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RESEARCH ARTICLE

Estimation of Protective Dose 50 of Chromatographically Purified Foot and Mouth Disease Virus Serotype A, Asia 1 and O and Immune Response of Trivalent Vaccine in Bovines

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Abstract

Foot and Mouth Disease (FMD) is an economically significant livestock disease in developing countries such as Pakistan, where serotypes A, Asia1, and O of Foot and Mouth Disease Virus (FMDV) are prevalent. Effective disease control relies on immunization using potent vaccines, and evaluation of vaccine potency in terms of protective dose 50 (PD_{50}) is a critical step during purified vaccine formulation. The present study aimed to prepare chromatographically purified vaccines of the three prevalent serotypes in different formulations and to determine PD_{50} values. Viruses were propagated in cell culture, biologically titrated, and chemically inactivated. To obtain highly purified antigens with reduced non-structural protein content and improved quantification compared with conventional methods, viral antigens were purified, and quantified using Size Exclusion Chromatography (SEC) with Sephacryl S-300 followed by purity check through SDS-PAGE. Five formulations of each serotype (16, 8, 4, 2, and 1 µg/dose) were prepared and inoculated into five animals per concentration. Serum samples were analyzed by SPCE to identify protected animals. PD_{50} values were calculated for each antigen and used to formulate a trivalent vaccine. The trivalent vaccine was evaluated for structural and non-structural proteins using SPCE and 3ABC ELISA, respectively. Results demonstrated that SEC efficiently removed NSPs, as confirmed by SDS-PAGE. PD_{50} values for serotypes A, Asia1, and O were estimated as 2.14, 1.90, and 1.63 µg/dose, respectively. Accordingly, trivalent vaccine concentrations of 6.42, 5.70, and 4.90 µg/dose ($3PD_{50}$) were formulated. The trivalent purified vaccine induced protective immune responses with less than 20% NSP content in SPCE and 3ABC ELISA.

Keywords: Size exclusion chromatography, Sephacryal S-300, SDS-PAGE, Nonstructural protein, PD_{50}, Vaccine

INTRODUCTION

Pakistan is an agricultural country and livestock (63.6% value addition to agro-sector) serve as the largest subsector contributing 14.97% to national gross domestic product (GDP) during year 2025-2026. More than eight million people are dependent with 35-40% income source from livestock sector. Food in the form of milk, meat & product as well skin & hides are obtained from livestock for the human consumption. It is also the mode of transportation and draught power in rural areas. There is diverse population of animals in Pakistan; buffaloes 47.7M (Million), cattle 59.7M, sheep 33.1M, goat 89.4M & camels 1.2M.

Economic growth, food security and poverty reduction are challenges of developing countries. These are due to the devastating diseases, natural disasters and climate change. Foot and Mouth Disease is one of the World Organization of Animal Health (WOAH) listed, trans boundary & contagious disease of more than 70 species of animals. The disease is devastating not in term of only production but also economic losses due trade restrictions. Various outbreaks have been reported in neighboring countries due to uncontrolled movements of animals. Pakistan is also facing outbreaks every year in cattle population with heavy economic losses [1].

The disease is caused by Foot and Mouth Disease Virus (FMDV), which is positive sense of single stranded RNA member of genus *Aphthovirus* of *Picornaviridae* family. The FMDV is non-enveloped, 146S particle comprised of 8500 bases and small icosahedral protein capsid of 25-30 nm in size made up of four structural proteins each having 60 copies from VP1 to VP3 of 25-34 kDa molecular weight

and VP4 of 8-10 kDa [2] . Virus produced ten nonstructural proteins during replication. The FMDV is extremely labile in vitro and converted into its monomers at above 56°C and pH below 6. The FMDV have seven distinct serotypes A, O, Asia1, C, SAT-I, SAT-II and SAT-III [3-5].

Foot and mouth disease can be controlled using various strategies including the forward and backward tracing of infection origin, strict farm quarantines, restriction on movement of animals & their products, identification & culling of infected animals and vaccination of livestock herd. Mass vaccination in FMD endemic areas is key adopted measure by many countries for controlling disease. Inactivated whole culture vaccines are being used to prevent disease. The presence of nonstructural protein (NSP) of FMDV leads to dedifferentiation of vaccinated from infected animals [6,7]. Purified vaccine free from NSPs is required for FMD controlled zone as NSP are produced by infection not by the vaccination. FMD vaccines are produced using baby hamster kidney (BHK-21) cell line. While replication in the cell the FMDV translated its genome into a polyprotein i.e. co- and post-translationally sliced into 14 mature proteins out of which 04 structural protein (SP) and 10 NSPs (Lpro, 2A, 2B, 2C, 3A, 3B1–3, 3Cpro, and 3Dpol), and some cleavage intermediates [8]. Then suspension of virus including cell debris, culture media proteins and NSPs are inactivated with binary ethyleneimine (BEI) or formalin and emulsified with adjuvant to form vaccine. These proteins along with virus produce the immune response and hallmark of differentiation between vaccinated and infected animals. As per WOAH requirement vaccine must not produce any anti-NSP antibodies after repeated immunization [9].

A potent vaccine formulation depends upon purity and quantity of antigen used. The immunogenicity of FMD vaccines is primarily associated with the presence of intact and purified 146S viral particles [10]. Therefore, concentration and purification of harvested virus are essential steps for efficient storage, quantification of antigen and development of potent vaccines. Various methods, such as precipitation of antigen using polyethylene glycol (PEG) and ammonium sulfate, and ultrafiltration through membrane with particular molecular weight cut off (MWCO) capacity, have been employed for the antigen concentration [11]. For purification and quantification of FMD virus, sucrose density gradient (SDG) ultracentrifugation has traditionally been reference method. This technique separate biomolecules on the basis of size and sedimentation characteristics using linear sucrose gradient approaches [12]. However, SDG ultracentrifugation require specialized equipment and highly skilled, involve lengthy process of 64 hours, has high operating and maintenance costs, and has a low sample volume capacity, making it difficult to adopt for vaccine production on an industrial scale [13].

