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Optimization of Size Exclusion Chromatography for the Purification and Quantification of Foot and Mouth Disease Virus Serotype "O"

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ABSTRACT

Foot and mouth disease (FMD) is economically devastating disease of livestock overwhelming the international trading of animal based products. The unpurified inactivated vaccine containing Foot and Mouth Disease Virus a (FMDV) and non-structural proteins (NSPs) used to control the disease stimulated the production of anti-NSP antibodies. Countries striving to establish FMD free status must ensure the absence of circulating FMDV and anti-NSP antibodies in vaccinated animals, which are produced in natural infection. Production of purified FMD vaccines contrary to other animal's vaccines required extra step of virus purification from its NSPs. Present project was designed to optimize different parameters of size exclusion chromatography (SEC) for the purification of local isolate of FMDV from NSPs. For this purpose Bio-rad Econo-Column 15/50 was packed with Sephacryl™ S-300 resin and fitted with BioRad BioLogic LP Chromatographic system. Mixture of blue dextran and bovine serum albumin used as biomarkers for FMDV and NSPs respectively was employed to optimize resin bed height in column based on resolution between curves, while that FMDV was used to optimize flow rate of mobile phase and sample volume based on column efficiency. The chromatographic fraction of FMDV was ensured for purity through protein analysis by SDS-PAGE and serologically by 3ABC-NSP ELISA. It was found that maximum resolution of 1.86 was achieved at 48cm of resin bed height followed by 1.5 and 1.27 at 40cm and 32cm respectively. FMDV was eluted earlier with retention time 138.02±2.02min at flow of 0.7ml/min than 165.76±1.28min at 0.5ml/min along with better column efficiency with sample volume at 4% of column volume. SEC could detect and quantify from 603.75µg/ml in PEG concentrated FMDV up to 3.33µg/ml in 1:64 dilution as lower limit of detection and quantification. Presence of two bands of structural protein with absence of NSPs bands on SDS-PAGE and negative NSP% in hyperimmune sera raised in goats revealed the successful elimination of NSPs contents from FMDV suspension by SEC.

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AR and IA conceptualized, designed, executed the experiment and wrote the manuscript. FNA and ARA interpreted the results. AAA critically revised the manuscript and approved the final conclusion.

Key words
FMDV, Size exclusion chromatography, Purified FMD vaccine, 3ABC-NSP ELISA

INTRODUCTION

Foot and mouth disease (FMD) is highly contagious disease of livestock and wild animals characterized by high fever, formation of vesicular lesions in the mucosa of mouth and inter-digital regions of feet (Arzt *et al*., 2011). FMD being an economically devastating disease has direct impact on livelihood of livestock farming communities in terms of decrease in production of milk (81%) in lactating animals, weight loss (5%), feeding improvement cost (4%), distress sales of animals (8%) and others (2%) including treatment cost, abortion, mortality and loss in draught power (Hussain *et al*., 2017). Several outbreaks of the FMD have occurred in different regions of Pakistan intermittently, but now it is prevailing in acute form throughout the year (Zahur *et al*., 2006). Developing countries in Aisa, Middle East and Africa endemically infected with FMD are facing bans on access to international lucrative market for export of animals and their byproducts (Waters *et al*., 2021).

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Foot and mouth disease virus (FMDV) causative agent of disease, is non-enveloped, 146S particle consisting of +ssRNA genome enclosed in icosahedral protein capsid (Alkhalefa *et al*., 2021). FMDV exist in seven distinct serotypes namely O, A, C, Asia-1, SAT-1, SAT-2 and SAT-3 lacking cross immunity (Mahmud *et al*., 2018). Upon replication, FMDV produces four types of structural proteins (SPs) forming the capsid along with ten types of non-structural proteins (NSPs), which are regulatory enzymes (Cao *et al*., 2016). Regular vaccination along with restrictions on movements of infected animals is effective tool for preventing the disease and decreasing the production losses (Lee *et al*., 2017). The Office International des Epizooties (OIE) has recommended the FMD vaccine purified from its NSPs contents for the control of disease (OIE, 2009) because anti-NSPs antibodies are produced in case of infection and used as potential target for serological diagnosis (Clavijo *et al*., 2004).

