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Comparative Efficiency of Polyethylene Glycol, Ammonium Sulphate, Methanol Precipitation, and Ultrafiltration Techniques for the Down Streaming of Viral Antigen

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Abstract

Background: Foot and mouth disease (FMD) is ubiquitous worldwide but endemic in many countries of Africa, Asia, South America, and the Middle East. Many reasons contribute to the incidence of viral diseases even in vaccinated animals. These reasons include low antigenic payload, low PD50, improper formulation, unstable vaccine containing antigen, and genetically different from field strain. Among these, the most important one is the low antigenic load per dose of the vaccine. Vaccine failure is mainly due to the direct use of virus suspension in the vaccine without the concentration of viral antigen. Another reason to concentrate the antigen is small volume storage in the vaccine bank. These issues are mostly concerned with developing countries like Pakistan which lack antigen concentration technology. The concentration of the virus is a major milestone to be achieved for the production of an effective vaccine as well as for the diagnostic tool.

Methods: Different techniques including precipitation with polyethylene glycol, ammonium sulfate, methanol, and filtration through an ultra-filter membrane were used for the concentration of viral suspension. Antigen quantification in terms of µg/ml was determined through size exclusion chromatography by using Sephacryl S-300 as a stationary phase.

Results: Percentage recovery of FMDV calculated through analysis of chromatograms found 77.80%, 59.75%, 32.50%, and 13.83% for polyethylene glycol, ammonium sulfate, ultra-filtration, and methanol treated samples respectively.

Conclusion: Classical polyethylene glycol precipitation showed a maximum percentage recovery of foot and mouth disease virus as compared to other concentration methods.

Introduction

Livestock is considered a prime subsector of agriculture as Pakistan is an agrarian country. It contributes 60.84% to the agriculture value addition and 14.36 % of the total gross domestic product (GDP). Animal diseases are a major cause of loss of gross livestock production which leads to a reduction in the export sector of the country. FMD is a chief concern for the world's livestock industry as disease is the foremost obstacle in exporting animals and their products like meat, meat products, milk and milk products [1,2]. The prevalence rate of FMD in cattle is high (37.1%) and low in buffaloes (28.7%) in Pakistan [3]. Socioeconomic impacts of foot and mouth disease (FMD) are high in Pakistan. Control of clinical disease is effective through vaccination [4]. About one-third of the total animal population resides in FMD-endemic countries [5,6]. Despite FMD being characterized by low mortality, frequent outbreaks cause economic losses every year [7]. In FMD endemic regions the disease in susceptible animals is controlled through inoculation of inactivated vaccine [8]. Vaccination can play an important role in the control of outbreaks of FMD by reducing the impact of clinical disease and the extent of virus transmission among susceptible animals [9]. Mostly inactivated vaccines containing different strains of the FMD virus are being used in the country in either immunization programs or to control outbreaks of specific strains. Serotypes and subtypes show no cross-protection and even sometimes the same serotype of the virus fails to induce total cross-protection [10]. The performance of vaccines depends upon factors like potency, antigen payload, antigen stability, formulation of vaccine, variable antigen strain, the timing of vaccination, and frequency of herd coverage. According to the World Organization for Animal Health (WOAH) potency is defined as "concentration of immunologically active component". The potency of the vaccine is measured by the manufacturer through quantification of the antigen so that a dose of vaccine delivers a known antigen 'Payload' [11]. The potency of vaccine is mainly affected by factors like choice of strain, a system in which the virus is multiplied, virus quality and quantity, inactivation process, and adjuvant(s) used [12]. The quantity and quality of the virus are affected by mainly production culture system and concentration methods. The concentration of a virus is an important step for potent vaccine production during the downstream process. Virus concentration by classical physical methods from cell culture suspension includes ultracentrifugation and ultrafiltration. Despite these techniques, some other techniques like sucrose density gradient, chemical precipitation, and chromatography are used for concentration as well as purification of FMDV [13]. Polyethylene glycol has been used in aqueous polymer two-phase systems, helpful for concentrating and isolating viruses from various samples. Among all chemical precipitation methods, concentration and purification of FMDV with polyethylene is the most efficient method [14]. Ammonium sulfate has been used for the concentration of many different fragile viruses and large viral suspensions. However, with the precipitation of ammonium sulfate, recovery of a virus is considerable, but virus stability issues are present [15]. Tangential flow filtration or ultra -filtration is a technique in which viral particles are separated based on membrane pore size compatible with that biomolecule of interest.

