



# Development and Therapeutic Potential of Foot-and-Mouth Disease Virus Specific Anti-Idiotypic Antibodies

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**Abstract** | The biological effects of idiotype antibodies in immune modulation against diseases have been described in various studies. Idiotype vaccine is a novel alternative approach to immunize animals against various contagious diseases. The recombinant subunit vaccines and antibody-based vaccine does not result in post-vaccine outbreaks as it is devoid of viral structural and non-structural proteins. Three inactivated serotypes of foot-and-mouth disease virus (A, Asia-1, O) were injected into the goats. The serum containing a high antibody titer was proceeded for the separation of idiotype antibodies via octanoic acid-ammonium sulfate precipitation. The fragment antigen-binding (Fab) components of idiotype antibodies were separated and purified. Two different concentrations of Fab idiotype were used as antigens in the layer birds for the development of anti-idiotype antibodies that were used as FMD virus surrogates in rabbits. The production of anti-idiotype antibodies in egg yolk was detected through the agar gel immunoprecipitation test (AGPT) and western blot. The birds immunized with 10 mg mL<sup>-1</sup> of idiotype perfectly produced anti-idiotype antibodies at day 14 post-immunization. Antibody titer of anti-idiotype antigen was significantly high with a peak titer of 83% at 45-day post-immunization. The caprine anti-FMD virus idiotype has the potential to successfully produce anti-idiotype in layer birds. Anti-idiotype antigens may be a good alternative as virus surrogates in vaccine development. Layer birds are the potential experimental source for the bulk production of anti-idiotype antibodies.

**Keywords** | Antibody, AGPT, Caprine, Foot-and-mouth disease, Idiotype

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## INTRODUCTION

Foot-and-mouth disease (FMD) is an endemic highly contagious disease in most parts of the world that affects broad host range including domestic and wild cloven-footed animals (Smith et al., 2004). Globally, an economic burden of six to twenty billion US dollars is associated with the FMD in terms of animal milk and meat production, mortality, preventive measures, and trade restrictions (Knight-Jones and Rushton, 2013). FMD outbreaks in 2001 in United Kingdom led to slaughtering of approximately 7 % of all cattle and 15 % of sheep while a US\$300 million loss from outbreaks of 2007 (Anderson,

2008; Rushton et al., 2002). FMD virus, a positive-sense single-stranded RNA virus is responsible for the disease outbreaks. The virus has seven major serotypes each having subtypes. There is no cross-protection between the serotypes (Waters et al., 2018). Different disease control policies have been adopted across the globe including restriction of animal movement and mass vaccination programs (Jamal and Belsham, 2013). Vaccination is considered an effective strategy to control the disease, but still, there are some uncertainties in the time of vaccination and type of vaccines. Mostly killed monovalent, bivalent, trivalent and polyvalent vaccines are used in vaccination programs (Paprocka, 2013). FMD virus is RNA virus, the genetic

variability due to genetic drift in the virus helps in escaping the virus from immune responses (Ahmed et al., 2017). Previous studies also reported the risk of viral escape during manufacturing from production units and vaccines may contain traces of viral non-structural proteins that can interfere with nonstructural protein (NSP)-based serological diagnosis to differentiate infected and vaccinated animals (Yimei et al., 2016). Continuous improvements in the vaccines are needed for the effective control of diseases (Mason and Grubman, 2009).

As, maintenance of cold chain is important issue that is difficult to manage in developing countries. Development of an idiotypic based vaccine is an alternate approach to immunize the animals. Currently, we developed anti-idiotypic antigen that does not require large amount of virus rather bulk quantity of antigen can be developed in the layer eggs from small dose of viral inoculations (Naveed et al., 2018). Furthermore, the problems associated with cold chain maintenance can be reduced using anti-idiotypic as surrogate antigens. Idiotypic (Ids) are the antigenic domains in the variable region of the antibodies (Gomez et al., 2013; Bachrach, 1985). Hence, non-infectious, protein in nature idiotypic antibodies are a good choice for vaccine development (Segundoa et al., 2017). The idiotypic antibody-based vaccine can elicit a strong immune response of fine specificity compared to live/ inactivated virus as antigen in vaccines (Naveed et al., 2018).