To overcome these limitations Size exclusion chromatography (SEC) has emerged as an alternative approach for purification and quantification of biomolecules [14]. Compared with SDG ultracentrifugation, SEC is a simpler, more economical, and less time-consuming technique that can be readily scaled up by increasing the sample-to-column volume ratio [15]. Furthermore SEC has been successfully utilized for both purification and quantification of FMDV, and has demonstrated a significant correlation value of 0.9669 with SDG-based measurement [16]. The technique utilizes a column packed with porous resin beads where smaller molecules enter pores and elute later, while larger molecules excluded from the pores and elute earlier. These characteristics make SEC a practical and promising method for purification and quantification of FMDV antigen during vaccine production.

Potency is key parameter for evaluating the vaccine that limit the spread of disease. According to Europen Pharmacopeia (Monograpgh 04/2005:0063), vaccine potency is determined by measuring the protective dose through challenge studies in animals vaccinated with different antigen payloads. Protective dose 50 (PD_{50}) value of vaccine is well-defined by number of protective doses of antigen which protects fifty percent vaccinated population, when challenged with live virus. Value of PD_{50} equal or greater than 3 is considered as acceptable for routine vaccination, while equal or greater than 6 is recommended in emergency vaccination in the case of FMD [17,18].

Despite being the gold standard, in-vivo potency test is highly expensive experiment, labor intensive, and associated with ethical concern in FMD endemic countries like Pakistan. These experiments require maintaining high bio-containment animal facilities to safeguard the premises from challenging infection, availability of susceptible host and involve exposure of animals to virulent virus. Furthermore, approximately 50% of unprotected animals may develop painful clinical manifestation of the disease. Even the animals with protective immunity will show lesions at live virus inoculation site and reduced in appetite and weight [19].

Consequently , there is growing interest in adopting substitute techniques that lessen the usage of animals and uphold the 3Rs: replacement, reduction, and refinement [20]. As alternatives to challenge-based potency testing, serological tests like the virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA) have been thoroughly studied. ELISA and VNT significantly lessen animal suffering while estimating the expected level of protection by evaluating antibody responses

against virulent pathogen. Potency of FMD vaccine could be estimated indirectly by serological tests capable of detecting neutralizing antibodies against structural proteins of FMDV [21]. Neutralizing antibodies directed against the structural proteins of FMDV, particularly intact 146S particles, are recognized as an important correlate of protection and can provide valuable information regarding vaccine performance. However, FMD protection is influenced by multiple factors, including antigenic matching between vaccine and field strains, vaccine formulation, adjuvant composition, duration of immunity, and cell-mediated immune responses. Therefore, although serological assays are valuable tools for monitoring immune responses and predicting vaccine efficacy [22], they cannot fully replace challenge-based potency studies, which remain essential for definitive evaluation of FMD vaccine protection

So therefore, to gain access to international market for the export of FMD free livestock, meat and other commodities, it is important to ensure that vaccines used in FMD control program/campaign should meet the purity requirement with regards to their NSP contents and it contain at least minimum required Protective Doses fifty ($3PD_{50}$). The study was design to formulate monovalent purified vaccine of local serotype A, Asia1 and O of FMDV with different concentration to estimate PD_{50} as well as to evaluate anti NSP antibodies.

Material and Methods

Ethical Approval

All animal experiments were reviewed and approved by the Ethical Review Committee, Office of Research Innovation and Commercialization, University of Veterinary and Animal Sciences (approval number: No.DR/157, April 29, 2025), and conducted in accordance with the institutional guidelines for the care and use of laboratory animals.

Sample Collection and Confirmation

Vaccine serotype 'A, Asia 1, O' of FMDV was collected from Quality Operation laboratory (QOL), University of Veterinary and Animals Sciences (UVAS) Lahore. The viruses were confirmed through antigen detection ELISA kit (FMDV antigen and serotyping ELISA, the Pirbright Institute UK, Lot# 01-2021210708a).

Revival of the Cells

Cryopreserved baby hamster kidney (BHK-21) cell line was obtained from Quality Operation Laboratory, University of Veterinary and Animal Sciences. The cells were revived by propagating in Glasgow's Minimal essential medium (GMEM) (Cassion USA) with 10% fetal bovine serum (FBS) at 37°C for 24 hours incubation in T-25 flask with 5% CO_2. Next day the cells were disintegrated through trypsin (Gibco, 0.25%) to check the viable count by dye exclusion method (Trypan blue) and 10^6 cells/ml were seeded into T-275 flasks and again the incubated at the above condition to develop monolayer. After 24 h cells were checked under inverted microscope for 80-90% monolayer.

Virus Propagation

After the establishment of confluent monolayer (80-90%) in flasks the media was removed. The cells were washed with sterile Phosphate Buffer Saline at least three times. The labeled cells culture flasks were inoculated with filtered FMDV serotype O, A and Asia separately under aseptic conditions for 37°C/40 min to adsorb the virus on cell surface. The flasks were seeded with infection medium (GMEM + 1% FBS) and incubated at 37°C for 24 h (O serotype), 36 h (A & Asia serotype). Following incubation time Cytopathic effects (CPEs) were observed under inverted microscope. After optimal CPEs the flasks were freeze thaw 2-3 time for the release of FMDV from cells. The suspensions containing the FMDV were centrifuge at 1000 rpm/10 min and supernatant were collected and stored at -40°C for further used.

