bp, which confirmed the molecular characterization of FMDV belongs to serotype O as shown in Fig. 1c.

### 3.2. Establishment of BHK-21 cells and propagation of FMDV type O

Initially the roux flasks (25 $cm^2$) were seeded at cell density of $10^5$ cells /ml and incubated for different time intervals. The cells

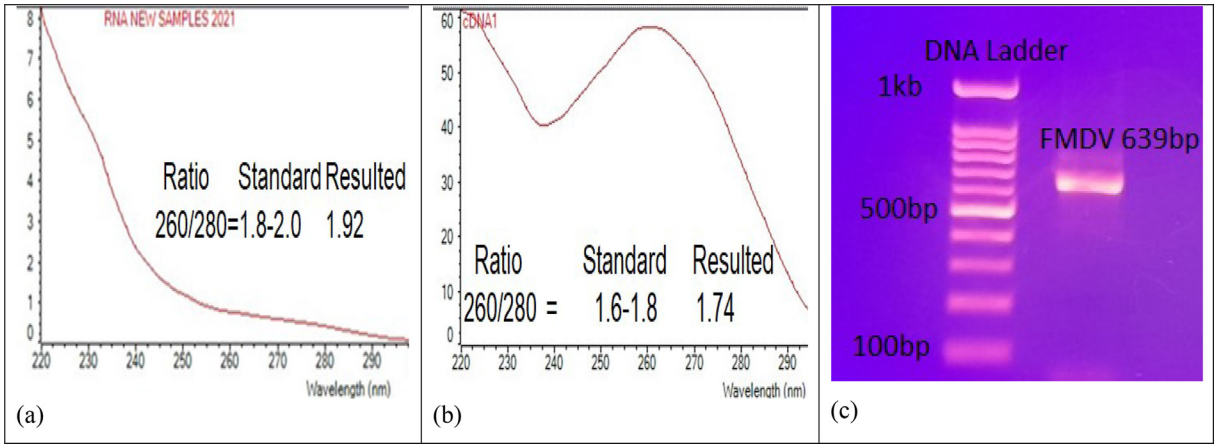


**Fig. 1.** 260/280 ratio for RNA (a); cDNA (b); RT-PCR amplicon of FMDV type O (c).

monolayer developed at specific incubated time periods were disaggregated and viability of cell suspension was determined through dye exclusion method. The viable cells didn't retain the dye and appeared colorless as shown in Fig. 2 (a). The viability of cells at zero time, 24hrs, 36hrs, 48hrs and 72hrs of incubation found 68 %, 74.31 %, 88.31 %, 78.57 % and 58.16 % respectively. The results were analyzed statistically through completely random design (CRD) and p value found 0.000 interpreting 36hrs of incubation has significant effect on viability.

The BHK-21 cells with maximum viability were transferred and propagated in roller bottles of 1900 $cm^2$. The confluent monolayers in roller bottles as shown in Fig. 2 (b) were infected with FMDV type O. Replication of FMDV in infected cells was ensured by appearance of CPEs including rounding & flattening of infected cells, breakdown of intracellular bridges and finally cell death within 15 h of infection and 80–90 % cell death observed in 24 h as shown in Fig. 2 (c). Following repeatedly freeze and thaw of FMDV infected bottles, the cell suspension was harvested and stored at −40 °C till further use.

### 3.3. Biological titration

Biological titer in terms of $TCID_{50}$ quantified the FMDV to produce CPEs in 50 % of infected BHK-21 cells. The infectious FMDV type O titer was calculated as shown in Table 1.

### 3.4. Confirmation of inactivation and concentration of FMDV

The inactivation of FMDV was ensured in-vitro by consecutive seven blind passages on BHK-21 cells. The monolayers were observed under inverted microscope and found no development of CPEs up to 7th blind passages. About 500 ml of inactivated FMDV was concentrated through precipitation with PEG and 500 ml through UF membrane. The FMDV in PEG pellet was eluted in 50 ml of elution buffer by reducing the 1/10th of original volume, similarly 50 ml retenate left in sample jar of UF assembly were collected and run through size exclusion chromatography (SEC) for purification and estimation of FMDV.

