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Evaluation of Different Methods for Conversion of Whole Virion Particle (146S) of FMDV into 12S Subunits and Application in Characterization of Monoclonal Antibodies

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A B S T R A C T

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven hoofed animals caused by FMD virus classified under genus Apthovirus and Picornaviridae family. Monoclonal antibodies (mAb) to FMDV provides best tool for the development of reliable diagnostics and there is a need for the development of mAbs that specifically recognize either 146S or 12S for the quality control analysis of FMDV vaccines. In the present study, we developed mAbs specific to FMDV serotype O and characterized their specificity in recognition of 146S or 12S particles. Three different methods were evaluated for the conversion of 146S into 12S subunits that includes heat method, strong acid and mild acid methods for testing the reactivity of mAbs with these antigens by double antibody sandwich ELISA. Mild heating of 146S antigens at 56^0C for 1hour and treatment of 146S antigen with weak acid (0.5M NaH_2PO_4, pH 4.5 - 5.5) resulted in complete conversion of 146S into 12S. Where as in strong acid method (1N HCl with pH below 4.5), 146S treated with 1N HCl resulted in loss of antigenicity of 12S subunits that reflected in absence of reactivity in ELISA. This may mislead to consider the mAb as 146S specific. This study revealed that mAb binding epitopes of all monoclonals are commonly shared among 146S and 12S antigens. Further it helps in understanding the specificity of mAbs to 146S and 12S particles which will be useful for application of mAbs in different diagnostic assays.

Keywords

Foot-and-mouth disease virus, Monoclonal antibodies, Whole virion particle, 12S Subunits

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Introduction

Foot-and-Mouth disease is a highly contagious, transboundary and emerging disease of wild and domesticated cloven hoofed animals. The etiological agent is a foot-and-mouth disease virus classified under the genus Apthovirus, Picornaviridae family. Total seven antigenically distinct serotypes were identified viz. O, A, C, Asia1, SAT-1, 2 and 3. Multiple genotypes and lineages are exists under each serotype. The Indian sub-continent consists of higher number of susceptible animal population, hence the disease is endemic in India. The serotypes O, A and Asia1 are more prevalent in India. As per the 2017-18 annual reports, majority of FMDV outbreaks in India are involved with

serotype O followed by Asia 1 and A (ICAR-DFMD Annual report, 2017-18).

The FMD virus consists of a single stranded positive sense RNA genome that encodes for four structural proteins VP1, VP2, VP3 and VP4 and eight non-structural (NSPs) 2A, 2B, 2C, 3A, 3B, 3Cpro, 3D (RNA dependent RNA polymerase) (Mason *et al.,* 2003; Carrillo *et al.,* 2005). The virion capsid is formed by four structural proteins VP1-4, where the capsid proteins VP1, VP2 and VP3 are exposed on the surface of the capsid while the VP4 lies internally. Each protomer of the capsid consists of proteins VP1, VP2 and VP3, five of such protomers forms a pentameric structure.

These pentameric structures have a sedimentation coefficient of 12S, hence so called 12S subunits (Denoya *et al.,* 1978, Sanger *et al.,* 1978). Twelve of these pentamers assembled to form an intact virion particles which have a sedimentation coefficient of 146S (146S particles) and identified as the highly immunogenic component (Doel *et al.,* 1982). The antigenic sites present on these intact virion particles are responsible for developing immune response in the natural hosts. The 12S particles are poorly immunogenic when compared to that of intact 146S antigens. However most of the epitopes are commonly shared between both 146S antigen and 12S antigens. Hence most of the monoclonal antibodies and polyclonal antibodies are cross reactive to both 146S and 12S antigens. The specificity of mAbs in recognition of epitopes present on 146S or 12S or both is an essential feature for considering the feasibility of a mAbs in developing a diagnostic assay.