Methods

Sample collection: Vaccinal serotype 'O' of FMDV was collected from the Quality Operation laboratory (QOL), University of Veterinary and Animals Sciences (UVAS) Lahore.

Sample confirmation: A sample of the virus was confirmed by 2-step RT-PCR using serotype-specific primer for serotype 'O' of FMVD. For this purpose, the genome of the FMDV (RNA) was extracted by the TRIzol method [16]. Complementary DNA (cDNA) was prepared by using RevertAid first strand cDNA kit (Thermo scientific # K1622). Then PCR was done using the above cDNA as a template with specific primers on standard PCR conditions. Amplicon was analyzed on 1% agarose gel by electrophoresis [17].

Media preparation: Glasgow's minimal essential media (GMEM) (Caisson, Smithfield, USA) with 10% fetal bovine serum was prepared and filtered for sterilization purpose by using a syringe filter (Millex GV 0.22µm).

Cell revival and virus propagation: The baby hamster kidney cell (BHK-21) line was revived and 80-90% monolayer of the cells was developed with a cell suspension of 10^6 cell/ml. The live virus was inoculated on BHK-21 cells with a multiplicity of infection (MOI, 0.01). The virus-infected cell culture suspension was harvested after 18-24 hours followed by the development of cytopathic effects as observed under an inverted microscope (BioBase, Shandong, China) [18]. The harvested FMDV cell culture suspension was clarified by centrifugation at 4000rpm, and the supernatant was stored at -4°C till further use.

$TCID_{50}$ Calculation: For live viral particle count $TCID_{50}$ was calculated according to the method described by [19], using reed & munch formulae [19].

Inactivation of virus: For inactivation of clarified live virus, 3mM binary ethyleneimine BEI, MP Biomedicals, Santa Ana, USA) was added into the culture recommended by WOAH. Virus suspension was

incubated at 26℃ for 24 hours and again the process was repeated for another 24 hours for double inactivation [20]. After 2nd inactivation, sodium thiosulphate (Thermo Fisher Scientific, USA) was added at the rate of 2% of the final volume to neutralize the effect of BEI [21].

Confirmation of Inactivation: Inactivation was confirmed by inoculating BEI inactivated virus on BHK-21 cell line up to seven passages of cell culture. The inactivated virus was filtered using a syringe filter (Millex GV 0.22µm) so that no media proteins and inactivating agent residues remained.

Concentration methods: About 500 ml inactivated virus was divided into 5 equal parts (100 ml each). One part was for the inactivated control and the other four were for the concentration of viruses by different methods. Each 100ml virus suspension was concentrated by different methods like polyethylene glycol precipitation (PEG), ammonium sulphate (AS) precipitation, methanol precipitation, and ultrafiltration (UF).

Concentration through Polyethylene Glycol (PEG): First of all 50% PEG (Daejung, Siheung, South Korea) stock solution was prepared in Tris buffer. About 100ml of FMDV was collected and added with PEG at the rate of 7.5% (final concentration w/v) [22]. The solution was stirred overnight at 4℃. After stirring centrifugation was done at 4000 rpm for 20 minutes and the pellet was obtained [14]. The virus from the pellet was eluted using phosphate buffer (0.01M, pH 7.2). 10ml antigen was eluted (10 times concentrated) and stored.

