

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.902.119>

## Comparative efficacy of different methods in the generation of 12S particles from 146S particles of FMDV serotype A and their detection using serotype (146S) specific monoclonal antibodies

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### ABSTRACT

Foot-and-mouth disease [FMD] is a highly infectious and contagious viral disease of domestic and wild cloven hoofed animals. The disease is controlled by vaccination using inactivated vaccine as one of the important options. The integrity of 146S plays an important role in the efficacy of the vaccine. The currently applied methods like density gradient to check the integrity of the 146S particles are besotted with certain disadvantages. Therefore, we generated monoclonal antibodies (mAbs) specific to 146S of FMDV serotype A [Indian strain]. To test these mAbs for their specificity to 146S, conversion of 146S into 12S is a must. Normally, three methods like strong [1N HCl] and weak acid [Na<sub>2</sub>HPO<sub>4</sub>] methods; and heat method are followed in the conversion of 146S into 12S. Upon comparison of these methods in the present study, consistent results were obtained using heat method at 56<sup>o</sup>C and 60<sup>o</sup>C each for half an hour and one hour. However, the former two methods were very inconsistent in yielding 12S from 146S particle due to slight variation in the pH. Hence, we optimized heat method for efficient conversion of 146S particle to 12S particle of FMDV serotype A [A/INDIA/40/00], an Indian vaccine strain. The results are ascertained applying serotype specific [146S] monoclonal antibody based double antibody sandwich ELISA. However, the method needs to be evaluated using more number of 146S samples.

#### Keywords

Viral disease  
antibodies  
monoclonal  
serotype

#### Article Info

Accepted:  
08 January 2020  
Available Online:  
10 February 2020

### Introduction

Foot-and mouth-disease [FMD] is a highly infectious and contagious viral disease of domestic and wild cloven hoofed animals causing an enormous economic loss in

agriculture and allied sectors throughout the world (Kandeil *et al.*, 2013). The causative agent of FMD is foot-and-mouth disease virus (FMDV), a picornavirus with a worldwide distribution (Brito *et al.*, 2017; OIE, 2012) and grouped under the genus *Aphthovirus* of

the family *Picornaviridae*. It was first recognised by Fracastorius (1546) in cattle. Loeffler and Frosch (1897) demonstrated for the first time that the causative agent of FMD is a filterable agent. FMD virus exists in seven immunologically different serotypes such as O, A, Asia 1, C, SAT1, SAT2, and SAT3.

FMD virus has a single stranded RNA genome which encodes four structural proteins (VP1, VP2, VP3 and VP4) and eight non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C pro and 3D). The virus particle is formed by four structural proteins VP1-4, where the capsid proteins VP1, VP2 and VP3 are present on the surface of the capsid while the VP4 lies internally.

The disease is enzootic to large areas of Africa, Asia, and South America and has the potential to cross international borders and creates epidemics in non-infected areas (Knowles *et al.*, 2001; Alexandersen *et al.*, 2003).

FMD is an economically devastating disease as it causes major losses like reduced milk yield and draught capacity of the affected animals. Recovered animals fail to reach optimum development. The indirect losses include trade embargo and fear of consumers and hence, prevention and control of this disease is very important in animals.

The virus capsid has 60 copies of each of the four structural proteins namely VP1, VP2, VP3 and VP4, which form into an icosahedral structure (Acharya *et al.*, 1989). Each promoter of a structural proteins (VP1, VP2, VP3 and VP4) assemble together to form a pentameric structure which have a sedimentation co-efficient of 12S (Denoya *et al.*, 1978; Sanger *et al.*, 1978).

Similarly, twelve of these subunits assemble

together to form 146S with sedimentation coefficient of 146S. The 146S is considered as virion particle which is highly immunogenic in nature (Doel *et al.*, 1982). The antigenic sites present on 146S particle act as a causative agent for the development of a disease in animals, whereas, 12S particles are less causative.