Biological Titration

The stored FMDV serotypes were serially diluted tenfold separately in medium with 1% FBS. Three 96 well plates with monolayer of cells were prepared. The exhausted medium was discarded and each plate was inoculated 100 µL of dilated FMDV in respective wells and up to 10^{th} well while 11 well with pure virus and 12 well with cell control. The plates were incubated at 37°C in CO_2 incubator. Following the incubation CPEs were recorded in 50% as an end point. The biological titer of FMDV serotypes were recorded as a 10_{log} Tissue culture infective dose in samples of FMDV as described by; 50% endpoint titer (TCID 50/0.1 mL) = 10_{log} total dilution above 50% + [PD*log (dilution factor)]

Inactivation of Virus

Clarified FMDV were chemically inactivated by using final concentration 3 mM binary ethyleneimine (BEI, MP Biomedical) and 0.04% formaldehyde (MP Biomedical). The residual of inactivation agents were neutralized by 2% Sodium Bis sulphate and sodium thiosulphate for BEI and Formaldehyde respectively. Sample from each inactivated virus were subjected to BHK-21 cells culture flask to check the live viral particle and incubated at 37°C for 24 & 36 h.

Concentration of Antigen Through Polyethylene Glycol (PEG-6000)

Inactivated FMDV serotype A, Asia 1 and O was divided two parts. One part of each serotype was concentrated using PEG-6000 (Deajung, Korea). 100 mL of each

virus was inoculated 7.5% PEG from 50% concentrated solution (Tris Buffer, pH 7.2) and suspension was placed at stirring at 4°C. The suspension was centrifuged and pelted. Supernatant was discarded and elution of antigen was done using PBS (0.011M, pH 7.2). 1/10 of the original volume of antigen of each virus was obtained and stored at 4°C for further action.

Purification and Quantification of FMDV by Size Exclusion Chromatography (SEC)

The antigen was purified and quantifed using BioRad Biologic Chromatogrph system 358-BR3506 with UV montor (2 mm path length) at 254 nm. Biorad Econo Column model 15/50 was filled with resin Sephacryl™ S-300 high resolution (Cytiva, Germany) using PBS 0.011M pH 7.2 and resolution was monitored using solution of Blue dextron (BD) and Bovine Serum Albumen (BSA) with optimized parameter like flow rate (FR = 0.75 mL/min), bed height (48cm), sample volume (W=3.5 mL), path length of flow cell (PL = 0.2 cm), chart recorder speed 12 cm/h (S = 0.2 cm/min). The concentrated sample of each virus was run on chromatographic system and eluted was collected and stored at 4°C for vaccine preparation used. The chromatogram (peaks) of each virus was were recorded 254 nm with $E_{cm}1\%$ =72; different sensitivity absorbance unit (FSD) used for the estimation of FMDV in µg/mL using formulae;

$$\text{FMDV in sample (µg/mL)} = \frac{FR \times PA \times FSD \times 1000}{S \times PL \times E \times W}$$ [23]

Purity Confirmation of FMDV

The chromatographic elute of FMDV was run on Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, resolving gel and stacking gel was prepared at concentration 15% & 5% respectively. Sample of chromatographic elute and concentrated was loaded with prestained protein marker (10-180kDa, Servicebio, China) onto well and with Tris-Glycine buffer at 190V for continuous current. The size of band pure and crude FMDV was compared with protein marker.

Vaccines Formulation and Quality Control

Monovalent purified inactivated vaccine of each serotype with different dilution was prepared. For this purpose, 50% of aqueous phase containing quantified contents of 146S fraction of FMDV with 50% of montanide oil ISA 50 (France) as adjuvant was emulsified for each antigen. Five different dilutions of each virus ('A' serotype as A_1 A_2 A_3 A_4 A_5), ('Asia 1' as B_1 B_2 B_3 B_4 B_5) & ('O' as C_1 C_2 C_3 C_4 C_5) at 16, 8, 4, 2 & 1 µg/dose was adjusted with final dose volume 2 mL sterile PBS. Thiomersal sodium was added at 0.01% as preservative. Each vaccine was mixed thoroughly with sterilized homogenizer at room temperature to get consistent vaccine suspension. The prepared purified inactivated vaccine was evaluated for its safety, sterility, Innocuity and potency testing as described by [9]. Physical parameters of the vaccines like viscosity, stability and emulsion type were validated as described by [24].

Selection of Calves and Inoculation of Vaccines and Serum Collection

A total of seventy-five healthy, adult and unvaccinated bovine calves (above 5 months of age, 25/group, 5/dilution/vaccine type) of randomly selected breed raised at private cattle farm at FMD free region Bahawalpur, Punjab Pakistan were selected randomly and used for the estimation of potency testing of purified monovalent FMD vaccines. The calves were screened for the presences of antibodies against FMDV type through solid phase competitive ELISA (SPCE). The animals found non protective against FMDV were selected for potency testing and divided into four main group A, B, C & D with each subgroup for each dilution ('A' serotype as A_1 A_2 A_3 A_4 A_5), ('Asia 1' as B_1 B_2 B_3 B_4 B_5) & ('O' as C_1 C_2 C_3 C_4 C_5) and D (Control group, 2/each group). Each group was inoculated with primary dose and following 28 days with booster dose of vaccine from each dilution. All animals of each group were kept under observation for five days post vaccination. Any kind of allergic reaction, edema formation, erythema, changes in body temperature, change in feed intake or any other symptoms were observed. The serum of all animals was collected after 28 days of booster inoculation and tested for the presence of antibodies.

Solid Phase Competitive ELISA (SPCE)

The commercially available blocking ELISA (PrioCHECK™ FMDV Type A, Type Asia 1 and Type O Ab Strip Kit) was used for the detection of antibodies against FMDV. Each ELISA micro plate was coated with non-infectious FMDV type antigen. The reaction between FMDV type and a specific monoclonal antibody (mAb) was blocked by specific antibodies that were present in the test serum of bovine calves and rests of the steps were performed according instruction. For serotype (A, Asia 1), PI = 100- (OD_{450} sample/OD_{450} Max.) *100 (OD450 max = control serum (Mean OD ≥1.00). For serotype O, PI = 100- (Corrected OD_{450} sample/Corrected OD_{450} Max.) *100 (Whereas Corrected OD450sample = (OD450 sample-Blank, Corrected OD450Max is control serum). PI<50% is considered negative for FMDV while PI≥50% is considered positive for FMDV.