It had been known for several years that mild heating of the 146S particles or lowering the pH of the buffer below 6.5 will disrupt the intact 146S into 12S subunits. The presence of high density of Histidine (which have a pKa of 6.8) residues at interphase of VP2 and VP3, are identified as the amino acids responsible triggering electrostatic repulsion between protonated imidazoles that led to dissociation of 146S to subunits (Acharya *et al.,* 1989). Two of these histidine residues were identified at VP3-141 and VP3-144 in inter-pentameric junction as potential destablilizers. Because these histidine, lysine and arginine residues greatly exceeds the number of negatively charged aspartic acid and glutamic acid residues at inter-pentameric interface, resulting in electrostatic repulsion at pH below 6.5. Moreover these residues are highly conserved among all the serotypes of FMDV.

The conversion of 146S particles into 12S subunits can be done by different methods viz. heat method and acid method. In heat method, the 146S particles heated at 56^0C for 1hour will led to the complete conversion of 146S to 12S subunits (Harmsen *et al.*, 2015). In acid method, citrate phosphate buffer, sodium phosphate buffer and hydrochloric acids were used for conversion of 146S to 12S (Barnett *et al.,* 1989; Samir *et al.*,2008; Crowther *et al.,* 1982; Smit saart *et al.*,1990; Steve *et al.,* 1986; Rao *et al.,* 1994; Yang *et al.,* 2007). However, in all acid methods the pH plays the critical role and need to maintain at pH 4.5. In addition, traditionally sucrose density gradient ultracentrifugation methods were also used for extraction of 12S particles (McCullough *et al.,* 1982).

In the present study, monoclonal antibodies were raised with FMDV serotype O and characterized the specificity of monoclonal antibodies in recognition of either 146S or 12S or both antigens. For this purpose, we evaluated three different methods, heat method and acid methods (strong acid and weak acid) for efficient conversion of 146S antigen into 12S subunits. Using these antigens in a double antibody sandwich

ELISA (DAS ELISA), the specificity of mAbs in binding to 146S and 12S particles were characterized.

Materials and Methods

Production of 146S antigen

The FMDV virus serotype O (R2/1975) strain available at Indian Veterinary Research Institute, Bengaluru was cultivated in bulk quantities, inactivated by Binary ethylene imine and concentrated by PEG-6000 method. The concentrated antigen was purified by CsCl ultracentrifugation method at 1,00,000g for overnight and the purified 146S antigen was dialyzed against PBS (pH 7.6). The concentration of the 146S antigen was estimated by multiplying the OD at 259nm with the extinction factor 131. The purified 146S antigen aliquots were prepared as per requirement and stored at -80^{0}C until use.

Development of monoclonal antibodies

The 146S antigen was immunized into 6 weak old Balb/C mice with a concentration of 20µg/mice with Fruends complete adjuvant through intra-peritoneal route. This was followed by booster doses at regular intervals of 30days with Freunds incomplete adjuvant. The immunized mice sera were screened in DAS ELISA for sufficient serum titres in comparison to that of Guinea pig polyclonal sera. Prior to fusion experiment three consecutive doses of 146S antigen were given intra-venously. The mice was sacrificed, spleenocytes were harvested and the fusion of spleenocytes with SP2/0 myeloma cells were carried as per the standard polyethylene glycol method. The supernatant from parental hybridomas were screened for their reactivity with 146S antigen by DAS ELISA. From reactive parental hybridomas, single cell clones secreting mAbs were developed by limiting dilution method.

Conversion of 146S into 12S by heat method

The 146S antigen with a concentration of 5µg in 0.5ml PBS (pH 7.6) was heated at 56^{0}C for 1hour in a circulating water bath. After incubation the antigens were diluted in 5ml of DPBS (pH7.6) and used immediately in ELISA at a concentration of 50ng per well.

Conversion of 146S into 12S by acid method

In acid method we used one strong acid, 1N HCl and one weak acid, 0.5 M NaH_2PO_4 for conversion of 146S into 12S. In strong acid method 10% v/v of 1N HCl was added to 5µg of antigen in a total volume of 0.5ml, adjusted the pH to 4.5 with 1N NaOH and incubated at room temperature for 30 minutes. The pH of the antigen was brought down to 7.6 using 1M Tris and dissolved in 5ml of DPBS pH7.6 and used immediately in ELISA. In weak acid method, 0.5M of NaH_2PO_4 was added to 5µg of 146S antigen in a total volume of 0.5ml and incubated at room temperature for 30minutes. After adjusting the pH to 7.6 with 1N NaOH, the antigen was dissolved in 5ml of DPBS and used immediately in ELISA.