The use of laboratory animals to produce vaccines has many advantages, but the associated debates on the unethical use of laboratory animals are not that noticed. The use of Freund's complete adjuvant is documented to induce pain and stress in the immunized animals (Hendriksen, 2005). The growing industry and experimental animals are a major concern at present. There is a dire need for research in the field of vaccine development and potency testing to find the best alternatives to laboratory animals (Caseya et al., 2011). The study was designed to develop anti-idiotypic antigen from the idiotypic antibodies of caprine origin. Furthermore, there is no need to kill the layers post experiments and animals can be consumed for food purpose after residual time.

## MATERIALS AND METHODS

### ETHICAL STATEMENT

Experimental birds/animals were purchased from private farms and maintained in the animal house of Institute of Microbiology under proper care according to the instructions of the institutional biosafety/bioethics committee (Ref. No. 4858/ORIC) of the University of Agriculture, Faisalabad, Pakistan. The animals were halal slaughtered giving a cut to the jugular vein, carotid artery, and wind-

pipe after completion of experiments.

### MOLECULAR CHARACTERIZATION OF FMD VIRUS

Three foot-and-mouth disease virus serotypes (A, Asia1 and O) with tissue culture infective dose 50 (TCID<sub>50</sub>) of the virus was 1X10<sup>4</sup> in BHK-21 cells were procured from the Veterinary Research Institute Peshawar, Pakistan. Molecular re-characterization of the FMD virus was done by PCR using oligonucleotide primer (forward P1-P32 5' CAGATGCAGGAGGACATGTC 3' and reverse P33 5' AGCTTGTACCAGGGTTTGGC 3') amplify 131 bp fragment FMD virus genome (Shin et al., 2000). The PCR confirmed FMD virus was further proceeded to competitive 3ABC non-structural protein ELISA (ID Vet ELISA Kit, IZSLER, Brescia, Italy).

### DEVELOPMENT OF POLYCLONAL IDIOTYPE ANTIBODIES

FMD virus specific anti-idiotypic antibodies were raised in goats. Ten month old 12 Teddy goats were kept in animal house facility under Institute of the Microbiology, University of Agriculture Faisalabad, Pakistan. The goats were tagged as GA (group of serotype A), GA1 (group of serotype A1), GO (group of serotype O) and GC (control group) and segregated into four groups based three in each. An increasing dose of 2-5 mL animal<sup>-1</sup> inactivated FMD virus serotypes O, A and Asia-1 were injected through intramuscular route in the neck region of respective groups after every week for three 3 weeks. Aseptic blood collection and serum separation was performed on day 14, 21, 28 and 35 of the post immunization. Positive samples with high antibody titers of all three serotypes O, A and Asia-1 was separately detected using competitive ELISA Kits (ID Vet, IZSLER: Brescia, Italy) according to the manufacturers' protocol. The value above 70% at 1/10th dilution was considered positive (Manzoor et al., 2015).

### SEPARATION OF POLYCLONAL IDIOTYPE ANTIBODIES AND PREPARATION OF ADJUVANTED IDIOTYPE ANTIGEN

The positive serum samples for all the three serotype Asia1, A and O were separately processed for separation of polyclonal idiotypic antibodies with minor modification (McKinney and Parkinson, 1987). Briefly, the IgG were separated through ammonium sulfate-octanoic acid double precipitation and purified IgG idiotypic was digested with pepsin and ensued to dialyzation against 50 volumes of phosphate-buffered saline at 7.0 pH. The Fab were assessed for their purity through Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in a discontinuous buffer system. The Fab protein concentrations were adjusted through the Nanodrop (Thermo Scientific, NanoDrop 8000) quantification technique. Two different doses (5 mg mL<sup>-1</sup> and 10 mg mL<sup>-1</sup>) of Fab protein were separately adjusted for all the three serotypes. The purified Fab component was emulsified in a 1:1 ratio of Montanide

**Table 1:** Idiotype protein concentrations in layer birds of respective groups

Total Birds	Groups	Sub groups	Idiotype protein concentrations	Intramuscular Dose
60	LI (30)	LIA1	5 mg mL <sup>-1</sup>	0.2mL/bird
		LIA		
		LIO		
	LII (30)	LIIA1	10 mg mL <sup>-1</sup>	0.2mL/bird
		LIIA		
		LIIO		

adjuvant for idiotype antigen preparation.