Determination of Mass Load of FMDV and Estimation of Protective Dose 50 (PD_{50})

The test sera of animals vaccinated with FMD vaccine having five different concentrations of the 146S fractions for each serotype were declared protective or non-protective based upon the percent inhibition of solid

phase competition ELISA. The minimum concentration of the 146S fraction which elicits 100% protective immune response in calves was determined. Apart from this number of protective doses 50 (PD_{50}) of monovalent FMD vaccine containing minimum concentration of FMDV Ag but eliciting 100% protection in group of animals based upon SPCE according to WOAH was estimated as described by Spearman-Karber's method.

PD_{50} (log10) = - [Xo – D/2 + D (Σ 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.

Trivalent Vaccine Formulation and Protective Immune Response

Trivalent vaccine ($3PD_{50.}$) containing purified antigen Serotype A, Asia 1 & O with concentration 6.42 µg, 5.7 µg and 5µg respectively was formulated with 50% montanide ISA 50 oil adjuvent with standard protocol by adjusting final volume 2 mL/dose. After quality control testing, vaccine was inoculated to five healthy, non-vaccinated calves with primary and booster dose at day 0, and 4th week. Serum was collected at 4th week post booster dose and evaluated for protective antibody titer through Solid Phase competitive ELISA. Anti-NSP antibodies were measured through commercial ELISA kit (IDEXX, 3ABC Bov-Ov). NSP<20% & ≥30% were subjected/considered negative and positive respectively.

Statistical Analysis

The percentage inhibition (PI) values obtained from SPCE ELISA for serotypes A, Asia 1, and O at day 0, 4th week, and 8th week post-vaccination were statistically analyzed by repeated measure ANOVA using SPSS (Version 20, IBM Corp., USA).

Results

Confirmation of Serotypes

FMDV serotyping was confirmed by antigen detection ELISA with positive (≥1.0) and negative control (≤0.1). All the sample were serotyped in their respective well and value for consider to be positive must be greater than 0.1. And for the test validity positive well must be ≥1.0 while negative must be ≤0.1 *(Table 1)*.

Table 1. *serotyping of FMDV through Antigen Detection ELISA*

Catching MAbs	Sample	POS Control	NEG Control
Type O	0.545	1.531	0.0332
Type A (1st MAb)	0.940	1.531	0.0332
Type A (2nd MAb)	0.031	1.531	0.0332
Type Asia 1	0.297	1.531	0.0332
Type C	0.027	1.531	0.0332
Pan O, A, C & Asia 1	0.517	1.531	0.0332

Cells Monolayer Development and Propagation of Virus

Cells were revived from stock under aseptic condition and monolayer of cells was first developed in T-25 flask. Following the process the viable cells were counted through dye exclusion method and cells were transferred to T-275 flasks for monolayer developed. After specific incubation time the 80-90% developed monolayers on specified flask were selected as shown in *Fig. 1-A, C, E*. Serotype A, Asia 1 & O of FMDV was propagated and incubated at 37°C for 18-24 h & 36 h for O and A, Asia serotype respectively for replication of virus. As virus replicate the specific changes on cells were observed in term of cytopathic effects as shown in *Fig. 1-B, D, F)*. After completion of incubation and observation of CPEs the flasks were freeze thaw for 2-3 time and centrifuge to collect supernatants and stored at -20°C.

Tissue Culture Infective Dose 50 Calculation of Serotype A, Asia 1 and O

Biological titration in term of Tissue Culture Infective Dose 50 (TCID50) for quantification of FMDV serotype A, Asia & O to produce fifty percent cytopathic effects on BHK-21 cells were estimated through reed and munch method *(Table 2)*.

Assessment of Inactivation and Concentration

Inactivated sample of each serotype was inoculated on BHK-21 cells to evaluate the inactivation of the viral antigen. Repeatedly seven blind passages were given to each serotype and CPEs were checked. Up to the 7th passage no development of CPEs indicated that viruses were inactivated successfully. Following the procedure the 100 mL of each serotype were concentrated 10X (1/10th of original) through PEG-6000 to obtain enough antigens after purification through SEC as it dilutes the sample. About 10ml of each concentrated virus was run on chromatography.

Purification and Quantification Through SEC and SDS-PAGE Analysis

Each concentrated sample was run on chromatographic system and elute was collected. Chromatogram of each virus *(Fig. 2-A, B, C)* was obtained and peak area was calculated to quantify the virus in sample and eluted in term of µg/mL in as described in *Table 3*. Elute of the sample A and Asia 1 was run on SDS-PAGE to check the purity. Structural proteins 64kDa (VP2=VP4), 25kDa (VP3) and 35kDa (VP1) and no bands of non-