### 3.5. Comparative efficiency of concentration methods on the recovery of 146S

The symmetric chromatograms of purified FMDV from non-concentrated as a control (Fig. 3a), UF retanate (Fig. 3b), PEG precipitated (Fig. 3c) appeared with retention time of 130.25, 134.52, 135 min were analyzed for calculation of area under peaks ($cm^2$) and found 5.25 at 0.2 FSD, 9.22 at 0.5 FSD, 12.42 at 0.7 FSD respectively. The concentration of FMDV in non-concentrated, PEG treated and UF retenate was calculated and shown in Table 2.

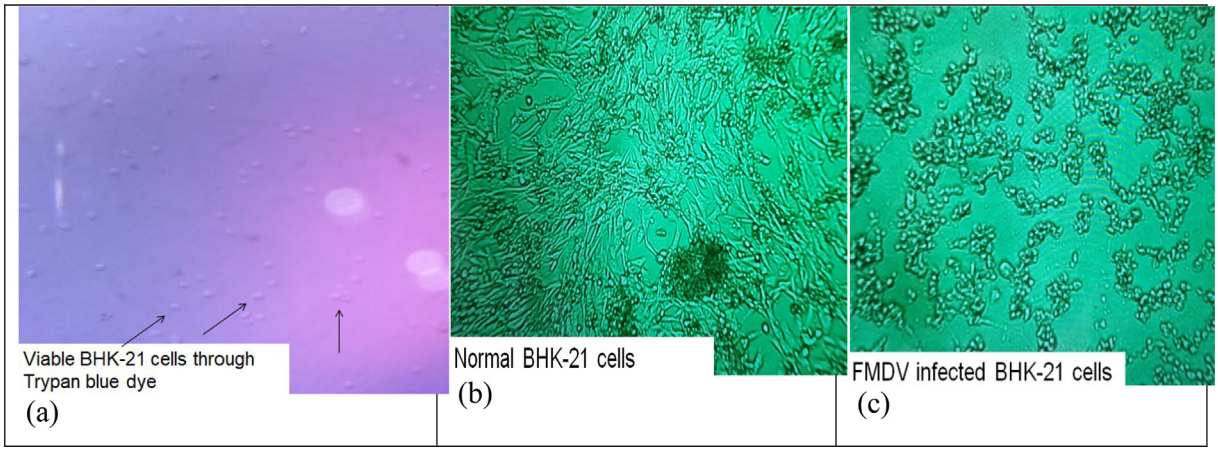


**Fig. 2.** Viable cells (a); Confluent monolayer (b); CPEs caused by FMDV infection (c).

**Table 1**
Biological titer of FMDV type O.

| Dilution of FMDV | No. of Wells with CPEs | No. of Wells without CPEs | Accumulated Numbers | | | % age of wells with CPEs | $TCID_{50}$/ml |
|---|---|---|---|---|---|---|---|
| | | | Wells with CPEs | Wells without CPEs | Total number of wells | | |
| $10^{-1}$ | 8 | 0 | 45 | 0 | 45 | 100 | $10^{7.23}$ |
| $10^{-2}$ | 8 | 0 | 37 | 0 | 37 | 100 | |
| $10^{-3}$ | 8 | 0 | 29 | 0 | 29 | 100 | |
| $10^{-4}$ | 7 | 1 | 21 | 1 | 22 | 95.45 | |
| $10^{-5}$ | 6 | 2 | 14 | 3 | 17 | 82.35 | |
| $10^{-6}$ | 5 | 3 | 8 | 6 | 14 | 57.14 | |
| $10^{-7}$ | 2 | 6 | 3 | 12 | 15 | 20 | |
| $10^{-8}$ | 1 | 7 | 1 | 19 | 20 | 5 | |
| $10^{-9}$ | 0 | 8 | 0 | 27 | 27 | 0 | |
| $10^{-10}$ | 0 | 8 | 0 | 35 | 35 | 0 | |