Concentration through Ammonium Sulphate: A saturated solution of ammonium sulphate (Thermo Fisher Scientific, USA) was prepared in distilled water. An equal volume of 50% AS and virus suspension was stirred overnight at 4℃. The pellet was obtained by centrifugation at 4000 rpm for 20 minutes and elusion of the virus was done by using phosphate buffer saline (PBS, 0.04M with 0.5 BSA). A total of 10ml volume was obtained after elution (10 times concentrated).

Ultrafiltration: For concentration through ultrafiltration polyether sulfone membrane with a nominal molecular weight limit (NMWL) of 300 kDa (Millipore Corporation, USA) was used to concentrate the virus [23,24]. 100 ml virus suspension was added into the filtration chamber and specific pressure was generated for proper filtration recommended by Millipore Corporation. Filtration was done until the retentate of UF reached 10ml volume (final concentration factor 10 or 10 times concentrated).

Concentration through Methanol: 100ml virus was taken and methyl alcohol (95% pure molecular biology grade methanol) was added at the rate of 20 % final volume according to Barlow [15]. The suspension was placed at -6℃ for overnight incubation. The next day centrifugation was done at 4000 rpm for 20 minutes and a pellet was obtained. The pellet was suspended in 0.04M PBS and elution was done until the eluted volume reached 10ml (concentration factor 10).

Quantification of antigen by size exclusion chromatography (SEC): Quantification of non-concentrated (BEI-inactivated) virus and concentrated virus by the above different method was done by size exclusion chromatography (SEC, Biologic LP chromatographic system 731-8350; Bio-Rad, Hercules, USA) using specific resin Sephacryl ™ S-300 high resolution (Cytiva, Marlborough, USA) [25]. First of all, resin slurry was prepared, and a Bio-Rad glass column of 50 cm was packed using phosphate buffer (0.01M, pH 7.2). Packing of the column was done according to the instructions using a pressure flow of 1-2minutes/ml. Already optimized parameters like flow rate (FR=0.75ml/minutes), bed height (48cm), sample volume (W=3.5ml), path length of flow cell (PL=0.2cm), chart recorder speed 12cm/h (S=0.2cm/minutes) were set on machine [26]. Non-concentrated samples were injected through the sample port and a chromatogram was recorded on the PC attached. All the other samples (duplicate of each) of the concentrated virus through different methods like PEG, AS, UF, and methanol precipitation were run, and peaks were recorded in the form of chromatogram at 254nm with $E_{cm}^{1\%}$ =72; different sensitivity absorbance unit (FSD) [27]. The peak area of each sample was calculated in cm^2 from the chromatogram. The concentrations of all samples were calculated by following formulae described by [28].

$$\text{Estimation of FMDV in the sample (µg/ml)} = \frac{FR \times PA \times FSD \times 1000}{S \times PL \times E \times W}$$

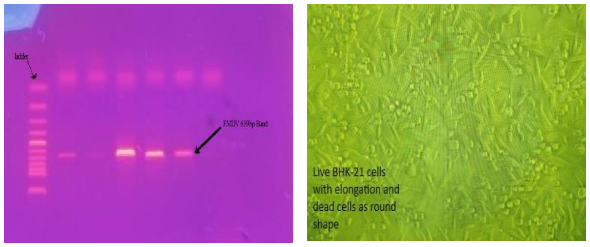
Concentrations of different samples (concentrated, non-concentrated) were compared to rule out the best method of concentration. Chromatography elutes of all samples were collected separately in falcon tubes and stored at 4℃ for further use in vaccine preparation or other testing.

Antigen Detection through ELISA: The presence of virus in the chromatographic eluted sample is confirmed by using commercially available ELISA kit (FMDV antigen detection and serotyping ELISA, The Pirbright Institute, UK). Each sample was diluted in diluent buffer and 50ul/well of each sample (PEG, AS, UF, and Methanol precipitated chromatography elute) was added into ELISA plate wells. For the validity of the test Positive inactivated control was expected to give an optical density (OD) value ⩾ 1.0 while negative control ⩽ 0.1

unit. For the positive sample, yellow color of the well is the first indication. Corrective OD (sample OD-NC) values were measured for the sample to declare positive/negative. If the corrective OD > 0.1 unit then the sample will be positive [29].