Monoclonal (mAbs) and polyclonal antibodies are antigen binding particles which neutralize the disease causing agents. Both 146S and 12S particles share common antigen binding sites.

Therefore, most of the monoclonal and polyclonal antibodies cross react with both 146S and 12S particles. The specificity of mAbs in recognition of epitopes present on 146S or 12S or both is an essential feature for considering the feasibility of mAbs in developing a diagnostic assay (Gollapalli *et al.*, 2019). The dissociation of 146S particle into 12S particles occurs through mild heating or by lowering the pH of the buffer below 6.5.

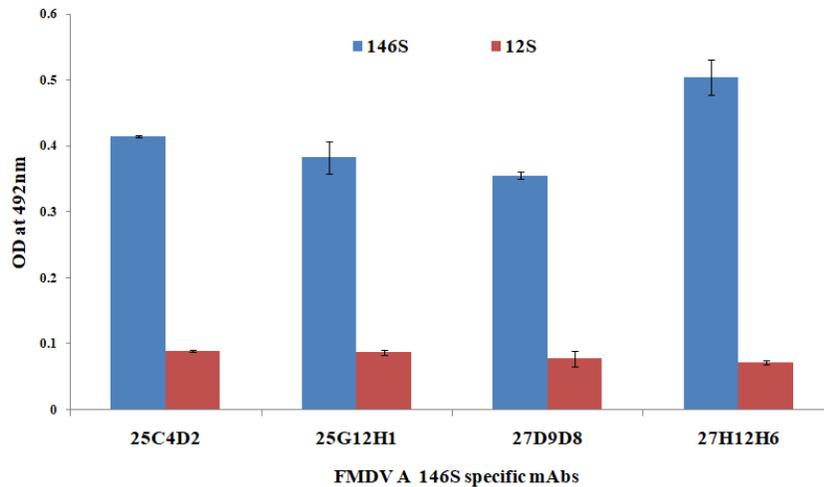
In this present study, we generated monoclonal antibodies (mAbs) specific to FMDV serotype A. In order to test the mAbs, whether they are specific to 146S or 12S, efficient conversion of 146S into 12S particle is a must. To generate, 12S from 146S, different methods like 1N HCl, NaH<sub>2</sub>PO<sub>4</sub> and heat methods are followed.

However, the former two methods are very inconsistent in yielding 12S from 146S particle. Hence, we optimized heat method for efficient conversion of 146S particle to 12S particle of FMDV serotype A [A/INDIA/40/00], an Indian vaccine strain.

The efficient conversion of 146S particles to 12S particles was ascertained by application of serotype specific mAb based double antibody sandwich ELISA (DAS-ELISA).

**Table.1** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S and 12S particles of FMDV serotype A in type specific DAS-ELISA

Monoclonal Antibodies	146S	STD DEV	%CV	12S	STD Dev	%CV
25C4D2	0.4154	0.0016	0.3915	0.0890	0.0019	2.1439
25G12H1	0.3829	0.0244	6.3896	0.0872	0.0032	3.7301
27D9D8	0.3554	0.0055	1.5720	0.0781	0.0117	15.029
27H12H6	0.5040	0.0270	5.3594	0.0725	0.0026	3.7062



**Fig.1**

**Fig.1** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S and 12S particles of FMDV serotype A in type specific DAS-ELISA

## Materials and Methods

### Preparation of 146S antigen of FMDV serotype A

The FMDV serotype A (IND 40/00) antigen stocks available at the Indian Veterinary Research Institute, Bengaluru were prepared in bulk quantities by infecting baby hamster kidney [BHK<sub>21</sub>] cultured cell monolayers using Glasgow's minimum essential medium [GMEM] media without tryptose phosphate broth (TPB) and then clarified by

centrifugation at 10000xg/30min/4<sup>0</sup>C. The clarified antigen supernatant was then inactivated using 3mM binary ethyleneimine (BEI) and concentrated using 8% [v/v] PEG-6000 (polyethylene glycol) method.