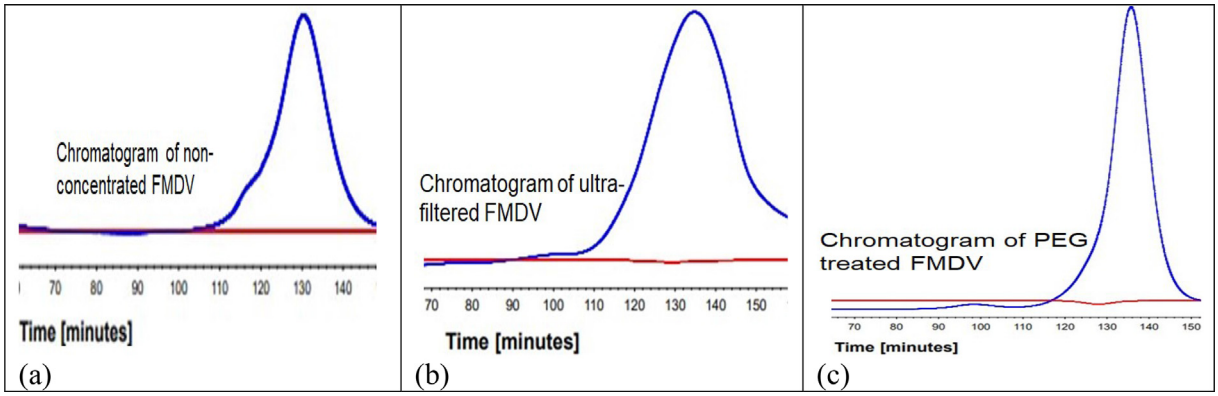


**Fig. 3.** Chromatograms of non-concentrated (a), UF retenate (b) and PEG treated FMDV (c).

The results were analyzed through CRD and depicted the significant effect of PEG precipitation concentration method with p value 0.007 over UF method.

### 3.6. Formulation of monovalent purified FMD vaccine

The chromatographic elute of PEG precipitated FMDV (603 μg/ml) was diluted to prepared 24 μg/ml stock. The stock was further serially diluted two fold and each dilution was used for the preparation of five different types of purified monovalent vaccine in following formulation as shown in Table 3.

### 3.7. Immune response of calves

Bovine calves of five different groups were inoculated with different types of vaccines and booster at 4 weeks intervals. Sera samples collected at zero day, 4th and 8th weeks post priming (WPP) were analyzed through SPCE and the results in terms of percent inhibition (PI) are shown in Table 4.

The vaccines containing different viral loads (24, 12, 6, 3 and 1.5 μg/dose) gave different protection %age as shown in Table 5.

**Table 2**
Comparative efficiency of FMDV concentration methods.

| FMDV sample | Concentration of FMDV (μg/ml) | Recovery % |
|---|---|---|
| BEI inactivated non-concentrated (1X) | 72.91 | – |
| PEG precipitated (10X) | 603.75 | 82.80 |
| UF concentrated (10X) | 320.14 | 43.90 |

The vaccine type $B_3$ having minimum viral load elicited 100 % protections in calves. The $PD_{50}$ of this vaccine type was calculated and found as per standard value (PD50 $\geq$ 3).

$PD_{50}$ (Log10) = —[0—0.3010/2 + 0.3010 (11/5)].

= 0.51.

= 3.23$PD_{50}$ / dose.

### 3.8. Protective potential of purified FMD vaccine type $B_3$

The bovine calves of G3 were kept under observation and serum sampling was performed at 0 times, 4th, 8th, 12th, 16th and 20th weeks post priming. Antibody titer was estimated through SPCE and VNT. The same samples were also tested for anti-NSP antibodies for the confirmation of purification of vaccinal seed lots.

The percent inhibition (PI) through SPCE, 10Log of GMT through VNT and NSP % through 3-ABC NSP ELISA are shown in Tables 6-8 respectively. The results were analyzed statistically through CRD and it revealed the significant increase in immune response with p value 0.000 up to 20th week post booster inoculation. The NSP % in serum remained below than cut off value (<20 %) hence found negative.