Results

The sample was collected and confirmed by RT-PCR (639bp) bands (Fig.1.a) of FMDV serotype 'O' observed on 1% agarose gel. BHK-21 cell was revived and a monolayer of cells (5.6×10^6 cells/ml) was developed on cell culture flasks. Elongated shape cells adherent to the surface of the flask appeared with a cell count of 5.6×10^6 cells/ml (Fig.1.b). Virus propagation on the cell leads to cytopathic effects (CPEs) after incubation which indicates the virus's presence. After harvesting of antigen, $TCID_{50}$ ($10^{7.5}$) of live virus was calculated by reed and munch method. Virus inactivation was done by BEI (double inactivation) followed by neutralization of the effect of sodium thiosulphate. Confirmation of inactivation was done, by inoculating the virus on cell culture up to seven passages. No CPEs were found on cell culture after seven passages that confirm the inactivation of antigen. 100 ml of virus for each method was separated; concentration was done by each method, PEG, AS, UF, and methanol. Quantification of antigen (µg/ml) (concentrated and non-concentrated/control) was done by size exclusion chromatography from chromatograms (Fig.2). The amount of antigen determined by column chromatography in the control antigen was 60.17±1.32 µg/ml. 7.5% PEG concentrated showed higher concentration of antigen (467.70±1.47 µg/ml) than 50% AS treated sample (359.16±1.18 µg/ml), UF treated sample (195.38±0.83 µg/ml), and methanol treated sample (83.19±0.19µg/ml) (Table. 1). Antigen detection was done after quantification of concentrate and the samples were positive for serotype 'O' of FMDV (Table. 2).



(a)FMDV Band on 1% agarose (b)BHK-21 cells

Figure 1: (a) RT-PCR amplicon (639bp) of FMDV on 1% agarose gel with 1kb ladder for confirmation of virus; (b) Baby hamster kidney cells (BHK-21) for growth of FMDV, live cells with elongation & dead cells with a rounded shape

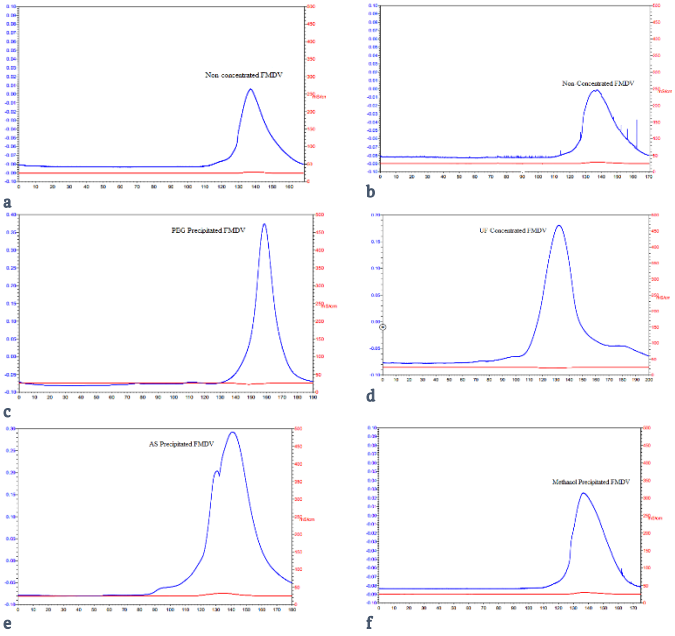


Figure 2: Chromatogram of FMDV obtained from Size Exclusion Chromatographic (SEC) system for quantification of FMDV; (a & b) Non-concentrated FMDV, (c) PEG precipitated, (d) UF precipitated, (e) AS precipitated, (f) Methanol precipitated FMDV.