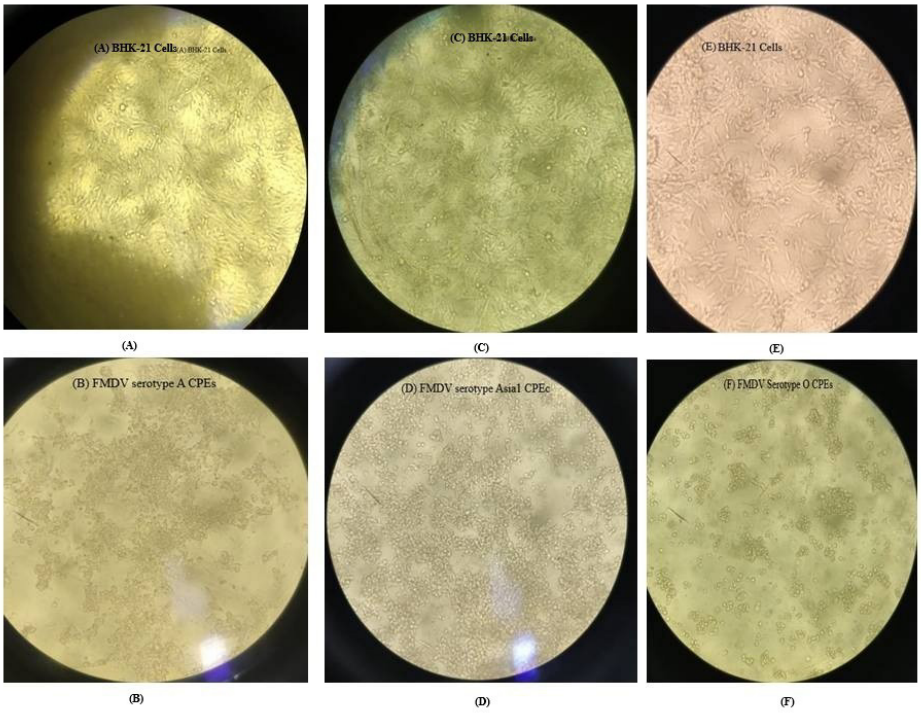

Fig 1. (A) confluent monolayer of BHK-21 Cells, **(B)** CPEs caused by FMDV serotype 'A' infection **(C)** confluent monolayer of BHK-21, **(D)** CPEs caused by FMDV serotype 'Asia 1' infection **(E)** confluent monolayer of BHK-21, **(F)** CPEs caused by FMDV serotype 'O' infection

structural protein at 53kDa (3ABC) and 56kDa (3D) with respect to prestained protein marker (10-180x, Servicebio, China) for purifies FMDV. While in crude sample NSP bands at 53-56kDa along with structural protein were observed *(Fig. 3)*. It is resulted that purified FMDV antigen were free from NSP contents.

Preparation of Monovalent Vaccines

The purified and quantified elute of antigens were diluted to prepared stock of 16 µg/mL of each virus *(Table 4)*. The purified monovalent vaccines (five concentration of each antigen) were prepared by emulsifying montanide oil ISA 50 and adjusted volume 2 mL/dose (15 doses/30 mL) with sterile PBS as shown in table. Quality testing was performed as per standard protocol.

Vaccine Inoculation and Animal Response

Vaccine of each antigen/concentration was inoculated to five bovine calves at day 0 & 21st (booster) and blood was collected at different interval day 0, 4th and 8th week of vaccination. Serum was collected and checked through Solid Phase Competitive ELISA for calculation of percentage inhibition (PI) (PI≥50% protected, PI<50% non-protected) as shown in *Table 5*. A P-value of less than 0.05 in Mauchly's test of sphericity indicated that the assumption of sphericity was violated, demonstrating significant differences in percentage inhibition (PI) across day 0, week 4, and week 8. Furthermore, the time × serotype interaction effect was also statistically significant (P<0.05), indicating that the pattern of antibody response over time differed significantly among serotypes A, Asia 1, and O.

Estimation of PD_{50}

On the basis of ELISA percentage inhibition interpretation, the animals declared protective (P) and non-protective (NP). Protective Dose 50 for FMDV type A was measure as lowest concentration of antigen (8 µg/dose) give 100 percent protection (PD≥3). Likewise, PD_{50} for Asia 1 and O was determined & describe in *Table 6*, respectively.

Trivalent Vaccine Preparation, Inoculation and Immune Response

Trivalent vaccine (equal to $3PD_{50}$) was prepared by containing antigen concentration (6.42, 5.7 and 4.90 µg/dose) as calculated by estimation of protective dose 50. The five animals were inoculated at day 0 and 4th week (booster dose). Blood was collected at different interval and serum was obtained to check the combined effect of

Table 2. Calculation of biological titration ($TCID_{50}$)

Serotype	Dilution	Wells with CPE	Wells without CPE	CP	CN	Total	% CPE	$TCID_{50}$/mL
Serotype A	10^{-1}	8	0	46	0	46	100	$10^{7.29}$
	10^{-2}	8	0	38	0	38	100	
	10^{-3}	8	0	30	0	30	100	
	10^{-4}	7	1	22	1	23	95.65	
	10^{-5}	6	2	15	3	18	83.33	
	10^{-6}	5	3	9	6	15	60.00	
	10^{-7}	3	5	4	11	15	26.66	
	10^{-8}	1	7	1	18	19	5.26	
	10^{-9}	0	8	0	26	26	0.00	
	10^{-10}	0	8	0	34	34	0.00	
Serotype Asia 1	10^{-1}	8	0	49	0	49	100	$10^{7.61}$
	10^{-2}	8	0	41	0	41	100	
	10^{-3}	8	0	33	0	33	100	
	10^{-4}	8	0	25	0	25	100	
	10^{-5}	7	1	17	1	18	94.44	
	10^{-6}	6	2	10	3	13	76.92	
	10^{-7}	3	5	4	8	12	33.33	
	10^{-8}	1	7	1	15	16	6.25	
	10^{-9}	0	8	0	23	23	0.00	
	10^{-10}	0	8	0	31	31	0.00	
Serotype O	10^{-1}	8	0	45	0	45	100	$10^{7.19}$
	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	20.00	
	10^{-8}	1	7	1	19	20	5.00	
	10^{-9}	0	8	0	23	23	0.00	
	10^{-10}	0	8	0	31	31	0.00	

trivalent vaccine and PI was calculated through SPCE. All Animals were protected against three antigens *(Table 7)*. Anti NSP antibodies were checked to assess the purified potential of the vaccine through NSP ELISA (IDEXX, 3ABC Bov-Ov). The entire animal population showed <20% NSP antibodies contend *(Table 7)*.

Discussion

FMD is devastating disease of animals which produced heavy economic losses in term of meat, milk and related products. These products have restriction on export to disease free countries. Potent vaccine is ultimate need to control the disease and spread of live virus. The endemic countries need to check the potency of vaccine to produce NSP free vaccine with DIVA strategy (to distinguish vaccinated from infected animals) to take access to global market for their animal products. To evaluate the potency of the vaccine in-vivo by challenging animals is an expensive task. Scientists are agreed upon Replacement, Reduction and Refinement (3R) concept to reduce the challenge phase of the testing for animal welfare and cost issues. Alternative methods like challenge free serological approaches are being used with relation of antibody titer to protection level [25].