Combination of heat and acid methods

In this method, after addition of acids to the antigen as mentioned above, the samples were heated at 56^{0}C for 15 minutes followed by adjusting the pH to 7.6, dissolved in 5ml of DPBS and used immediately in ELISA.

Standardization of double antibody sandwich ELISA (DAS ELISA) for identification of specific reactivity of monoclonals with 146S and 12S antigens

The 96-well ELISA plates (Nunc, Maxisorb) were coated with 1:8000 dilution of anti-FMDV polyclonal Rabbit sera in carbonate

bicarbonate buffer (pH-9.6) and incubated at 4^0C for overnight. The plates were washed thrice with wash buffer (PBS + Tween 20, pH 7.6). The antigens (146S & 12S) were added to the plates at a concentration of 50ng/well in 50µl and incubated at 37^0C for 1hour. After washing the plates for thrice with wash buffer, the monoclonal antibodies were added to respective wells and incubated at 37^0C for 1hour. At the end of incubation, the plates were washed thrice and anti-mouse HRPO conjugate prepared in 1:4000 dilution in wash buffer was added 50µl per well. The plates were incubated at 37^0C for 45 minutes. The plates were washed thrice in wash buffer and O-phenylene diamine with H_20_2 substrate was added to the plates and incubated at 37^0C for 15 minutes. The colored reaction was stopped by adding 1M H_2SO_4. Immediately the optical density reading was taken in a automated ELISA reader at 492-620nm wave length. The data was transformed into preformed excel sheet and analyzed for percentage reactivity of mAbs with 146S and 12S antigens.

Results and Discussion

Purification of 146S antigen

The FMDV serotype O virus produced in bulk quantities was inactivated and concentrated by standard methods. The purification of 146S antigen was done in linear gradient of CsCl density ultracentrifugation method revealed a clear band at F4 fraction of the gradient. The ratio of OD at 259nm and 239nm was close to 1.40 for the fraction F4, confirming the sedimentation coefficient of purified antigen, which is expected close to 140S. The spectrogram revealed a smooth curve with two peaks which reflects the purity of the 146S antigen in that particular fraction (Fig. 1). The antigen concentration per ml of fraction was estimated by multiplying the OD at 259nm with extinction factor 131. The antigen concentration of the fraction was estimated as 255µg/ml. This purified antigen was used throughout the study for immunization of mice, screening of hybridomas, for preparation of 12S subunits and in DAS ELISA.

Production of monoclonal antibodies

The titration of immunized mice sera prior to fusion revealed sufficient sera titres in comparison to that of Guinea pig polyclonal sera (Fig. 2). Total 61 parental hybridomas were obtained from which 85 single cell clones secreting monoclonal antibodies were developed. The figure 3 represents the reactivity of 85 monoclonals with the cell culture virus antigen in DAS ELISA which confirming the reactivity of all monoclonals with serotype O cell culture antigen.

Evaluation of different methods for conversion of 146S antigen into 12S subunits

The intact whole virion particles (146S), consist of four structural capsid proteins VP1, VP2, VP3, and internally located VP4. Mild heat treatment or lowering the pH below 6.5 resulted in dissociation of intact virion particles into 12 pentameric subunits, which have a sedimentation coefficient of 12S, so called 12S subunits. In the present study, three different methods were evaluated for efficient conversion of 146S antigen into 12S sub units. In heat method, mild heating of 146S antigen was done at temperature 56^0C for 1hour. The antigen was captured by rabbit anti FMDV serotype O polyclonal coating sera and tested the reactivity of mAbs with captured 12S antigen in ELISA. The results indicated that all mAbs are equally reacting to both 146S and 12S antigens prepared by heat method, suggesting the common representation of mAb binding epitopes in both 146 and 12S (Figure 4). In acid method, a strong acid (1N HCl) and a weak acid (0.5M NaH_2PO_4) were used for