In Pakistan, whole cell cultured vaccines containing both intact virus particle and NSPs are produced by propagation of FMDV on BHK-21 cells. Following inoculation, antibodies produced against SPs neutralizes the infection, while against NSPs considered as hallmark of infection and causes false positive results during the DIVA (differentiation of infected from vaccinated animals) strategy (Paton *et al*., 2009; King *et al*., 2015). Pakistan is striving hard for the establishment of disease free zone through inoculation of imported purified FMD vaccine with the collaboration of Food and Agriculture Organization (Lyons *et al*., 2021).

Several chromatographic techniques are conventionally used for the purification of biomolecules and advancement in size exclusion chromatography (SEC) has allowed the purification of diversified viruses in shorter time (Zhu and Chen, 2017). Previously affinity chromatography and receptor ligand binding have been employed for the purification of FMDV (del Cañizo *et al*., 1997).

Present study has been designed to optimize the several parameters of SEC for the elimination of NSPs and quantification of FMDV up to lowest quantity from cell culture suspension. Purity of FMDV chromatographic elute was ensured through serologically and protein analysis.

MATERIALS AND METHODS

The experiment was conducted in Quality Operation Laboratory (QOL), Institute of Microbiology UVAS Lahore during the year 2020-21 and approved by the UVAS's Ethical Committee.

Source of FMDV, serotyping and propagation on BHK-21 cells

The FMDV was collected from Quality Operation Laboratory, UVAS and confirmed for serotyping against O, A, C, Asia-1and SAT-1, SAT-2 and SAT-3 through commercially available antigen detection ELISA kit (ISZLER Biotechnology). The wells of ELISA microplate supplied with pre-coated catching anti-FMDV monoclonal antibodies (MAbs), were added with FMDV sample. The test was performed as per manufacturer instructions, and the optical density (OD) of each well was measured by multiskanIt ELISA reader at 450nm. The OD of serotype specified wells equal or greater than 0.1 was considered as positive.

The BHK-21 cells were propagated at density of 10^5cell/ml in roller bottles of 1900cm^2 capacity containing GMEM growth media with 10% fetal calf serum. The confluent monolayers were infected with FMDV serotype O and kept in CO_2 incubator at 37ºC for 24-36h. The infected bottles showing 80% carbapenemase-producing Enterobacteriaceae (CPEs) caused by FMDV were repeatedly frozen and thawed to release the virus and pooled the cell culture suspension in sterilized container. (Mahmud *et al*., 2017).

The tissue culture infectivity dose ($TCID_{50}$) of harvested FMDV was determined as described by Muench (1938). BHK-21 cell monolayers grown in 96-well micro titration plate were infected with 10-fold serially diluted FMDV and the end point dilution of the assay quantifies the amount of virus required to produce CPEs in 50% of infected cells of monolayer.

$$PD = \frac{\%\text{positive at or above } 50\% - 50\%}{\%\text{ positive at or above } 50\% - \%\text{ positive below } 50\%}$$

$$50\%\text{ endpoint titer } \left(\frac{TCID50}{0.1ml}\right) = 10^{\text{Log total dilution above } 50\% + [PD * \text{Log (dilution factor)}]}$$

The FMDV suspension was inactivated with 3mM binary ethylene imine (OIE, 2009) and concentrated up to 10 times by precipitating with PEG-6000 (7.5% w/v) (Kim *et al*., 2019).

Optimization of size exclusion chromatography

Slurry of Sephacryl™ S-300 (GE Healthcare) was prepared and packed in Econo-Column 15/50 (Bio-rad) fitted with BioLogic LP Chromatographic system 358-BR3506 having UV detector at 254nm of 2mm path length of flow cell. The UV-absorbance of biomolecule appeared in form of chromatogram. The system was run and equilibrated with phosphate buffer until the UV tracking line become continuous as base line.