ELISA OD values for A, Asia1 & O were 0.545, 0.940, and 0.297 respectively (≥0.1) [26]. The cells monolayer with 80-90% was developed on flat bottom flasks with inoculation of 10^6 cells/ml at 37°C for 24-36 h. The virus was propagated

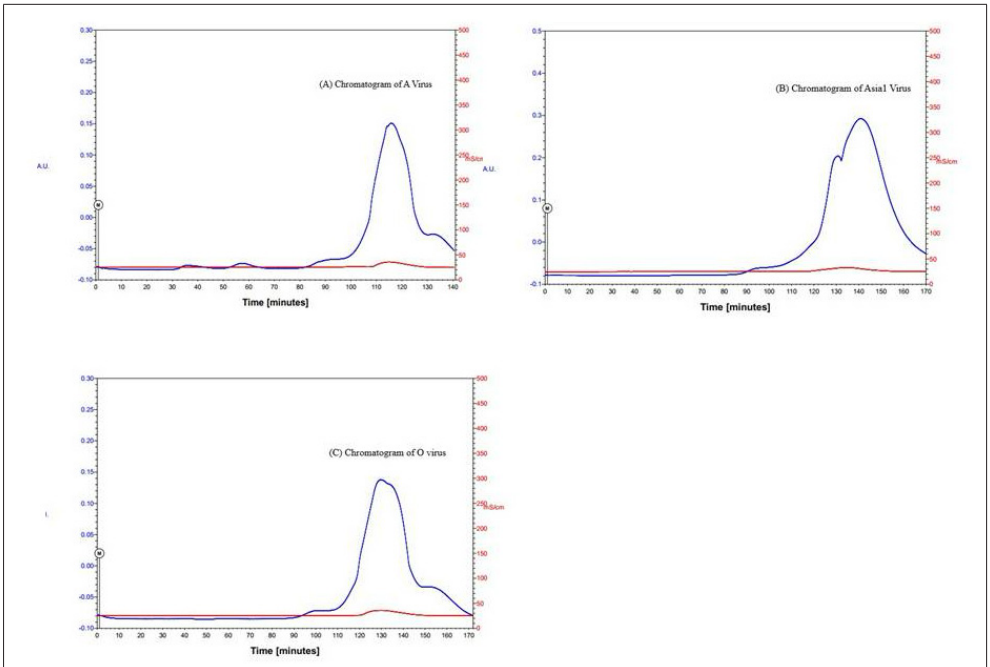

Fig 2. (A) Chromatogram of FMDV serotype 'A', **(B)** Chromatogram of FMDV serotype 'Asia1', **(C)** Chromatogram of FMDV serotype 'O'

and after CPEs development virus was harvested and biological titer $10^{7.29,}$ $10^{7.61,}$ $10^{7.19}$ TCID50 for serotype A, Asia1 & O was calculated using reed and much method. The results were consistent accordance with the study by study [27]. Harvested culture of each serotype virus was double inactivated by BEI and formaldehyde with 3mM and 0.04% respectively followed by ensuring the inactivation process by inoculation on cell culture upto the 7 blind passages. The results are supported by investigation [28].

SEC is efficient technique to purify the 146S content of the FMDV from nonstructural and media protein. Sephacryle S-300 have exclusion limit 10kDa-1500kDa and all the protein from media and NSP are trapped while FMDV (8200kDa) can pass between resin beads; therefore, eluted rapidly [16]. Peak area from UV detector in the form of chromatogram is obtained that is directly related to virus concentration. The optimized condition according to the referenced article [16] were used to calculate the

Table 3. *Chromatographic attributes of FMDV serotypes and antigen load (µg/mL)*

Chromatographic Attributes	**Serotype A**	**Serotype Asia 1**	**Serotype O**
Flow rate of mobile phase (FR)	0.7 mL/min	0.7 mL/min	0.7 mL/min
Sample volume loop (W)	3.5 mL (4% of CV)	3.5 mL (4% of CV)	3.5 mL (4% of CV)
Speed of chart recorder (S)	12 cm/h	12 cm/h	12 cm/h
Extinction point of FMDV €	72	72	72
Path length of flow cell (PL)	0.2 cm	0.2 cm	0.2 cm
Full scale absorbance unit setting (FSD)	0.3	0.5	0.3
Elution volume	28 mL (40 x 0.7)	37.5 mL (54 x 0.7)	37.5 mL (54 x 0.7)
Dilution factor (DF)	28/3.5 = 8	37.5/3.5 = 10.8	37.5/3.5 = 10.8
Length of peak	5.4 cm	5.4 cm	5.0 cm
Base of peak	4.5	5	4.7
Area under peak	12.37 cm^2	13.5 cm^2	11.75 cm^2
Crude concentration * =	= 257	= 468	= 244
Elute Concentration (µg/mL) = Crude/Dilution factor	257/8 = 32.125	468/10.8 = 43.75	244/10.8 = 22.59

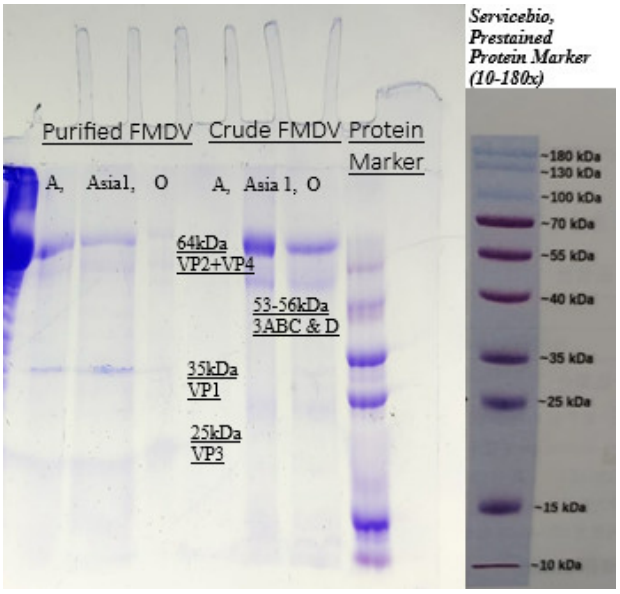

Fig 3. 64kDa (VP2+VP4), 25kDa (VP3) and 35kDa (VP1) Bands of purified FMDV serotypes on SDS-PAGE after elution from Size Exclusion Chromatography (SEC)