## 4. Discussion

FMD causes heavy economic losses in in terms of decline in milk production, reduced working capability of draught animals, reduction in weight gain leading to reduced production of meat. The byproducts of animal origin including milk, meat, skin and hides from FMD endemic countries are not allowed to export into disease free countries (Singh et al., 2013). Inoculation of susceptible animals with highly potent vaccine is limiting factor to control and

**Table 3**
Formulation of purified monovalent FMD vaccine.

| Type | FMDV (µg)/dose | Ingredients (ml) | | | Total volume of vial / doses |
|---|---|---|---|---|---|
| | | Antigen | Montanide Oil | PBS | |
| $B_1$ | 24 | 15 | 15 | – | 30/15 |
| $B_2$ | 12 | 7.5 | 15 | 7.5 | 30/15 |
| $B_3$ | 6 | 3.75 | 15 | 11.25 | 30/15 |
| $B_4$ | 3 | 1.9 | 15 | 13.1 | 30/15 |
| $B_5$ | 1.5 | 0.95 | 15 | 14.05 | 30/15 |

**Table 4**
Pre and post vaccination percent inhibition (PI) through SPCE.

| Vaccine Type / (µg/dose) | Group of Animals | Animal Tag No. | SPCE (PI) | | |
|---|---|---|---|---|---|
| | | | 0 day | 4WPP | 8WPP |
| $B_1$ /24 | $G_1$ | 11 | 36.29 | 59.35 | 84.31 |
| | | 12 | 34.67 | 55.41 | 81.47 |
| | | 13 | 29.58 | 56.72 | 83.89 |
| | | 14 | 31.58 | 53.92 | 78.35 |
| | | 15 | 33.86 | 54.28 | 85.25 |
| $B_2$ /12 | $G_2$ | 21 | 28.65 | 52.9 | 78.46 |
| | | 22 | 30.98 | 57.36 | 81.72 |
| | | 23 | 39.81 | 53.48 | 79.53 |
| | | 24 | 26.96 | 56.22 | 82.39 |
| | | 25 | 27.97 | 54.59 | 81.91 |
| $B_3$ /6 | $G_3$ | 31 | 32.53 | 56.27 | 82.93 |
| | | 32 | 29.64 | 55.9 | 86.69 |
| | | 33 | 28.52 | 56.31 | 75.64 |
| | | 34 | 25.87 | 54.28 | 79.58 |
| | | 35 | 32.47 | 57.82 | 84.37 |
| $B_4$ /3 | $G_4$ | 41 | 24.82 | 32.37 | 41.92 |
| | | 42 | 36.79 | 53.29 | 74.8 |
| | | 43 | 22.78 | 55.79 | 76.35 |
| | | 44 | 27.94 | 34.82 | 60.37 |
| | | 45 | 31.68 | 55.47 | 73.29 |
| $B_5$ /1.5 | $G_5$ | 51 | 35.93 | 43.31 | 68.94 |
| | | 52 | 39.54 | 52.37 | 72.62 |
| | | 53 | 29.8 | 37.82 | 48.74 |
| | | 54 | 26.97 | 30.59 | 40.94 |
| | | 55 | 30.73 | 34.07 | 39.87 |
| Control | $G_6$ | C1 | 38.69 | 32.4 | 39.26 |
| | | C2 | 35.83 | 31.59 | 28.72 |

**Table 5**
$PD_{50}$ of FMDV type O.

| Group ID | FMDV Type / (µg/dose) | PC | NPC | CPC | CNPC | Protection % |
|---|---|---|---|---|---|---|
| $G_1$ | $B_1$ /24 | 5 | 0 | 21 | 0 | 100 |
| $G_2$ | $B_2$ /12 | 5 | 0 | 16 | 0 | 100 |
| $G_3$ | $B_3$ /6 | 5 | 0 | 11 | 0 | 100 |
| $G_4$ | $B_4$ /3 | 4 | 1 | 6 | 1 | 85.71 |
| $G_5$ | $B_5$ /1.5 | 2 | 3 | 2 | 4 | 33.33 |

(PC: protected calves, NPC: non-protected calves, CPC: cumulative protected calves, CNPC: cumulative non-protected calves).