Resin bed height

Initially resin bed height varied at three levels *i.e.*,

32cm, 40cm and 48cm in column was optimized by using mixture of blue dextran (2mg/ml, MW-2000kDa) and bovine serum albumin (5mg/ml, MW 67-kDa) as biological markers for intact FMDV (MW 8200kDa) and NSPs (53-56kDa), respectively (Park *et al*., 2020b) achieving maximum resolution between separated curves as described by Ravisankar *et al*. (2019). The chromatographic runs were performed at flow rate of 0.65ml/mint with chart recorder speed at 12cm/h.

$$\text{Resolution (Rs)} = \frac{(TR_2 - TR_2)}{0.5 \times (W_1 + W_2)}$$

Whereas T_{R2}, T_{R1} are retention time and W_2, W_1 are peak widths of second and first curves of biomarkers respectively measured in same units.

Flow rate of mobile phase and sample volume

Flow rate of mobile phase at three levels *viz*. 0.5ml/min, 0.7ml/min and 0.9ml/min were optimized to achieve earliest possible retention time against the maximum of sample volume at 2%, 4% and 6% of column volume by keeping the previously optimized resin bed height. PEG precipitated FMDV (1:5 dilutions) was injected into injector valve at said conditions separately and chromatograms were analyzed to find out column efficiency as described by Ravisankar *et al*. (2019).

Column efficiency was determined by comparing the values of number of hypothetical plates (N) against standard > 5000/m, height equivalent to a theoretical plate (HETP), reduced plate height (h) against < 3.0, peak asymmetric and tailing factors (A_s and T_f) between the standard 0.8-1.8 as per resin manufacturer instructions.

$N = 16\times (T_R/W_h)^2$

$HETP = L/N$

$h = HETP/d_{50v}$

$A_s = b/a$

$T_f = (a+b) / 2a$

where L, a, b and d are length of resin bed height, distance measured at 10% of peak height from leading, tailing edges and median particle size, respectively.

Purification and quantification of FMDV

The PEG precipitated FMDV was serially two fold diluted and each dilution was injected at previously optimized conditions (4% of column volume, flow rate at 0.7ml/min and resin bed height of 48cm). Chromatograms of each dilution were analyzed for estimation of FMDV as described by Rweyemamu *et al*. (1989).

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

whereas FR is flow rate, PA is area under the peak; FSD is full scale absorbance unit setting, S is chart recorder speed, PL is path length of the flow cell, E is extinction point for FMDV, and W is sample volume.

Confirmation for purity of FMDV

The chromatographic fraction of purified FMDV was collected and subjected to SDS-PAGE at 15% of resolving and 5% of stacking gel with constant supply of 190V for 30min (Loureiro *et al*., 2018). Protein standard (PageRuler, MW 10–180kDa) was run parallel as the molecular weight marker. The size of bands obtained on gel following staining were compared with protein standard (Park *et al*., 2020a).

Apart from this, the chromatographic fraction of purified FMDV was emulsified with montanide oil (1:1) to final concentration of 24µg/dose and injected into three healthy goats on six alternate days. Serum samples prior inoculation and fourteen days post last injection were collected and checked for anti-NSPs antibodies through commercially available test kit (IDEXX FMD 3ABC Bo-Ov). The wells of test plates pre coated recombinant 3ABC FMDV antigen were added with diluted serum. Test was performed and NSP% in serum samples was calculated as per manufacturer instructions.

$$\text{Sample NSP\%} = \frac{\text{Sample A (450)} + \text{NCx}}{\text{PCx} - \text{NCx}}$$

where PCx and NCx are net ODs of positive and negative controls. The serum samples with NSP% < 20 were considered as negative.

Statistical analysis

The results were analyzed statistically by applying one way analysis of variance (ANOVA) followed by Tukey post-hoc test. All analysis was performed by using Minitab 17 Ink.

RESULTS

Propagation and biological titer of FMDV

FMDV was confirmed for serotyping through antigen detection ELISA. It was found that mean OD_{450} values of wells specified for O and Pan-O-A-C-Asia1 types were greater than cut-off 0.1, hence found positive for type O and negative for types A, Asia-1 and C as shown in Table I.