virus concentration 32.125, 43.75 and 22.59 µg/mL for A, Asia1 and O serotype antigen respectively. The result are supported by the study by [16]. Elute was run on SDS-PAGE to check the purity of FMDV with crude virus as a control. Bands of 64, 35, & lighter 25kDa of VP2+VP4, VP1 and VP3 respectively of purified FMDV were seen using 10-180x prestained protein marker. While in crude FMDV the 53-56kDa band of 3ABC & 3D was seen which was absent in the Purified product. These findings are accordance to study which use purified FMDV in SDS-PAGE from SDG method [29].

The vaccine containing 16 & 8 µg/dose of A serotype showed 100%protection while 4, 2 and 1µg/dose animal group were 87.5, 42.85, & 12.5% respectively. The protective dose in group of animals with 100% percent protection and lowest concentration of virus (8 µg/dose) while was $3.73PD_{50}$ /dose. These are supported by study [9]. Minimum concentration of 2.14 µg was calculated which protect 50% animal in the group. For A serotype $3PD_{50}$ = 6.42 µg/dose was estimated in this study. 100% animal protection was observed in vaccine type Asia1 with concentration 16 & 8 µg/dose while remaining 4, 2 & 1 µg/dose were 88.8, 57.14, and 14.28% that mean lowest concentration of 8 µg/dose showed 100% percent protection with PD_{50} = 4.21/dose. Minimum concentration for $1PD_{50}$ was 1.90 µg/dose while $3PD_{50}$ = 5.7 µg/dose. In the case of O serotype 100% animal protection were calculated in 16 & 8 µg/dose; however, the 4, 2, and 1 µg/dose showed the 90.0, 62.5 & 25.5% protection with calculated as $4.92PD_{50}$/doses of the lowest concentration of 8µg/dose (100% protection). Estimated minimum concentration conferring PD_{50} was 1.63 µg/dose with $3PD_{50}$ = 4.89 µg/dose. These above findings are accordance with studies [19,30,31] $\geq 3PD_{50}$ is international requirement for routine vaccine and $6PD_{50}$ for emergency vaccination [32].

Trivalent vaccine of FMDV equal to $3PD_{50}$ concentration 6.42, 5.7, and 4.90 µg/dose was prepared and inoculated to healthy, with no prior vaccine history of animals (n=5) with primary and booster dose at day zero and 28th week. After 4th week of booster dose serum was collected and checked for SPCE & NSP ELISA. All the animals were protective at week 4 & 8 to three serotypes with mean PI for serotype A; 62.49±4.90 &79.28±6.95, Asia165.96±4.29, 83.11±7.43 and O 57.71±2.25, 84.97±4.7 while NSP contents in all animals were (Mean±SD) 8.83±1.16, 8.87±0.77, 7.78±1.67 at day zero, week 4&8 respectively, were less than the cutoff value (20%). Same NSP-ELISA Approach was used and results are accordance with referenced study [33].

In conclusion, present study determined the PD_{50} values of chromatographically purified FMDV serotypes A, Asia1, and O using serological techniques and evaluated their application in the formulation of a trivalent vaccine in bovines. These findings demonstrate that serological techniques can be alternatively applied for the estimation of PD_{50} values of purified FMD vaccines, providing a practical approach for vaccine potency assessment and antigen payload standardization in resource setting

Table 4. *Formulation of Purified Inactivated Monovalent FMD Vaccines, against A, Asia 1 and O serotype (Monovalent vaccine for each serotype with same layout)*

B #	146S Fraction (µg)/Dose	Ingredients (mL)				Total Volume of Vial/Dose
		Antigen	Montanide Oil	Thiomersal Sodium (µL)	PBS	
B1	16	15	15	3	--	30/15
B2	8	7.5	15	3	7.5	30/15
B3	4	3.75	15	3	11.25	30/15
B4	2	1.875	15	3	13.125	30/15
B5	1	0.9375	15	3	14.065	30/15