This concentrated antigen was purified using cesium chloride (CsCl) ultracentrifugation method [1.38g/ml and 1.42g/ml] at 100000xg over night to obtain 146S particle. The purified 146S antigen was collected and diluted using PBS. The concentration of 146S antigen was estimated by multiplying the OD

at 259nm with an extinction factor of 131. The purified 146S antigen was made into small aliquots as per the requirement and stored at -80°C.

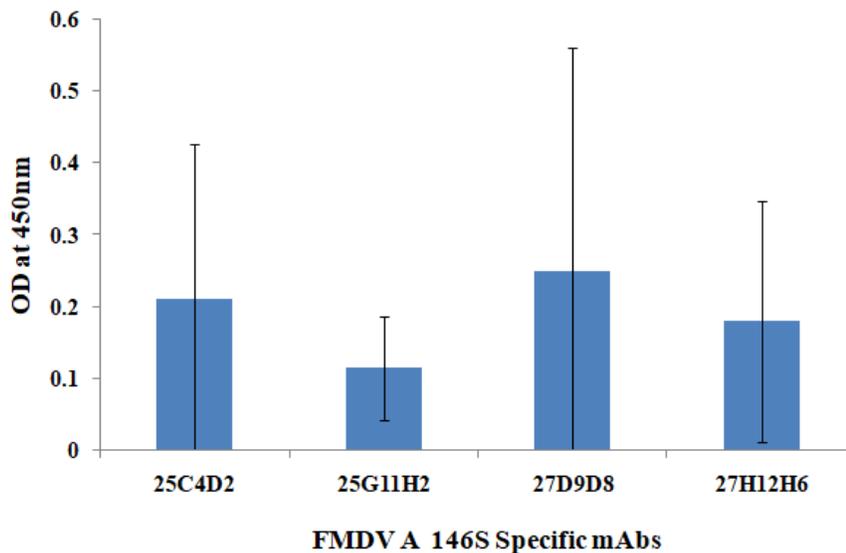
**Raising of monoclonal antibodies**

Two Balb/C mice were immunized with FMDV serotype A (A40/00) purified 146S antigen at 25µg intra peritoneally with Freund's

complete adjuvant [FCA] in a volume of 100 µl, followed by two boosters with the same antigen concentration/volume homogenised with Freund's incomplete adjuvant [FIA]. Prior to fusion experiment, three consecutive injections of 146S antigen in phosphate buffered saline were given intravenously. Fusion experiments were carried out as per the standard protocol (Yokayama *et al.*, 2013).

**Table.2** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated with 1N HCl to generate 12S particles in type specific DAS-ELISA

Monoclonal Antibodies	Trial 1	Trial 2	Trial 3	Average	STD Dev	CV %
25C4D2	0.0208	0.4437	0.17	0.2115	0.2144	101.41
25G11H2	0.03555	0.1293	0.179	0.11462	0.0728	63.554
27D9D8	0.02535	0.6045	0.117	0.24895	0.3113	125.048
27H12H6	0.01925	0.3529	0.1675	0.17988	0.1671	92.9321



**Fig 2**

**Fig.2** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated with 1N HCl to generate 12S particles in type specific DAS-ELISA

**Conversion of 146S into 12S particle**

The following three methods were followed for conversion of 146S antigen to 12S antigen of FMDV.