The BHK-21 cells were seeded at density of 10^5 with viability 68% determined through tyrpan blue dye exclusion method. The maximum viability 88.31% was observed at 36h then declined to 58.16% at 72h of incubation. Significant difference among the means of viability% ($P<0.05$) was observed among all groups of incubation period. The confluent monolayer of maximum viability was infected with FMDV type O and characteristic CPEs including rounding of cells and detachment of monolayer were developed at 15-18h as shown in Figure 1.

Table I. Serotyping of FMDV through antigen detection sandwich ELISA.

Serotypes	Replicate			Mean OD	Control	
	1	2	3		+ve	-ve
Type O	0.357	0.239	0.365	0.320	1.801	0.033
Type A (1st MAb)	0.041	0.032	0.038	0.037	1.650	0.029
Type A (2nd MAb)	0.036	0.034	0.027	0.032	1.555	0.026
Type Asia-1	0.035	0.029	0.026	0.030	1.950	0.030
Type C	0.024	0.029	0.031	0.028	1.763	0.023
Pan-O-A-C-Asia1	0.405	0.349	0.379	0.377	1.943	0.039

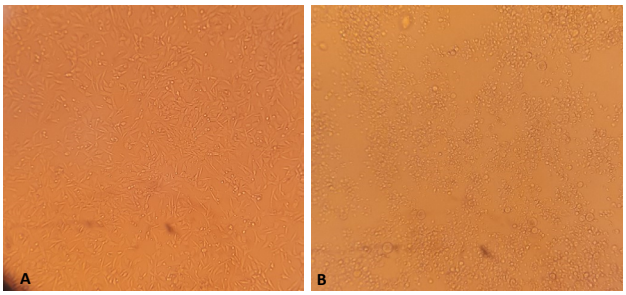

Fig. 1. Normal BHK-21 cells (A); CPEs developed due to FMDV (B).

The biological titer of serially 10-fold diluted FMDV type O was calculated by means of CPEs developed in 50% of inoculated wells and found $10^{7.20}$/ml as shown in Table II.

Table II. Biological titration of FMDV type O.

Dilu-tion of FMDV	No. of wells		Accumulated number of wells			%age of CPEs	$TCID_{50}$/ml
	with CPEs	without CPEs	with CPEs	without CPEs	Total		
10^{-1}	8	0	45	0	45	100	$10^{7.20}$
10^{-2}	8	0	37	0	37	100	
10^{-3}	8	0	29	0	29	100	
10^{-4}	6	2	21	2	23	91.30	
10^{-5}	6	2	15	4	19	78.94	
10^{-6}	5	3	9	7	16	56.25	
10^{-7}	3	5	4	12	16	25	
10^{-8}	1	7	1	19	20	5	
10^{-9}	0	8	0	27	27	0	
10^{-10}	0	8	0	23	23	0	

CPE, Cytopathic effects; $TCID_{50}$, tissue culture infectious dose.

The cell culture suspension of FMDV was inactivated by treating with 3mM of BEI and ensured through re-inoculating on freshly grown BHK-21 cells. No CPEs were developed up to 7th blind passages.

The volume of inactivated FMDV suspension (500ml) was reduced ten times by precipitating with PEG-6000 (7.5% w/v) followed by elution in 50ml of eluting buffer.

Optimization of size exclusion chromatography

Initially resin bed height was optimized by injecting mixture of blue dextran (BD) and bovine serum albumin (BSA) at 2% of CV as biomarkers for FMDV and NSPs, respectively. The asymmetric curves of biomarkers with poor resolution of 1.27 and 1.5 appeared at the bed height of 32cm and 40cm, respectively; while symmetric curves with higher resolution of 1.86 appeared at 48cm (Fig. 2). The co-relation co-efficient between bed height and resolution found R^2 0.9841. The attributes of Rs are summarized in Table III.