### IMMUNIZATION OF LAYER BIRDS

The idiotype antigens were injected into different groups of 26-week old layer birds (NOVOgen white) to raise anti-idiotype antibodies. Sixty-layer birds of the same age were equally divided into LI and LII groups. The immunization schedule of layer birds with idiotype is given in Table 1, eggs were collected after 2 weeks of the last injection. Anti-idiotype antibodies in the eggs were detected at 2, 3, 4, and 5 weeks' post-immunization.

### AGAR GEL IMMUNOPRECIPITATION TEST

Presence of anti-idiotype antibodies in the egg yolks were confirmed using agar gel immunoprecipitation test (AGPT) and western blot analysis. The overlay method was adopted for the experiment, a 2 mm of 4% agar was covered with a 4 mm layer of 0.9% agarose gel (Sigma-Aldrich) prepared in borate buffer (pH 8.8) with the addition of 2% sodium azide (SIGMA-ALDRICH ≥ 99.5%). Egg yolks were separated and washed thoroughly with distilled water to remove the membrane and 9-fold dilution was prepared (Sudjarwo et al., 2017). The idiotype antigens were added in the central well while the diluted egg yolks were added in all the three wells keeping the fourth well as control. The process was separately repeated for all three serotypes and plates were incubated at 37°C for 48 hrs.

### WESTERN BLOT ANALYSIS

The specificity of anti-idiotype to FMD virus antigen was assessed through western blot analysis. Briefly, 80 µL of 5-10 mg/mL anti-idiotype protein was mixed with equal volume of electrophoresis sample buffer. The prepared samples for all three serotypes were subjected to SDS-PAGE in a 12 % slab polyacrylamide gel separated by a 4% stacking gel at 100 V for 2.5 hours at RT. After electrophoresis, the gels were electrically transferred onto a nitrocellulose membrane for 2 hours at 80 V at 4 °C. The membranes were blocked for 1 h in 5% skim milk in Tri- buffered saline supplemented with tween 20 (TBST). The membranes were washed thrice with TBST and incubated over night at 4 °C anti-idiotype antibodies to FMD virus (mimicked FMD virus) in 1:500 dilutions in TBST containing 5% skim milk. After washing with TBST, the membrane was

incubated with secondary antibody to FMD virus structural protein VP1 (procured from veterinary research institute Peshawar, Pakistan) at 37°C for 1h. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using primary Anti-GAPDH antibody (ab8245) while secondary goat anti mouse antibody (Invitrogen, Cat. A32723) as loading control. The bound HRP was detected by enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare Life Sciences, Buckinghamshire, England). The ECL signals were captured by Hyper film ECL (GE Healthcare Life Sciences, Buckinghamshire, England) (Zhu et al., 2019).

### IMMUNE RESPONSE OF ANTI-IDIOTYPE ANTIGEN IN RABBITS

Anti-idiotype antibodies to caprine anti-FMD virus idiotype antigen for serotype A, O and Asia-1 were separately adjuvanted (1:1) in Montanide.

Montanide adjuvanted anti idiotype antibodies were injected subcutaneously (0.5 ml/animal) in 9 rabbits divided into three groups (RA, RO and RA1) for serotype A, O and Asia-1 respectively, while 4<sup>th</sup> group (RC) was kept as Montanide control.

The second immunization of 0.5 ml/ animal was given 2 weeks after 1<sup>st</sup> injection. Blood samples were collected at 0, 15, 30- and 45-day post immunization for serum separation and ELISA titer through FMD virus competitive ELISA kit (ID Vet; Izslar, Brescia, Italy). The mean antibody titer was statistically analysed using origin pro software.

## RESULTS

### MOLECULAR CHARACTERIZATION AND SEROTYPING OF FOOT-AND-MOUTH DISEASE VIRUS

Molecular reconfirmation of FMD virus was done using conventional PCR (data not shown). Results were further confirmed via ELISA test for three serotypes (Asia-1, A, and O) of FMD virus. OD value of sample > 0.1 at 450 nm attained after subtraction from the control negative was considered as positive values (data not shown).

**IDIOTYPE ANTIBODY TITER IN GOATS**

FMD virus serotypes Asia-1, A, and O were separately injected in three groups of goats to raise idiotypic antibodies. The group GA showed inhibition titer ranging from 77 % to 91%, while the maximum mean inhibition titer of 88.33 % was observed on day 28 post-immunization (PI). The titer declined to 87.33 % on the day 35 PI. Group GA1 exhibited ELISA titer ranging from 86 % to 93 % while mean inhibition titer of 91 % was attained on day 21 PI followed by a slow decrease to 90 % and 89 % on days 28 and 35, respectively. The ELISA inhibition titer in group GO ranged from 79 % to 92 % and the maximum mean inhibition titer of 87.33 % was achieved on day 14 PI that was sustained until day 21 PI. Then decreased to 84.33 % on the day 28 PI and to 83.33 % on the day 35 PI (data not shown).