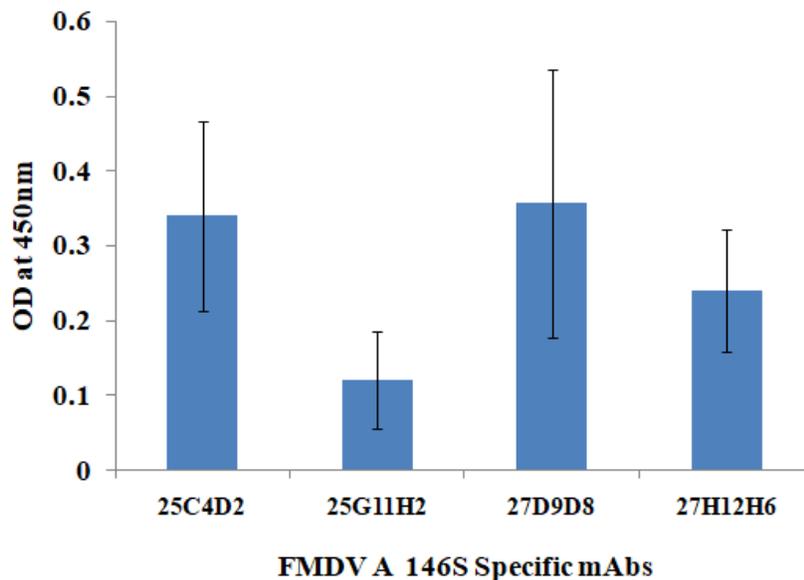
**1N HCl Method**

In this method, strong acid i.e., hydrochloric acid (1N HCl) was used for conversion of

146S to 12S, where, 10% [v/v] of 1N HCl was added to 5µg of 146S FMDV serotype A antigen in a total volume of 0.5ml. The pH was adjusted to 4.5 with 1N NaOH and incubated at room temperature for 30 min. The pH of the antigen was brought down to 7.6 using 1M Tris and made up to 4.5ml with Dulbecco’s phosphate buffered saline [D-PBS], pH 7.6 and used immediately in the ELISA (Rana *et al.*, 2008).

**Table.3** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated with 0.5M NaH<sub>2</sub>PO<sub>4</sub> to generate 12S particles in type specific DAS-ELISA

Monoclonal Antibodies	Trial 1	Trial 2	Trail 3	Average	STD Dev	CV %
25C4D2	0.41850	0.4392	0.1615	0.3397	0.1263	37.1800
25G11H2	0.03455	0.1404	0.1895	0.1215	0.0647	53.2228
27D9D8	0.42635	0.5325	0.1105	0.3565	0.1792	50.2822
27H12H6	0.22350	0.3479	0.1515	0.2410	0.0811	33.6667



**Fig 3**

**Fig.3** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated with 0.5M NaH<sub>2</sub>PO<sub>4</sub> to generate 12S particles in type specific DAS-ELISA

### **NaH<sub>2</sub>PO<sub>4</sub>**

In this method, weak acid i.e., sodium dihydrogen phosphate, 0.5 M NaH<sub>2</sub>PO<sub>4</sub> was added to 5µg of 146S FMDV serotype A antigen in a total volume of 0.5ml of D-PBS and incubated at room temperature for 30 min. After adjusting the pH to 7.6 with 1N NaOH, the antigen was made up to 4.5ml with D-PBS and used immediately in ELISA (Yang *et al.*, 2008).

### **Heat method**

In the this method, 5µg of 146S antigen was diluted in 0.5ml PBS (pH 7.6) and was heated at 56°C for 30 minutes, 56°C for 60 minutes, 60°C for 30 minutes and 60°C for 60 minutes in a circulating water bath. After incubation, the antigens were diluted in 4.5 ml of D-PBS (pH 7.6) and used immediately in ELISA at a concentration of 50ng/well [Harmsen *et al.*, 2015]

### **Double antibody sandwich ELISA**

Ten monoclonal antibodies viz., 25C4D2, 25C4G1, 25G12F12, 25G12G1, 25G12H1, 27D9D8, 27D9E8, 27G2E2, 27G2F3 and 27H12H6 specific to FMDV serotype A [IND/A/40/00] were initially screened for their specificity to FMDV A 146S antigen. However, out of ten, only four mAbs namely, 25C4D2, 25G11H2, 27D9E8 and 27H6H1) which were showing high reactivity and specificity were selected.