**Table 6**
Pre and post vaccination percent inhibition determined through SPCE.

| Animal Group | Weeks post primary vaccination | Animals inoculated | Animal Tag #/ PI % | | | | | Mean PI % |
|---|---|---|---|---|---|---|---|---|
| $G_3$ | Zero day | 5 | 31/32.53 | 32/29.64 | 33/34.79 | 34/25.87 | 35/32.47 | 31.06 |
| | 4 | 5 | 31/56.27 | 32/55.90 | 33/56.31 | 34/54.28 | 35/57.82 | 56.11 |
| | 8 | 5 | 31/82.93 | 32/86.69 | 33/75.64 | 34/79.58 | 35/84.37 | 81.84 |
| | 12 | 5 | 31/86.49 | 32/90.38 | 33/80.62 | 34/85.89 | 35/87.42 | 86.16 |
| | 16 | 5 | 31/78.79 | 32/83.27 | 33/76.29 | 34/82.66 | 35/80.48 | 80.29 |
| | 20 | 5 | 31/72.84 | 32/78.52 | 33/70.57 | 34/77.82 | 35/74.32 | 74.81 |

eliminate the spread of virus. The endemic countries need to maintain highly concentrated FMDV antigen banks to formulate vaccine with suitable potency to combat the disease outbreak. The vaccine manufacturer units are pressurized to produce purified vaccine free from NSPs to distinguish vaccinated from infected animals. Potency testing of vaccine is an expensive matter based on protection in vivo such as toxin-neutralization, immunization-challenging or immunization-serological tests. Scientific consideration and animal welfare concerns have established 3R-concept discouraging extensive use of animals in challenging phase which

**Table 7**
Post vaccination immune response determined through VNT.

| Animal Group | Weeks post primary vaccination | Animals inoculated | Animal Tag #/ VNT titer | | | | | 10Log GMT |
|---|---|---|---|---|---|---|---|---|
| $G_3$ | Zero day | 5 | 31/20 | 32/10 | 33/10 | 34/10 | 35/20 | 1.12 |
| | 4 | 5 | 31/80 | 32/40 | 33/80 | 34/80 | 35/80 | 1.78 |
| | 8 | 5 | 31/160 | 32/160 | 33/160 | 34/160 | 35/80 | 2.14 |
| | 12 | 5 | 31/160 | 32/160 | 33/80 | 34/160 | 35/80 | 2.08 |
| | 16 | 5 | 31/80 | 32/80 | 33/80 | 34/160 | 35/80 | 1.96 |
| | 20 | 5 | 31/80 | 32/40 | 33/40 | 34/80 | 35/40 | 1.72 |

**Table 8**
Post vaccination NSP% determined through 3ABC NSP ELISA.

| Animal Group | Weeks post primary vaccination | Animals inoculated | Animal Tag #/ NSP% | | | | | Mean NSP% |
|---|---|---|---|---|---|---|---|---|
| $G_3$ | Zero day | 5 | 31/6.94 | 32/7.42 | 33/5.71 | 34/6.82 | 35/6.90 | 6.75 |
| | 4 | 5 | 31/7.12 | 32/8.51 | 33/6.38 | 34/7.18 | 35/7.61 | 7.36 |
| | 8 | 5 | 31/8.26 | 32/9.39 | 33/8.92 | 34/8.27 | 35/7.31 | 8.43 |
| | 12 | 5 | 31/7.97 | 32/9.16 | 33/7.34 | 34/7.53 | 35/7.25 | 7.85 |
| | 16 | 5 | 31/6.38 | 32/6.24 | 33/7.18 | 34/6.81 | 35/7.50 | 6.82 |
| | 20 | 5 | 31/6.17 | 32/6.64 | 33/6.43 | 34/6.71 | 35/6.29 | 6.44 |

causes distress, pain and supporting use of alternative serological tests for assuring vaccine potency (Romberg et al., 2012). Several researchers have developed alternative approaches by using live animals and in-vitro models to find correlation between 146S content load in vaccine and protection elicited (Alkan et al., 2008).