**SEPARATION OF POLYCLONAL IDIOTYPE ANTIBODIES (IgG) FROM GOAT SERUM**

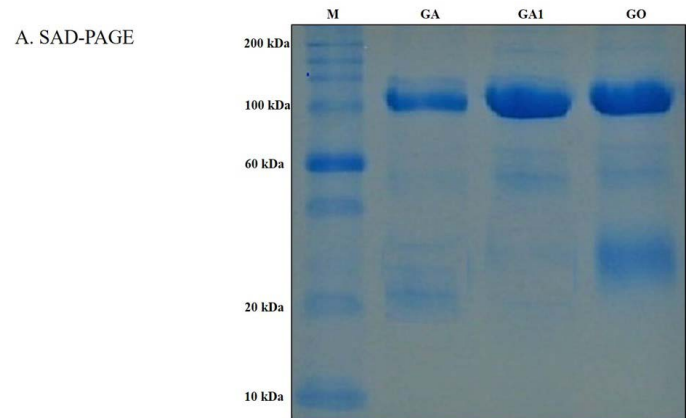
Goat serum to FMD virus were processed via double precipitation and dialyzation to purify Fab components of polyclonal idiotypic antibodies. The initial treatment with octanoic acid resulted in precipitation of the lipids and non-IgG proteins within a half-hour of incubation. The second treatment of the supernatant with ammonium sulfate resulted in the formation of the clear white pellet of IgG proteins. The pooled serum protein (IgG) purified through ammonium sulfate precipitation and pepsin digestion revealed the specific idiotypic protein contents of 110 kDa which was separately identified in all the serum samples collected from three respective groups. Moreover, the Fab component was maximum in the GA1 group followed by GO and GA as identified through the density bands appeared on the SDS-PAGE gel (Figure 1).

**CONFIRMATION OF ANTI-IDIOTYPE ANTIBODIES IN THE EGG YOLK**

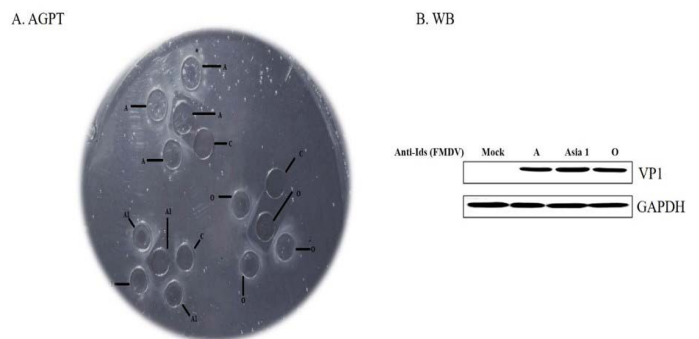
Purified Fab components of polyclonal idiotypic antibodies to FMD virus were adjuvanted and injected in layer birds according to the methods described previously. Eggs were collected at indicated time points and the presence of anti-idiotypic antibodies in the egg yolks was confirmed through AGPT. A clear line of precipitation indicated the presence of anti-idiotypic antibodies in the egg yolk specific for each of the idiotypic antigen (Figure 2A). While no line of precipitation was observed against control well. The AGPT results indicated the presence of anti-idiotypic antibodies in the egg yolk collected from the layer birds injected with caprine anti-FMD virus idiotypic antigen.

We further analysed anti-idiotypic antibodies that mimic original FMD virus using western blot analysis. Presence of mimicked FMD antigen was detected at 27 KDa for anti-idiotypic to all three serotypes of FMD virus, whereas

no band was detected in the control samples (Figure 2B).



**Figure 1:** Goat serum containing highest antibody titers to FMD virus serotypes were processed separately via double precipitation and dialyzation. Fab components were purified and SDS PAGE was performed for confirmation. SDS-PAGE profiling of fragment antibody binding components of idiotypic antibodies (IgG) after staining with Coomassie brilliant blue. L is for protein ladder while lane GA (group serotype Asia), GA1 (group serotype Asia1) and GO (group serotype O) are Fab components obtained from goat serum immunized against inactivated FMD virus serotypes. Band densities are indicating presence of more Fab components in the group injected with FMD virus serotype Asia 1 while least in group representing serotype O.