conversion of 146S into 12S. In case of 10% v/v of 1N HCl method there is little reduction in the reactivity of all mAbs in comparison to that of heat method and weak acid methods. The reduction in reactivity may possibly due to relative loss of antigenicity of 12S subunits by strong acid nature of HCl (Fig. 3). In weak acid method, 0.5M NaH_2PO_4 pH 4.5-5.0 was used. The 12S subunits synthesized by weak acid treatment and the untreated 146S antigens, both are recognized equally by all mAbs and the results are highly correlated with that of heat method. However, a combination of strong acid treatment along with heating of 146S antigen at 56^0C for 15 minutes resulted in complete loss of reactivity of all mAbs with that antigen. This possibly may occur due to strong acid nature of HCl in combination with heat aggravate the complete denaturation of proteins, that resulted in loss of antigenicity of the 12S subunits (Fig. 4A and 4B). On comparison of all methods, the strong acid method is not an ideal method of conversion of 146S antigen into 12S subunits, as it was causing complete denaturation of capsid proteins of virion. Hence, during characterization of monoclonals where it involves the preparation of 12S subunits by strong acid method may mislead to false results. In such instances, one may consider the mAb as 146S specific, instead of characterizing it as mAb recognizing both 146S as well as 12S antigens.

Characterization of mAbs binding epitopes by using 12S and 146S antigens

Total 85 monoclonal antibodies developed in the present study were evaluated for their specific recognition of 146S antigen and 12S antigens in DAS ELISA. All mAbs are reacting equally with both 146S and 12S antigens prepared by heat method and weak acid method. The results indicated that none of the mAbs are either 146S specific or 12S specific but reacting with both. Hence it was predicted that all mAb binding epitopes were commonly shared between 146S and 12S antigens.

In developing country like India, the control and eradication of foot and mouth disease is only by vaccination of susceptible animals with inactivated FMDV vaccine. The success of vaccination is largely depends on intactness of the 146S antigen. The whole virion particle (146S) is highly immunogenic and dissociation of 146S antigen into 12S subunits resulted in poor immunogenicity (Doel *et al.,* 1982). The quality control analysis involving the identification of 146S antigen requires the use of monoclonal antibodies specifically recognizing 146S antigen. Hence, there is a need to develop monoclonal antibody which recognizes only the epitopes present on the intact virion particle but not the 12S subunits. However, mAbs that specifically detect 146S antigens are rarely isolated (Van Maanen *et al.,* 1990; Yang *et al.,* 2008, Harmesen *et al.,* 2017).

The intact virion particle (146S) consists of three capsid proteins viz.VP1, VP2 and VP3 which are exposed on the surface of the capsid. The VP4 capsid protein is located over the internal surface of the capsid. The major physical property of the virus is that mild heating at 56^0C and lowering the pH below 6.5 resulted in loss of infectivity of the virus (Randrup 1954). The underlying fact was analyzed in detail by using radioactively labelled purified capsid proteins which evidenced that the four structural proteins encompassing the capsid were transformed into 12 pentameric subunits (New Man and Brown, 1997). The key factors responsible for this dissociation of 146S antigen to 12S were ratified by X-ray crystallographic studies. The presence of high density of histidine residues at the interface of VP2 and VP3 capsid proteins are involved in dissociation of 146S into 12S (Acharya *et al.,* 1989).