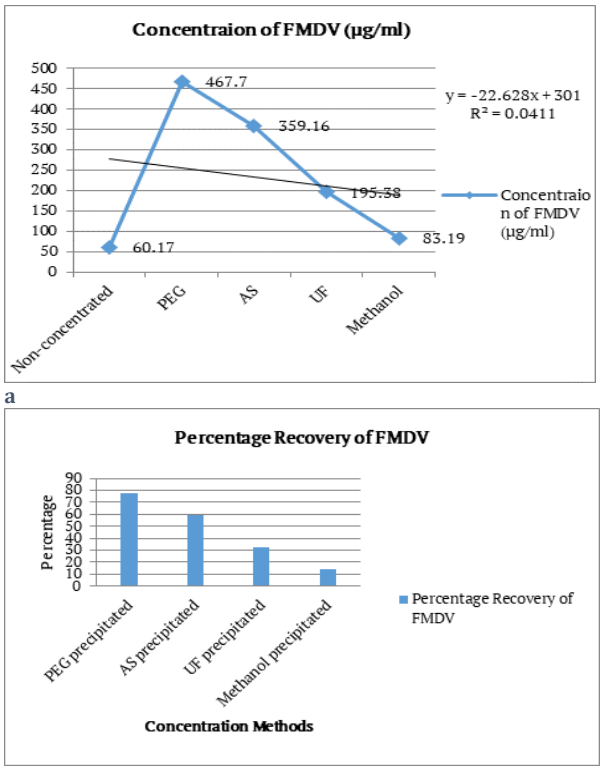


Figure 3: (a) Graph showing a comparison of the concentration of FMDV vs. methods; (b) Percentage recovery of virus vs. concentration methods.

FMDV Sample	Expected Concentration (µg/ml)	Concentration of Antigen (µg/ml)	Loss of Antigen (µg/ml)	Percentage Recovery (%)
Non-Concentrated	--	60.17	--	100
PEG Concentrated	601.10	467.70	133.4	77.80
AS Concentrated	601.10	359.16	241.93	59.75
UF Concentrated	601.10	195.38	405.72	32.50
Meth Concentrated	601.10	83.19	517.91	13.83

Table 1: Concentration of antigen (µg/ml) from different methods and percentage recovery of FMDV after application of concentration methods.

Analysis of variance (ANOVA) was applied on concentration methods vs. concentration of antigen which shows P = 0.000 which is less than 0.05, statistically concentration methods are different, Paired t-test was also applied between methods, and p <0.05 was recorded for all the pairs which also indicate that methods are significantly different.

Sample Type	Sample OD	Positive Control (PC)	Negative Control (NC)	Corrective OD (Sample-NC)	Results
PEG precipitate	3.344	3.603	0.091	3.253	+
AS	2.786	3.603	0.091	2.695	+
UF	2.469	3.603	0.091	2.378	+
Methanol	0.569	3.603	0.091	0.478	+

Table 2: Antigen detection ELISA results for confirmation of FMDV in samples from different concentration methods; OD>0.1 indicates the positive sample for FMDV serotype "O".

Discussion

Foot and Mouth is an infectious disease caused by FMD virus. The disease has major effects on the economy due to its transboundary nature. The annual economic loss due to FMD is estimated at almost 6.5 billion US dollars. Endemic areas are restricted for trading animals and their products. The disease is on the priority list among veterinary infectious diseases in the world. FMD is endemic in many countries of the world including Pakistan. The disease is a major problem for beef and dairy industries because of the trading issues as this is described as a vesicular lesion causing disease leading to ulcerative lesion [30]. FMDV serotype 'O' is more prevalent in Pakistan and 60% of the disease as compared to other serotypes [31]. A major source of the spread of disease in livestock is the transportation of animals and animal products in a legal or illegal manner from one country to another or more commonly between neighbor-joining countries [32]. Vaccine failure is the term where a population of animals cannot get proper vaccine whereas vaccination failure leads to either vaccine end or host end issues. Vaccine efficacy depends upon many factors like choice of strain, low antigen mass, formulation, improper strains, timing of vaccination, and herd coverage. Vaccine quality is an important factor for vaccination programs to succeed. Downstream processing of vaccine production is crucial for good quality vaccine [12].