All the four selected mAbs were tested for their specificity to 146S and/or 12S particles using serotype specific double antibody sandwich ELISA according to OIE [2012] with modifications. The selected four mAbs were highly specific to 146S of FMDV serotype A 40/00. The method followed is described below;

In brief, 96-well ELISA plates (Nunc, Maxisorb) were used for the assay. Initially, plates were coated with selected mAbs at a dilution of 1:20 (1µl of mAb in 20µl of coating buffer) of each of mAb using coating buffer [carbonate-bicarbonate buffer, pH9.6] and kept overnight at 4°C.

The plates were washed with washing buffer (phosphate buffer saline with Tween 20, pH 7.6) thrice. The purified antigens (both 146S and 12S) diluted in D-PBS were added to all the wells separately, at 50ng/50µl/well, incubated at 37°C for one hour. Again the plates were washed thrice with washing buffer.

Then, FMDV serotype A specific guinea pig serum (GPS) diluted in blocking buffer [washing buffer with 5% adult bovine serum (ABS)] at 1:8000 [50 µl/well] was added. The plates were incubated at 37°C for one hour.

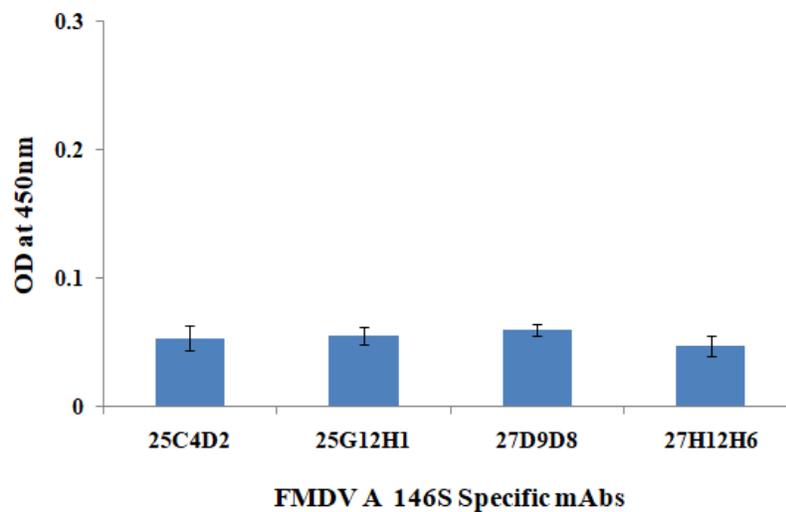
After incubation the plates were washed thrice as above, and anti-guinea pig HRPO conjugate was diluted in blocking buffer at 1:3000 dilutions and added at 50µl/well. The plates were incubated for 45 minutes at 37°C. At the end of incubation, the plates were washed thrice and TMB (Tetramethyl benzidine) buffer was added at 50µl/well and incubated at dark in room temperature for 15min for colour development.

The reaction was stopped at the end of incubation period using 1M H<sub>2</sub>SO<sub>4</sub> at 50µl/well. Then, the plate was read at 450nm wave length using ELISA reader (M/S Infinite 50-TECAN ELISA reader).

The obtained optical density readings were transformed to Excel sheet and required calculations were done to find out the percentage reactivity of mAbs with 146S and 12S.

**Table.4** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated at 56°C for 30min to generate 12S particles in type specific DAS-ELISA

Monoclonal Antibodies	Trial 1	Trial 2	Trial 3	Average	STD Dev	CV %
25C4D2	0.0428	0.061	0.0575	0.0538	0.0097	17.961
25G12H1	0.0493	0.054	0.0630	0.0554	0.0070	12.558
27D9D8	0.0580	0.065	0.0560	0.0597	0.0047	7.9203
27H12H6	0.0560	0.041	0.0450	0.0473	0.0078	16.410