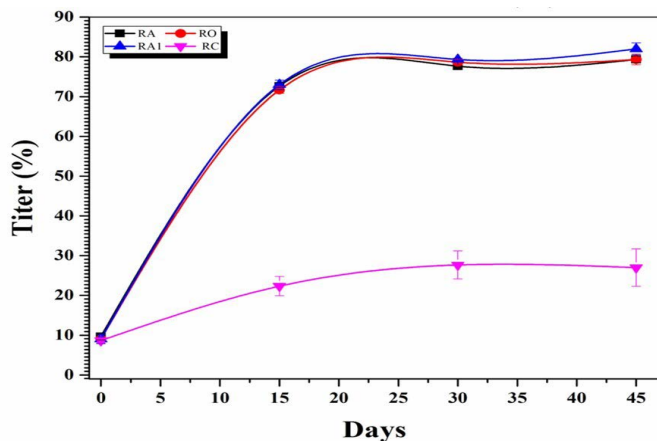


**Figure 2: (A) Detection of anti-idiotypic antibodies:** Purified idiotypic Fab components from respective groups were adjuvanted in Montanide and injected in layer birds. Eggs were processed for purification of anti-idiotypic antibodies to Fab components of polyclonal idiotypic antibodies against three FMD virus serotypes. Presence of anti-idiotypic antibodies were confirmed via agar gel immune precipitation test. A clear Line of precipitation in the figure between idiotypic antigen placed in the central well and egg yolk containing anti-idiotypic antibody on the 0.9% agarose gel plate after incubation of 48 hrs from the group II received the idiotypic antigen containing protein concentration of 10mg/mL. **(B). Confirmation of anti-idiotypic antibodies:** The anti-idiotypic proteins mimicking FMD virus were disrupted on SDS-PAGE and

transferred onto the nitrocellulose membrane. The anti-idiotypic proteins mimicking FMD virus were detected at 27KDa using VP1 antibody to FMD virus and GAPDH was used as loading control.

### IMMUNE RESPONSE OF ANTI-IDIOTYPE ANTIGEN IN RABBITS

The whole anti-idiotypic antibodies purified from egg yolks were adjuvanted in Montanide and injected in experimental rabbits to find out the viral inhibition titers. Anti-idiotypic antigen elicited robust humoral immune response in the rabbits. An increased antibody titer to anti-idiotypic antigen was observed from 15 to 45-day post vaccine. Significantly (4 log<sub>10</sub>) high inhibition titer percentage of 83% was recorded in the rabbits vaccinated with anti-idiotypic antigens compared to the control group, whereas, the inhibition titer among all the anti-idiotypic antigens developed for three serotypes was statistically non-significant (Figure 3).



**Figure 3:** Rabbits were divided in four groups as RO (Group of rabbits for serotype O), RA (Group of rabbits for serotype A), RA1 (Group of rabbits for serotype Asia 1) and RC (Group of rabbits for control). Anti-idiotypic bodies purified from egg yolks were adjuvanted in Montanide and injected in rabbits. Viral % inhibitory were measured via C-ELISA and results are expressed indicating time dependent inhibitory levels.

### DISCUSSION

PCR is a rapid, sensitive, and reliable technique for the detection of viral RNA. In this research, PCR was used to amplify the product of 131bp to confirm the FMD virus regardless of serotype. The results of the PCR were in line with the work done by (Alamdari et al., 2006). In another study, detection of FMD virus through RT-PCR was done using primers acting on high conserved regions of the virus, since a single point mutation in conserved sequences may inhibit primer binding (Locher et al., 1995). However, simple PCR analysis for the presence of FMD