In Pakistan vaccines are being produced by the traditional method of cell culture . The virus is propagated on BHK-21 cells and harvested. The antigen is either stored or used in vaccines. For a long-time storage, the virus degradation issue is ignored, and the vaccine is prepared which does not meet the requirement of antigenic mass and ultimately leads to vaccine failure. Another issue related to vaccine failure is the direct preparation of vaccine after harvesting the antigen without concentration of the antigen. The concentration of antigen is an important step of downstream processing during vaccine production. Through the concentration process issues like low mass of antigen either stored or harvested at the time of formulation of vaccine can be solved. Many techniques and methods are being used like ultrafiltration, ultracentrifugation, chemical precipitation, chromatography, and sedimentation [31].

The study was designed to investigate the best possible, cost-effective, and efficacious method to concentrate the antigen after harvesting from cell culture which gives maximum percentage recovery. The sample was confirmed by RT-PCR in a two-step process, as RNA of the virus was extracted initially by TRIzol method and then cDNA was prepared. PCR was done by using serotype-specific primers for FMDV. Amplicon of 639bp bands was observed on gel electrophoresis when a 1kb ladder was run. A molecular technique like RT-PCR was used for the detection of antigens as well as disease diagnosis [33,34].

In our study, BHK-21 cells were used for the cultivation of FMDV. Cells were revived by freeze-thawing mechanism followed by the addition of GMEM media with 10 % fetal bovine serum as described by [35]. Live cells were observed fibroblast, elongated in 70-80% monolayer after incubation at 37°C for 24-72 hours [36]. Cell counting was done by using a haemo-cytometer (glasslike grid). An equal volume of 0.4% trypan blue solution was mixed with cells. 10-20ul of the solution was poured into a glass chamber and average cells were counted in four large corners. 5.6×10^6 cells/ml was counted. The final density of 10^5 cells/ml was poured into cell culture flasks and media was added for further culturing purposes. FMDV serotype 'O' confirmed by RT-PCR as described above was propagated on cell BHK-21 cell line as done by [18]. Specific cytopathic effects (CPEs) were observed after incubation. Those CPEs included rounding of cells, multinucleated cells formation, flattening of cells, breaking of intracellular bridges, and ultimately cell death [37]. TCID50 was calculated according to the Reed and Munch method adopted by [38]. In the present study, a $10^{7.5}$ TCID50 titration value was calculated.

The inactivation of a virus is an important step in the preparation of inactivated vaccine. Many chemicals are used like 37% formaldehyde solution at 37°C, Binary Ethylimine (BEI) at 26°C, and propiolactone. In the present study, BEI was used to inactivate antigen according to the recommendation of WOAH. Freshly prepared BEI at the rate of 2% was added in viral antigen and flasks were incubated for 24-48 hours as the method [20]. The effect of BEI was neutralized with sodium thiosulphate. Inactivation was confirmed by propagating inactivated antigens on cell culture. No CPEs were observed on cell culture after incubation time as the same results were supported by [39].

Sucrose density gradient analysis is the suggested technique for measuring inactivated antigens. Over the past three decades, this method has been a tremendously useful tool, but it is highly operator-dependent and challenging to automate. Another method to quantify the FMDV during the vaccine manufacturing process is based upon the separation of components by size exclusion chromatography (SEC) and measurement of antigen by absorption at 254nm [25]. In the present study, a Bio-Rad chromatographic system with specific resin Sephacryl ™ S-300 was used to estimate the antigen (146S) in µg/ml from non-concentrated and concentrated samples. Similar studies were done by Spitteler and his colleagues to quantify the virus by two different methods SE-HPLC and SDG and correlation was measured which showed that chromatography can be used to quantify the FMDV. Another study was done to quantify the virus-like particles by size exclusion chromatography [40].