Fig.1 Purification of 146S antigen by cesium chloride ultracentrifugation method

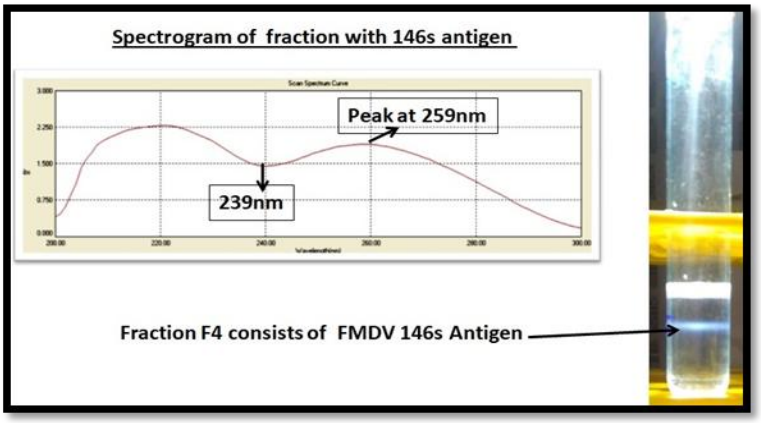


Fig.2 Titration of immunized mice sera in DAS ELISA

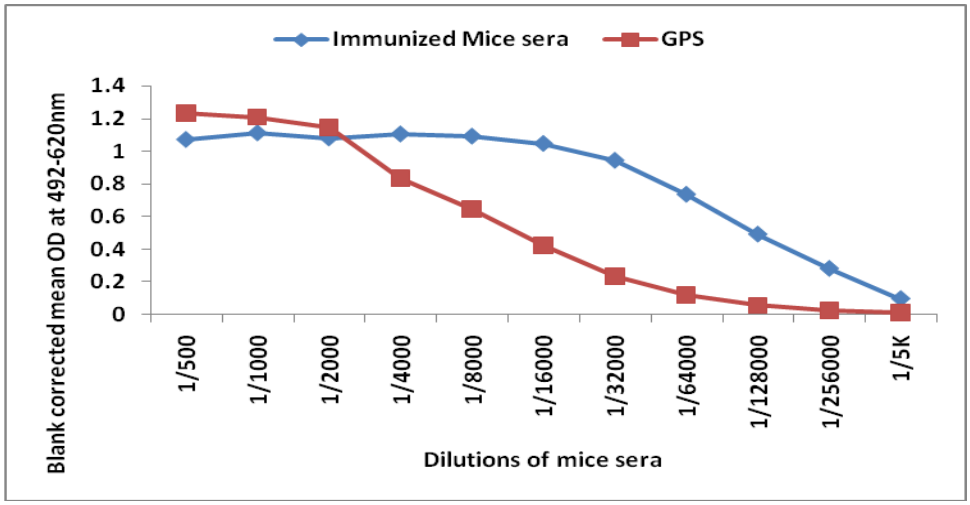


Fig.3A Reactivity of monoclonal antibodies in DAS ELISA with 146S antigen and 12S antigen prepared by different methods

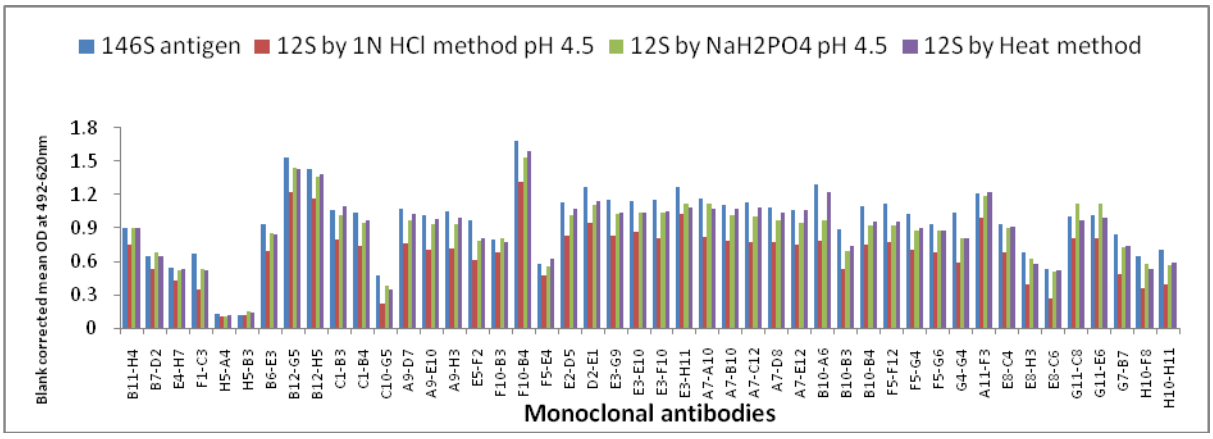


Fig.3B Reactivity of monoclonal antibodies in DAS ELISA with 146S antigen and 12S antigen prepared by different methods