virus in the samples is a far easier and rapid method. The detection of virus from the sample using Pan Serotype primers is more reliable as compared to a serotype-specific set of primers (Reid et al., 2014). Although reverse transcriptase PCR and real-time reverse transcriptase PCR are more rapid and sensitive methods for the detection of the virus genome in the samples. Often the results from these methods end in cross-contamination of the product and do not accurately distinguish among the serotypes (Morioka et al., 2009). The serotyping of the virus in the present study was conducted through ELISA for three serotypes: A, Asia-1 and O. The antigen captured ELISA by IgY and monoclonal antibodies are 100% sensitive means for the serotyping of FMD virus except for serotype A (Veerasami et al., 2008). Monoclonal ELISA (MELISA) was also compared to the traditional ELISA in studies for the detection of FMD virus serotype O with 100% specificity making it a more convenient method for the rapid diagnosis of serotypes (Chen et al., 2013). The serological analysis of non-structural protein through Is-ELISA is an effective tool for serotyping of FMD virus and results of the present study were similar to the study performed in Australia for the detection of FMD virus through 3ABC NSP (Colling et al., 2014). The 3ABC NSP ELISA is a broad test able to detect antibodies elicited by all FMD serotypes (Bronsvort et al., 2004).

The idiotypic antibodies in the present research were prepared from the goat serum immunized with inactivated FMD virus serotypes A, Asia-1, and O. Octanoic acid-ammonium sulfate method is an efficient way to purify antibodies from serum. OA-AS precipitation method is superior to affinity chromatography and HPLC in terms of yield of antibodies (Temponi et al., 1989). The results of IgG purification agreed with a two-step procedure adopted by Boushaba (Boushaba et al., 2003) for purification of IgG through the CA-AS precipitation method. The lipids and non-IgG fragments are removed by CA while IgG is salted out by AS precipitation. The digestion of purified IgG from serum can be efficiently done using pepsin in comparison with whole serum digestion (Jones and Landon, 2002). The digestion of idiotypic antibodies for purification of Fab was performed using pepsin and results of digestion were like the research conducted by (Bendandi, 2011). Idiotypic antibodies specifically recognize the invading antigen and they can be used as the immune-potentiating agent. In most of the human cancers as human B cell lymphoma, idiotypic based vaccines are used for their antigenic potential (Rodkey, 1974). The anti-caprine antibodies exhibit non-responsiveness to the self-antigen if these are prepared in the same animals used in the preparation of idiotypic antibodies (Schade et al., 2005). The same animal was not used for the development of anti-caprine antibodies and research was conducted with layer birds.

IgY antibodies are expected to produce few immunological side effects. The presence of a high protein concentration of 10 mg mL<sup>-1</sup> in idiotype based antigenic suspension leads to the development of anti-caprine FMD virus idiotype antibodies in layer birds at an early course of immunizations. The caprine anti-idiotype was developed in the layer birds at selective concentrations of idiotype antigens. Anti-idiotype antibodies thus produced can effectively induce humoral immune response in rabbits as observed in present study. A peak antibody titer to anti-idiotype antigen generated FMD virus serotype O was 83 %. Similar robust humoral immune response generated from anti-idiotype antigen was observed by (Wang et al, 2005). Anti-idiotype antigen mimic the similar molecular structure of antigen and can be used as viral antigen surrogate (Coelho et al., 2004).

## CONCLUSION

The idiotype based vaccine strategies can be adopted for the immunotherapy of animal diseases. The anti-idiotype antibodies may have the potential to serve as surrogate of viral antigens. Use of chicken egg for collection of antibodies has several advantages as they reduce the painful procedure of blood collection from animals. The utilization of animals in terms of their number is reduced because more IgY can be produced from the egg yolk. High idiotype protein concentrations can induce the early development of anti-antibodies in layers. Layer birds can be used to develop antibodies required in vaccine production instead of killing animals they can be used for food purpose. Animal welfare issues and ethical concerns associated with the use of laboratory animals may be minimized by reducing the number of animals involved in the production and testing units of the vaccines as more eggs can be produced from single animal.

## LIST OF ABBREVIATIONS

Foot-and-mouth disease (FMD); Idiotype (Id)/(Ab1); Anti-idiotype (Ab2); Antibodies (Ab); Antigen (Ag); Octanoic acid (OA); Ammonium Sulphate (AS); Immunoglobulin G (IgG); Non-structural proteins (NSP); High-performance liquid chromatography (HPLC); Enzyme-linked immunosorbent assay (ELISA); Reverse transcriptase Polymerase chain reaction (RT-PCR); Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE); Fragment antigen-binding (Fab); Agar gel immunoprecipitation test (AGPT); Optical density (OD)

## CONFLICT OF INTEREST

None of the authors declared any conflict of interest.

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## AUTHORS CONTRIBUTION

All the authors contributed equally.

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