**Fig 4**

**Fig.4** Reactivity [average OD of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated at 56°C for 30min to generate 12S particles in type specific DAS-ELISA

**Results and Discussion**

**FMDV serotype A 146S specific monoclonal antibodies**

In the present study, four monoclonal antibodies which are specific to 146S FMDV serotype A of Indian vaccine strain [IND/A 40/00] were used. The mAbs selected in this study were 25C4D2, 25G12H1, 27D9E8 and 27H12H6. The reactivity of these mAbs with 146S and 12S of FMDV serotype A Indian

vaccine strain in double antibody sandwich ELISA is represented in Fig 1 and Table 1. It is clear from the figure 1 and table 1 that the mAbs are highly specific to 146S of FMDV type A with least reactivity with 12S. The reactivity of these mAbs with 146S of FMDV type A was consistent upon repetition of the assay on different days with standard deviation and % coefficient of variation, respectively ranged from 0.39 to 5.35 and 2.14 to 15.02. These variations were within the acceptable limits.

## Conversion of 146S into 12S particle

### 1N HCl Method

The efficiency of this method was checked for conversion of 146S into 12S particle of FMDV serotype A Indian vaccine strain [IND/A/40/00]. These mAbs were used as capture antibodies. It is clear from the Table 2 and Figure 2 that these mAbs showed high reactivity towards 146S particles but showed some reactivity against 12S particles. This indicates that all the 146S particles have not converted into 12S. The coefficient of variation [%] varied from 63.554 to 125.048, which is not an acceptable limit.

### 0.5M NaH<sub>2</sub>PO<sub>4</sub> method

The efficiency in conversion of 146S particles to 12S particles was observed in FMDV serotype A Indian vaccine strain [IND/A/40/00] using four 146S specific mAbs in DAS-ELISA. Here also, the mAbs were used as coating antibodies. It is clear from the Table 3 and Figure 3 that the mAbs though they were specific to 146S but still

showing average reactivity against 12S ranged from 0.1215 to 0.3565 between the mAbs. This implies that there was no efficient conversion of 146S particles into 12S particles. The coefficient of variation [%] was varied from a minimum of 33.6667 to 53.2228 among the mAbs, which is not in an acceptable range.

### Heat method

The conversion of 146S into 12S particle of FMDV serotype A Indian vaccine strain [IND/A/40/00] was also tested using heat method at 56°C for 30 min using 146S specific mAbs in DAS-ELISA. The mAbs were used as coating antibodies.

It is clear from the Table 4 and Figure 4 that all the four mAbs are showing average reactivity ranged from 0.0473 to 0.0597 between the mAbs. This infers that there was an efficient conversion of 146S particles into 12S particles. The coefficient of variation [%] varied from a minimum of 7.92036 to 17.9615 across the mAbs, which is in the acceptable range.

**Table.5** Reactivity [average OD ± SE of duplicate wells] of FMDV A 146S specific monoclonal antibodies with 146S of FMDV serotype A treated at different temperatures to generate 12S particles in type specific DAS-ELISA

Heat Method	FMDV A 146S specific monoclonal antibodies			
	25C4D2	25G12H1	27D9D8	27H12H6
146S standard antigen	0.4664±0.035	0.5136±0.095	0.5572±0.019	0.5662±0.069
56°C, 30 min	0.0797±0.048	0.0864±0.005	0.0923±0.005	0.0959±0.005
56°C, 60 min	0.0698±0.014	0.0707±0.002	0.0842±0.001	0.0773±0.007
60°C, 30 min	0.0543±0.014	0.0528±0.001	0.0603±0.002	0.0620±0.009
60°C, 60 min	0.0465±0.000	0.0444±0.001	0.0572±0.002	0.0513±0.003
Blank	0.0441±0.006	0.0432±0.001	0.0437±0.009	0.0414±0.002

Further, we compared the efficacy of different combinations of temperatures at different time intervals separately for conversion of 146S into 12S. The different temperature variations used were 56°C for 30, 56°C for 60 minutes, 60°C for 30 and 60°C for 60 minutes. Both the temperature ranges were equally working well as shown in the table 5.