Preparation of cell cultured based purified vaccine is a basic need to control the disease in endemic regions for which propagation and maintenance of cell line is necessary. The BHK-21 is a continuous cell line widely used for the adaptation, propagation and cultivation of FMD Virus for vaccine production. In this study, viability of cells and development of monolayer reached maximum 88.13 % & 80–90 % at 36 h, later on it starts to decline and reached 58.13 % & 50–60 % at 72 h of incubation respectively. The results were in accordance with that of (Harvey et al., 2022). The FMDV type O was propagated on BHK-21 cells. The FMDV caused disruption of cell monolayer and more than 80 % CPEs were observed after 15 h of infection. These findings were in accordance with that of (Alam et al., 2015; Shahiduzzaman et al., 2016). The biological titer of harvested FMDV serotype O was determined and found $10^{7.23}$/ml. The results were similar to that of (Chowdhury et al., 2016), who estimated the biological titer $10^{8.5}$/ml.

Following the propagation, FMDV suspension was harvested and inactivated with combination of BEI (3 mM) and formaldehyde (0.04 %). The inactivation was ensured with inoculation on freshly grown cells till seven blind passages. These results were in accordance with that of (Aarthi et al., 2004; Soliman et al., 2013), who used 0.1 M to 1.6 M BEI with combination of formaldehyde for inactivation of FMDV.

The inactivated harvested cell culture suspension of FMDV was concentrated ten times by 7.5 % PEG precipitation and UF system with 300KDa membrane. Using BEI inactivated non-concentrated supernatant as a control, these two concentration methods were analyzed and comparative efficiency was determined through SEC based on recovery of 146S contents for the FMD vaccine production. The purification of FMDV through ion exchange and affinity chromatography has become strenuous because these techniques could not distinguish the intact 146S from disassembled aggregates (Jangam et al., 2018). SEC is one of the suitable techniques for purification and quantification of 146S from cell culture suspension. UV absorption peak shown on chromatogram is proportional to concentration of 146S (Spitteler et al., 2011). The 146S contents quantified in BEI-FA inactivated sample, 7.5 % PEG treated and UF through SEC found 72.91 μg/ml, 603.75 μg/ml and 320.13 μg/ml respectively. The PEG precipitation method for concentration of FMD found more efficient 82.80 % than UF 43.90 % on the recovery of 146S content. These results were in accordance with that of (Spitteler et al., 2011), who used SEC for the quantification of FMDV and (Chang et al., 2012) used 300KDa membrane in UF system for the concentration of enterovirus 71 particles similar to that of 146S of FMDV. Kim et al., (2019) claimed PEG concentration method removes 99.8 % of other proteins with highest yield of 146S contents (85.4 %) than UF with low yield (33.50 %).

The chromatographic elute of PEG concentrated FMDV type O was collected, quantified and diluted with sterile PBS to obtain desired concentration. Five types of purified monovalent vaccines with different concentration of 146S were prepared and found sterile and safe during in house quality testing. No signs of toxicity or pyrexia were noted after inoculation of health and previously un-vaccinated animals. The animals inoculated with 24, 12 and 6 μg/dose of 146S found 100 % protected through SPCE, while animals inoculated with 3 & 1.5 μg/dose showed 85.71 % and 33.33 % protection respectively. In our study minimum mass of FMDV antigen equal to 1.98 & 6 μg/dose found cut off for the 50 % & 100 % protection of inoculated animals respectively to meet the requirements of WOAH to prepare vaccine with $3PD_{50}$. These findings were close to finding of (Abu-Elnaga et al., 2015; Bazid et al., 2016; Goris et al., 2008b), who suggested indirect serological tests e.g SPCE to assess the potency of vaccine rather than challenging phase and claimed 3 & 2.5 μg/dose of 146S contents for in-vivo protection closest to our findings 1.98 μg/dose. Our results of $PD_{50}$ in monovalent vaccines estimated 9.85 were in accordance with that of (Zeb et al., 2015), who calculated $7.99PD_{50}$ of FMDV type A and Hardham et al., (2020) concluded $16PD_{50}$ per dose of FMD vaccine.