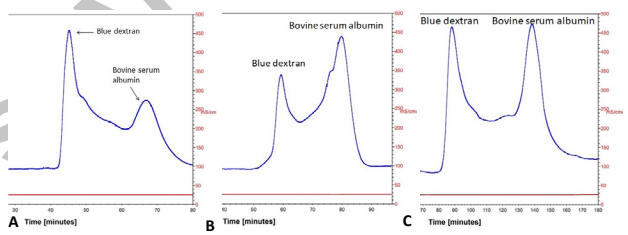

Fig. 2. Resolution between biomarkers at bed height 32cm (A), 40cm (B) and 48cm (C).

Table III. Comparative effect of resin bed heights (32, 40, 48 cm) on resolution between biomarkers.

Attributes	32cm		40cm		48cm	
	BD	BSA	BD	BSA	BD	BSA
R_t (min/cm)	45.07/ 9.0	67.05/ 13.41	59.25/ 11.85	79.80/ 15.96	78/ 15.60	128.48/ 25.69
Curve width (cm)	3.42	3.53	1.68	3.78	5.69	5.12
Time difference between curves (min)	3.20		3.58		4.16	
R_s	1.27		1.50		1.86	

BD, blue dextran; BSA, bovine serum albumin; Rs, resolution.

Flow rate of mobile phase and sample volume to be injected were optimized simultaneously by injecting PEG-6000 precipitated FMDV (1:5 dilutions) samples at 2%, 4% and 6% of column volume against 0.5ml/min, 0.7ml/min and 0.9ml/min of flow rate keeping the

resin bed height at 48cm constant. It was observed that retention time of sample was reduced from 165.76 ±1.28 min at 0.5ml/min to 138.02±2.02 min at 0.7ml/min, respectively (Fig. 3). Flow rate at 0.9ml/min was found not suitable due to filling of dead space over resin bed, inflow and out flow of mobile phase was not equilibrated causing wastage of sample; hence not applied for further experiments. The attributes related to column efficiency were fulfilled by injecting sample at 4% of (CV) as shown in Table IV. Significant difference among the means of N, HETP, h, As, Tf, dilution factor, retention time, retention volume and elution volume ($P<0.05$) and non-significant difference among the means of yield of FMDV ($P>0.05$) was observed in all groups of flow rate of mobile phase and sample volume.

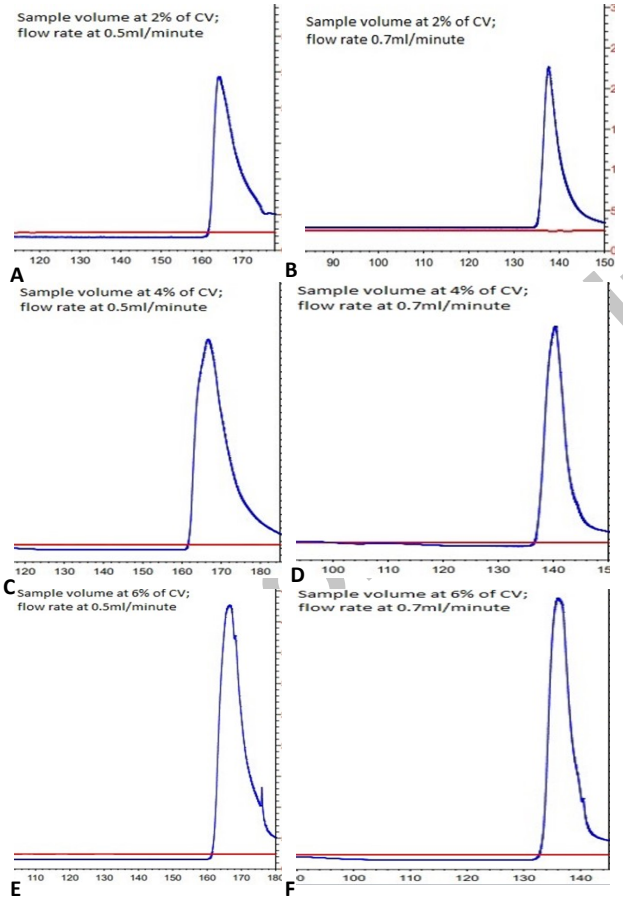

Fig. 3. Chromatogram of samples at 2% CV, flow rate at 0.5ml (A) and 0.7ml (B); 4% CV at 0.5ml (C) and 0.7ml (D); 6% at 0.5ml (E) and 0.7ml (F).