It is very clear from the table that all the mAbs are showing high reactivity with standard 146S of FMDV type A; whereas, the 146S treated at different temperatures with different time intervals were showing least reactivity.

This indicates that the heat method is very efficient in the conversion of 146S into 12S particles. The mean OD values and CV (%) of treated samples respectively ranged from 0.0431 to 0.0885 and 7.8333 to 11.4269, which are very much in the acceptable range.

FMD is one of the economically important, transboundary and OIE notifiable diseases. It affects cloven footed animals. The disease is controlled by restriction of animal movements, vaccination and test and slaughter methods.

The disease is enzootic in many countries of the world including India. Enzootic countries generally follow periodic vaccination to contain the disease. Vaccination is carried out using an inactivated vaccine (OIE, 2012).

The active principle in the inactivated vaccine is the 146S particle. It is a ribonucleoprotein comprising capsid and inactivated RNA. The integrity of 146S in the vaccine determines the efficacy of the vaccine (Doel *et al.*, 1982).

The integrity of the 146S is ascertained by vaccination of animals and measurement of immune response and density gradient ultracentrifugation either by using caesium

chloride or sucrose. These methods are cumbersome and time consuming. Therefore, we have generated four FMDV serotype A 146S specific mAbs [25C4D2, 25G11H2, 27D9E8, 27H12H6] by fusion experiments and they are ascertained by serotype specific double antibody sandwich ELISA [DAS-ELISA] in separate experiments.

In order to test the mAbs, whether they are 146S or 12S specific, we need to have both 146S and 12S particles. Basically, three methods viz., strong acid (Crowther *et al.*, 1982; Van mannen and Terpstra, 1990; Rana *et al.*, 2008), weak acid (Yang *et al.*, 2008) and heat (Harmsen *et al.*, 2011) methods are followed to convert 146S into 12S particles of FMDV. Of late, even thiomersal has been applied for the aforesaid purpose (Harmsen *et al.*, 2011).

Researchers have reported variable results with these methods. Therefore in the present study, we have compared the efficacy of the former three methods in the conversion of 146S particle into 12S of FMDV serotype A of Indian vaccine strain, A/IND/40/00. The results indicate that the acid methods are inconsistent in converting 146S into 12S of FMDV A 40/00 compared to heat method.

These results are in conformity with recent report of conversion of 146S particle of FMDV serotype O (R2/1975) (Sivaramakrishna *et al.*, 2019). Most of the researchers suggest that the 56°C for one hour is optimum for complete conversion.

However in our experiments, both 56°C and 60°C each for half an hour and one hour performed equally well in the conversion 146S of FMDV A into 12S particles.

In conclusion, among the three methods followed in the conversion of 146S of FMDV A [Indian strain] into 12S particles, heat

method performed well in the present study. However, the performance of the method needs to be evaluated using ample number of 146S samples.

### **Acknowledgement**

All the authors would like to thank the Director, ICAR-IVRI, Izatnagar and Principal Investigator of the project titled “*Development and evaluation of monoclonal antibody based ELISA systems for the detection of antigen/antibody of FMDV*”, ICAR-IVRI, Bengaluru.

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**How to cite this article:**

Shanmuganathan. S, Gollapalli Sivarama Krishna, S.R. Nagasupreeta, K. Kavya, Shreya Gopinath, R.P. Tamil Selvan, B.P. Sreenivasa, Bhanuprakash. V. 2020. Comparative efficacy of different methods in the generation of 12S particles from 146S particles of FMDV serotype A and their detection using serotype (146S) specific monoclonal antibodies. *Int.J.Curr.Microbiol.App.Sci.* 9(02): 1016-1026. doi: <https://doi.org/10.20546/ijcmas.2020.902.119>