In the present study, cell culture harvest was inactivated with BEI (double inactivation) and antigen was divided into five parts (100ml each). One part was non-concentrated control and the other was used for concentration by methods described above. PEG precipitation, AS precipitation, methanol precipitation, and UF were compared and analyzed to find the most efficient concentration method for FMDV. 100ml of antigen in each method was concentrated about 10 times (10X). All the samples were passed through column chromatography for quantification by chromatograms (Fig.2). Non-concentrated antigen was used as a control group for the comparison among the four methods described above. The amount of antigen determined by column chromatography in the control antigen was 60.17±1.32 µg/ml. 7.5% PEG concentrate showed higher concentration of antigen (467.70±1.47 µg/ml) than 50% AS treated sample (359.16±1.18 µg/ml), UF treated sample (195.38±0.83 µg/ml), and methanol treated sample (83.19±0.19µg/ml) (Table. 1, Fig.3). Analysis of variance (ANOVA) was done by using Minitab software on table between concentration method and concentration of FMDV. The p=0.000 from ANOVA shows that all concentration methods are significantly different (Table. 1).

Based on these results, respective percentage recovery from all the concentration methods were calculated as follows: PEG treated sample, 77.80%; AS treated sample: 59.75%; UF treated sample: 32.50% and methanol treated sample: 13.83% (Table. 1). In the previous study, Kim and his colleagues measured the percentage recovery of PEG, AS, and UF: 83.36%, 63.59%, and 33.5% respectively [14]. In the present study, 7.5% PEG showed maximum percentage recovery of antigen (77.80%) which is significantly higher than AS, UF, and Methanol (Table. 1). A pairwise t-test was applied to data (Table. 1) by using Minitab 17 software to compare the PEG-AS (p=0.000), PEG-UF (p=0.001), PEG-Meth (p=0.001), AS-UF (p=0.001), AS-Meth (p=0.002), and UF-Meth (p=0.003), As the p-values for all the groups were $p<0.05$, which shows that each method is significantly different (Table. 1). It means each method has its particular effect on FMDV recovery respective other methods.

An ELISA kit was used to detect antigens (The Pirbright Institute, UK). In antigen detection ELISA, eluted chromatography samples (concentrated using various techniques) were employed. The test is valid as specified above, as evidenced by the positive control (PC) values of $3.603 \geqslant 1.0$ and the negative control (NC) values of $0.091 \leqslant 0.1$. The ELISA kit specifies that corrective OD values for positive samples should be > 0.1 units. As can be seen in the table, all samples tested positive for FMDV serotype 'O' (Table. 2). The same study was done to detect FMDV antigen for confirmation [16].

In conclusion, the optimal approach for concentrating FMDV serotype "O" for local vaccine production is suggested based on the current findings, which point to 7.5 percent PEG precipitation. The current study demonstrates that the PEG-based approach of virus concentration recovers 77.80% of the virus, suggesting that this method is inexpensive, simple, rapid, and high-recovery for the concentration of FMDV. Due to differences in biophysical stability, different serotypes and strains may respond to these approaches differently.

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Author Contributions

Conceptualization: Faisal A., Imran A., Abdul R., Afifa S. & Muhammad A.D., Data curation: Faisal A., Imran A., Saad B.S., Tayyab K., Formal analysis: Faisal A., Imran A., Abdul R., Funding acquisition: Mahnoor R., Umair A.,

Zeeshan K. Investigation: Faisal Ayub., Imran A., Abdul R., Afifa S. Methodology: Faisal A., Imran A., Abdul R., Supervision: Imran. A. Writing-original draft: Faisal A., Aemin R., Zeeshan K., Hafiz M.M.A., Muhammad S., Muhammad A.R. Writing-review & editing: Faisal A., Imran A., Abdul R., Hafiz M.M.A., Muhammad A.D.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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