Sample volume at 4% of CV with flow rate at 0.7ml/min and resin bed height at 48cm fulfilled the standard values of column efficiency; hence considered as base line for further experiments.

Purification and quantification of FMDV

Samples from each of two fold serial dilutions of PEG-6000 precipitated FMDV (10x) were run at 4% of CV with 0.7ml/min flow through column. The chromatograms of each dilution as shown in Figure 4A were analyzed for estimation of FMDV in sample and chromatographic elutes eluted under peak were collected in fractions of 5ml in sterilized tubes. The concentration of FMDV in 10x sample found 603.75µg/ml and SEC detected FMDV in sample diluted up to 1:64 and quantified as 3.33 µg/ml as lower limit of detection and quantification, respectively (Table V). The co-relation co-efficient between dilutions and concentrations of FMDV found R^2 1.0.

Table IV. Optimization of flow rate and sample volume based on column efficiency.

Sample volume	Flow rate (ml/min)	N	HETP	h	As	Tf	DF	Yield (µg/ml)
2%	0.5	4097	0.024	4.88	4.01	2.5	4.47	103.27
	0.7	7381	0.013	2.6	2.18	1.59	3.82	97.34
4%	0.5	3847	0.025	5	2.06	1.48	2.21	89.28
	0.7	7352	0.013	2.6	1.45	1.22	1.91	98.26
6%	0.5	3018	0.033	6.6	2.43	1.71	1.72	97.75
	0.7	5987	0.016	3.2	2.17	1.58	1.39	99.13

Table V. Lower limit of SEC for the detection and quantification of FMDV.

Dilution of FMDV	Area under curve (cm^2)	Elution volume (ml)	Dilution factor	Concentration of FMDV in sample (µg/ml)
10x FMDV	8.69	21	6	603.75
1:2	3.56	21	6	247.20
1:4	2.60	35	10	180.5
1:8	1.26	28	8	87.44
1:16	0.43	32	9	28.8
1:32	0.126	28	8	8.75
1:64	0.048	23	6.4	3.33
1:128	Not detected			

SEC, size exclusion chromatography.

Confirmation for purity of FMDV

The purity of eluted FMDV fraction was confirmed by SDS-PAGE. Two bands of structural proteins appeared at 35KDa (VP_1) and 25KDa (VP_3) and no bands of 3ABC-NSP and 3D at 56KDa and 53kDa, respectively with respect to protein marker as shown in Figure 4B. The results revealed that SEC have purified the intact FMDV

from NSPs and other contaminating proteins.

Apart from this, hyperimmune sera raised against purified FMDV emulsified with montanide oil in three goats were processed through 3-ABC NSP ELISA kit. The values of controls found within validity criteria and NSP% prior inoculation were 2.39, 2.73 and 2.48; fourteen days post inoculation were 6.52, 6.94 and 6.71. The NSP% in sera were less than cut-off value of 20%, hence considered negative revealing the absence of NSPs contents in chromatographic elute of FMDV.

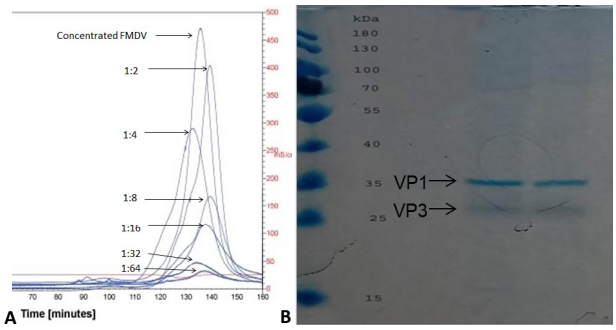

Fig. 4. Chromatograms of 2-fold dilutions of FMDV and Analysis of chromatographic elute of FMDV through SDS-PAGE.