The immune response elicited by FMD vaccine $B_3$ containing minimum quantity of FMDV Ag was evaluated through SPCE, VNT and NSP % through NSP ELISA. It was observed that mean of PI of $G_1$ animals was increased from 31.26 % prior inoculation to 56.11 % at 4 weeks and 81.86 % at 8 weeks post priming. The PI reached maximum 86.16 % at 12 weeks and then started to decline up to 80.29 % and 74.81 % at 16th and 20th weeks post inoculation. Although mean PI post primary inoculation was above the cut off value of 50 % post primary dose but PI was enhanced in response to booster inoculation. Same trend was observed, when sera samples were analyzed through VNT. The Log10 of GMT prior vaccination was 1.12, which was enhanced in response to primary inoculation up to 1.78. The booster inoculation significantly increased the GMT up to 2.14 at 8th weeks and started to decline 2.08, 1.96 and 1.72 at 12th weeks, 16th weeks and 20th weeks post

inoculation. Although primary inoculation elicited the protected immune response more than cut off value 1.65 but booster inoculation enhanced the immune response. Our results were similar to that of (El-Sayed et al., 2012b), who observed that FMD vaccine formulated with montanide ISA 206 adjuvant elicited higher immune response in calves than protection titer 1.5log10 and 1.9log10 and supported our results 1.78log10 and 1.83log10 at 4th week of primary inoculation as determined through VNT and ELISA respectively. Similar results were also put forwarded by (Peta et al., 2021), who noted antibody titers more than 2log10 and protective herd immunity up to 12 months when one or more booster doses are inoculated within six months of primary inoculation. Our results regarding VNT and SPCE were in accordance with that of (Peta et al., 2021), who observed that protective antibody titer against FMDV O type started to rise up to 1.53log10 after 14 days of vaccination, reached peak up to 2.241log10 after 32 days and then started to decline after 36th week of post vaccination. They also observed that SPCE titer of protective antibody titer started to rise up to 1.8log10 after 2nd week and reached peak up to 2.4log10 at 8th weeks of post vaccination. Same findings regarding levels of neutralizing antibody to be higher than protective titer 1.5log10 determined through VNT and 1.8log10 through SPCE respectively were observed by (Abd El-Rahman et al., 2007; Barteling and Vreeswijk 1991; Moussa et al., 1976).

The mean NSP % in claves of G3 prior vaccination at zero day found 6.75 %. In response to primary inoculation the NSP % increased non-significantly up to 7.36 % and 8.43 % in response to booster inoculation. It was observed that NSP % started to decline up to 6.44 % from 8th weeks to 20th weeks post priming inoculation. Our results were found in accordance with that of (Jangam et al., 2018), who stated that SEC could purify FMDV up to 94 % from its NSPs. our results regarding NSP % in chromatographic elutes were similar to that of (Park et al., 2020), who declared that heparin affinity chromatography purified the FMDV and vaccine prepared from purified fraction could not provoke anti-NSPs antibodies even after 5th vaccination.

## 5. Conclusion

Based upon the findings, it is concluded that PEG precipitation (7.5 % w/v) is more efficient down streaming method with higher recovery of FMDV antigen (82.80 %) than ultra-filtration with lower recovery rate (43.90 %). Size exclusion chromatography is most suitable technique for the purification of FMDV from its NSPs. Ex-vivo in-vitro serological test e.g SPCE and VNT are reliable for estimating the $PD_{50}$ of FMD vaccine. Based upon percent inhibition, it was observed that minimum concentration of 6 μg of FMDV / dose in vaccine is necessary for preparation of quality vaccine with recommended $PD_{50}$ eliciting 100 % protection. Booster dose of FMD vaccine could enhance the immune response, which persist up to 20th weeks post priming with non-significant increase in anti-NSPs antibodies.

### Author's contribution

Abdul Razak and Imran Altaf conceptualize, designed, executed the experiment, compiled the results and wrote the manuscript. Ali Raza awan analyzed the results. Aftab Ahmad Anjum interpreted the results, critically revised the manuscript and approved for submission.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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