Table 5. *PI of SPCE for Serotype A, Asia1 and O, at day zero, 4th & 8th week*

Group A (Serotype A)				Group B (Serotype Asia 1)				Group C (Serotype O)			
Animal Tag No.	Day 0	4th Week	8th Week	Animal Tag No.	Day 0	4th Week	8th Week	Animal Tag No.	Day 0	4th week	8th Week
A_{11}	41.53	58.78	75.31	B_{11}	26.52	56.14	82.84	C_{11}	23.97	55.72	84.57
A_{12}	31.62	50.95	69.88	B_{12}	33.15	54.55	79.39	C_{12}	26.20	51.73	81.41
A_{13}	40.97	69.72	72.76	B_{13}	38.19	52.69	88.94	C_{13}	20.04	53.18	83.64
A_{14}	44.32	57.58	76.91	B_{14}	39.25	61.27	87.53	C_{14}	22.11	52.56	78.51
A_{15}	41.29	61.42	78.19	B_{15}	49.04	57.02	88.94	C_{15}	27.34	50.28	85.60
A_{21}	36.26	78.99	74.52	B_{21}	38.28	55.87	83.28	C_{21}	19.78	53.96	77.68
A_{22}	30.99	67.41	72.44	B_{22}	40.40	59.23	90.80	C_{22}	15.27	53.75	81.67
A_{23}	33.62	56.94	62.38	B_{23}	43.85	57.73	87.97	C_{23}	23.14	53.08	76.44
A_{24}	36.82	66.21	74.92	B_{24}	38.01	59.85	90.18	C_{24}	21.23	52.56	82.08
A_{25}	38.89	61.74	76.11	B_{25}	32.36	65.07	82.40	C_{25}	32.72	50.70	82.81
A_{31}	35.06	58.14	67.49	B_{31}	34.30	61.62	81.16	C_{31}	25.16	53.65	82.96
A_{32}	21.00	51.27	53.43	B_{32}	39.08	54.46	85.05	C_{32}	22.26	37.86	45.83
A_{33}	32.74	60.14	78.43	B_{33}	28.20	62.68	88.15	C_{33}	27.60	50.08	73.54
A_{34}	38.97	48.80	49.28	B_{34}	31.83	57.73	86.03	C_{34}	18.12	50.28	77.01
A_{35}	28.03	62.85	82.82	B_{35}	38.90	45.88	48.98	C_{35}	27.03	54.89	84.41
A_{41}	14.61	47.36	48.16	B_{41}	34.92	59.77	84.52	C_{41}	17.55	56.60	36.30
A_{42}	25.31	58.22	85.14	B_{42}	26.17	37.04	38.19	C_{42}	29.67	51.42	74.31
A_{43}	47.36	42.65	46.24	B_{43}	41.73	58.53	86.29	C_{43}	15.06	52.46	76.44
A_{44}	8.86	44.16	46.96	B_{44}	39.52	63.92	85.05	C_{44}	22.06	27.91	48.99
A_{45}	41.45	55.43	73.16	B_{45}	35.19	47.03	48.18	C_{45}	9.94	51.89	72.14
A_{51}	32.26	57.90	65.73	B_{51}	26.79	33.24	36.51	C_{51}	29.41	37.86	41.22
A_{52}	23.64	46.40	49.28	B_{52}	41.99	46.41	46.15	C_{52}	14.39	53.34	70.17
A_{53}	34.10	30.35	46.00	B_{53}	36.25	40.84	40.40	C_{53}	22.09	51.73	59.14
A_{54}	25.07	48.00	49.12	B_{54}	34.74	55.08	68.70	C_{54}	19.26	23.25	35.53
A_{55}	28.19	44.48	47.84	B_{55}	38.19	45.62	47.39	C_{55}	12.42	27.29	33.66
Control	37.69	38.55	38.01	Control	34.10	35.54	36.02	Control	29.47	35.68	30.50
	38.54	40.31	44.82		38.33	39.88	43.92		33.45	38.58	37.55

areas. Furthermore, the study supports the use of SEC as an efficient method for the production of purified FMD vaccines with reduced NSP content and improved consistency for vaccine formulation.

Declarations

Availability of Data and Materials: The data supporting the findings of this study are available from the corresponding author (I.A.) upon reasonable request.

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Ethical Approval: All animal experiments were reviewed and approved by the Ethical Review Committee, Office of Research Innovation and Commercialization, University of Veterinary and Animal Sciences (approval number: No.DR/157, April 29, 2025).

Conflict of Interest: The authors declare that they have no relevant financial or non-financial interests to disclose.

Declaration of Generative Artificial Intelligence (AI): The authors declare that no artificial intelligence or AI-assisted technologies were used in the preparation of this article, including the creation of text, tables, or figures except to improve the readability and language of the article.

Author Contributions: FA and IA conceptualize, designed, executed the experiment, compiled the results and wrote the manuscript. ARA analyzed the results. AG interpreted the results, critically revised the manuscript and approved for submission.

Table 6. *Estimation of protective dose (PD_{50})*

Serotype	G. ID	146S con. (µg/dose)	Dilution	PC	NPC	CPC	CNPC	Total Animals	Protection %	Min. Con. of Ag. (µg/dose) for 50% Protection
Serotype A	A_1	16	Stock	5	0	17	0	17	100	2.14
	A_2	8	1:2	5	0	12	0	12	100	
	A_3	4	1:4	4	1	7	1	8	87.5	
	A_4	2	1:8	2	3	3	4	7	42.85	
	A_5	1	1:16	1	4	1	7	8	12.5	
	PD_{50} (Log10) = [0 - 0.3010/2 + 0.3010 (12/5)] = 0.5719 = 3.73 PD_{50}/dose									
Serotype Asia 1	B_1	16	Stock	5	0	18	0	18	100	1.90
	B_2	8	1:2	5	0	13	0	13	100	
	B_3	4	1:4	4	1	8	1	9	88.8	
	B_4	2	1:8	3	2	4	3	7	57.14	
	B_5	1	1:16	1	4	1	7	8	14.28	
	PD_{50} (Log10) = [0 - 0.3010/2 + 0.3010 (13/5)] = 0.6321 = 4.21 PD_{50}/dose									
Serotype O	C_1	16	Stock	5	0	19	0	19	100	1.63
	C_2	8	1:2	5	0	14	0	14	100	
	C_3	4	1:4	4	1	9	1	10	90.0	
	C_4	2	1:8	3	2	5	3	8	62.50	
	C_5	1	1:16	2	3	2	6	8	25.5	
	PD_{50} (Log10) = [0 - 0.3010/2 + 0.3010 (14/5)] = 0.6923 = 4.92 PD_{50} /dose									

PC: *protected calves,* ***NPC:*** *non-protected calves,* ***CPC:*** *cumulative protected calves,* ***CNPC:*** *cumulative non-protective calves*

Table 7. *Trivalent vaccine ($3PD_{50}$) evaluation through SP & NSP ELISA*

Time Interval	Percentage Inhibition for SPCE			
	A	Asia1	O	Control
Day zero	38.18±10.16	37.79±8.16	30.16±6.61	27.11±6.51
4th week	62.49±4.90	65.96±4.29	57.71±2.25	29.44±6.23
8th week	79.28±6.95	83.11±7.43	84.97±4.77	32.99±4.42
Percentage for 3ABC NSP				
Day zero	8.83±1.16			
4th week	8.01±0.77			
8th week	7.78±1.67			

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