DISCUSSION

For making the livestock a profitable business model, it is known fact that quality production is directly proportional to the efficient marketing of the livestock products. Unfortunately, market aspect of the livestock products with exploring new international markets had been neglected in the past due to non-compliance of requisite standards for the trading. In Pakistan, FMD vaccine production units are producing unpurified whole cell culture FMD vaccine containing both intact FMDV and NSPs. Following inoculation of unpurified vaccine, anti-FMDV antibodies produced are promainly involved in neutralization of infection, while anti-NSPs antibodies are used as potential target for serological diagnosis of infection in DIVA testing. Presence of antibodies against NSPs of FMDV in livestock commodities being hallmark of infection causes a big hurdle in trading with FMD free countries. So, in order to improve the quality of vaccine, and get access to the international market for export of livestock products, Pakistan is striving hard to establish FMD free zone through inoculation of imported purified FMD vaccine.

In current study FMDV type O was propagated on BHK-21 cells for because cell culture technique has been widely used for isolation, identification and propagation of different types of viruses. Upon replication, FMDV caused the development of characteristic CPEs involving rounding of cells and disruption of monolayer within 15-18h. Our results of biological titer in terms of $TCID_{50}$ $10^{7.20}$/ml were similar to that of (Chowdhury *et al*., 2016), who calculated $TCID_{50}$ $10^{8.5}$/ ml for serotype O. The cell culture suspension of FMDV was completely inactivated by 3mM of BEI within 24h of incubation and inactivation was ensured by re-infecting the freshly grown BHK-21 cells up to 7th blind passages. These results were confirming the findings of Aarthi *et al*. (2004), who recommended 0.4mM to 1.6mM BEI for complete inactivation of FMDV suspension. Down streaming of larger volume of FMDV suspension into lesser volume is necessary for easy storage and rescue of vaccine antigen from impurities. Our results agreed to findings of Kim *et al*. (2019), who used 7.5% w/v PEG for concentration of FMDV with higher yield up to 85.4%.

Various attempts have been made to optimize different methods for the purification of 146S fraction of FMDV from NSPs. It has been previously reported that ion-exchange and affinity chromatography has limitations to distinguish the intact 146S FMDV particles and disassembled large aggregates and purification becomes strenuous. SEC is one of the approaches to purify the FMDV from viral suspension used in vaccine production on industrial scale. When sample of FMDV is passed through column packed with resin and detected through UV detector. UV absorption shows chromatogram corresponding to 146S fraction of FMDV. The area under curve is directly proportional to the quantity of 146S fraction in sample (Barteling and Meloen, 1974).

In this study, we have optimized different chromatographic parameters for the purification of 146S fractions from viral suspension. The factors which ensured the success of SEC to separate biomolecules are selection of chromatographic resin, resin bed height in column, sample volume and flow rate of mobile phase. In this study Sephacryle S-300 as chromatographic resin depending upon its exclusion limit 10kDa-1500kDa was used. Smaller molecules of media proteins, NSPs and disassembled proteins trapped into resin beads and larger FMDV (8200kDa) can pass between resin beads; therefore, eluted rapidly. Spitteler *et al*. (2011) used SEC for the quantification of FMDV during vaccine production and Hosseini *et al*. (2016) selected the Sephacryle S-300 resin for the purification of FMDV from concentrated viral suspension.

Resolution measures the efficiency of resin beads and column to achieve separation between two peaks of interest. At low bed height of resin at 32cm, there was poor resolution of 1.27 between curves of biomarkers.

As the resin bed height was increased up to 40cm and 48cm, resolution was also improved from 1.5 to 1.86, respectively. These findings were in accordance with that of Kong *et al*. (2018), who stated that the packed bed height of column influenced the resolution and separation time. Longer the bed height, higher will be the resolution. Results of R_s were in closed to that of Ravisankar *et al*. (2019) who recommended minimum value of R_s for measurable separation is 1 and 1.6 considered as baseline separation for accurate quantification. Flow rate of mobile phase at 0.5ml/min irrespective of sample volume showed asymmetric chromatograms with higher retention time and poor column efficiency rather than flow rate at 0.7ml/min showed reduced retention time with better column efficiency. The column efficiency was improved as the number of theoretical plates increased by minimizing HETP. Volume of sample to be injected played a critical role on dilution of eluted sample; it was found that sample volume at maximum of 4% of CV fulfilled the standard criterion of column efficiency as per manufacturer instructions (GE Healthcare, 71500242AF) than at 2% or 6% of sample to CV ratio. These findings were supported with the findings of Meurs (2016) and Ó'Fágáin *et al*. (2017), who recommended optimal flow rate 0.65ml/minute and 1-5% of sample to column volume ratio to the optimization of liquid, gas and fluid chromatography for good resolution. The change in the shape of peak is indication of resin bed deterioration. In our study, value of peak asymmetric and tailing factors found 1.45 and 1.22 at 0.7ml/min with 4% of sample volume. These findings were satisfactory with the recommendations of manufacturer that asymmetric and tailing factor should be greater than 0.8 and less than1.8. The results are in accordance with Song and Wang (2003) who stated that value of $T_f > 1.00$ is tailing effect and $T_f < 1.00$ represents fronting effects.

Sucrose density gradient (SDG) ultra-centrifugation technique based on sedimentation velocity of biomolecules has been used for the purification and quantification of 146S fraction of FMDV from the tissue culture harvest. The limitations of SDG like highly complex equipment dependent technique, requiring highly skilled personal, time consuming up to 64 h, high operating and maintenance costs as well as low sample volume capacity make this technique difficult to adopt for vaccine production on industrial scale (Fernandez-Martinez *et al*., 2016; LaCava *et al*., 2016; Yu *et al*., 2016). On the other hand, SEC is simple, cheaper technique having low time duration and can easily be scale up by increasing sample volume to column volume ratio. The SEC could be used for both purification as well as quantification of FMDV and has strong co-relation coefficient 0.9669 with that of SDG (Spitteler *et al*., 2011).

When the efficiency of SEC was determined by finding lower limit of detection and quantification of FMDV, it was found that the area under curve proportional to the concentration of virus. Quantified 146S fraction of FMDV was twofold serially diluted and run through SEC. It was found that the area under curve was decreased as the dilution of 146S increased accordingly with the lower limit of detection and quantification up to 1:64 dilutions and 0 3.33µg/ml of 146S fraction, respectively. These results are in accordance with that of Spitteler *et al*. (2011), who claimed SEC could measure the 146s fraction over linear range up to 5µg/ml.

The purity of 146S fraction of FMDV can be ensured by SDS-PAGE as well as detecting antibodies against NSPs in hyper-immune sera raised against purified FMDV elutes. The purified fraction of FMDV processed through SDS-PAGE showed the absence of 3-ABC NSPs band at 56kDa and 3D bands on 53kDa as well as presence of structural proteins bands at 35kDa (VP_1) and 25kDa (VP_3). The results revealed that SEC has successfully eliminated the NSPs from FMDV suspension. The results were in accordance with that of Jangam *et al*. (2018) who declared that size exclusion chromatography could remove 94% of NSPs from the FMDV pool.

When the purity of eluted 146S fraction was checked by means of detecting antibodies against NSPs by commercially available 3ABC-NSP kit (IZSLER Biotechnology) it was found that hyper immune serum raised against chromatographic elute of FMDV was negative for antibodies against NSPs indicating the purifying efficiency of SEC. These findings were in accordance with Park *et al*. (2020a), who stated that purified fraction by heparin affinity chromatography did not provoke production of antibodies against NSP even after the fifth vaccination.

CONCLUSION

On the basis of above said findings, it is concluded that size exclusion chromatograph operated at optimized conditions like sephacryle S-300 resin as a stationary phase, resin bed height in at 48cm, sample volume injected at 4% of column volume and flow rate of mobile phase at 0.70ml/minute could be used for the purification and quantification of 146S fraction of FMDV for the production of purified vaccine against FMD.

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IRB approval

The experimental manipulations in this study were undertaken in compliance with institutional guidelines of Ethical Review Committee, UVAS.

Ethical statement

Purified FMD vaccine was inoculated into goats and blood samples were collected as per standard procedures without harming or giving stress to the animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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