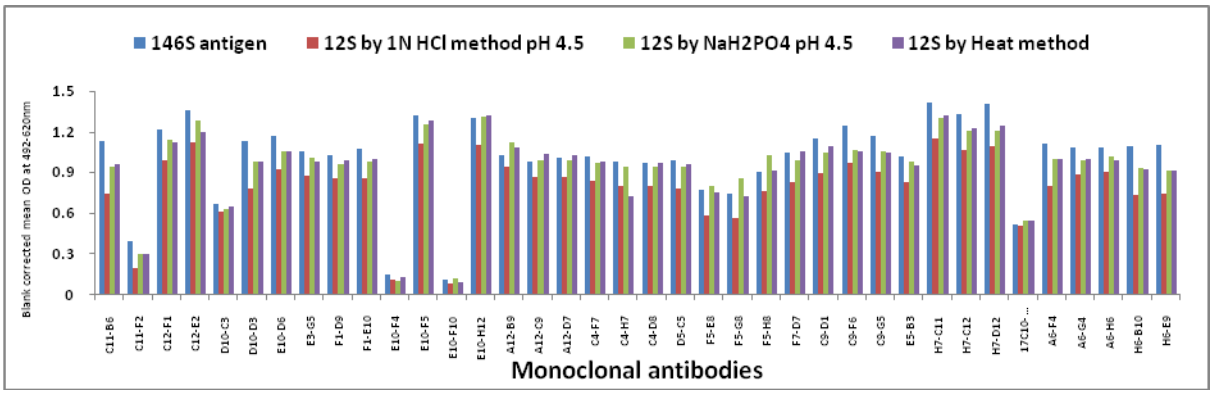


Fig.4A Reactivity of monoclonal antibodies with 146S antigen and 12S antigens prepared by combination of heat and acid method

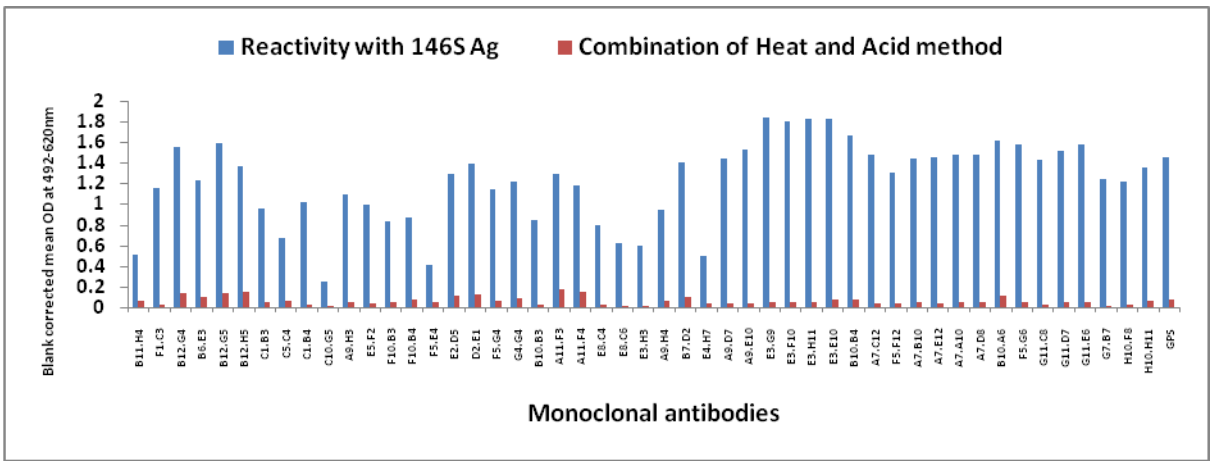
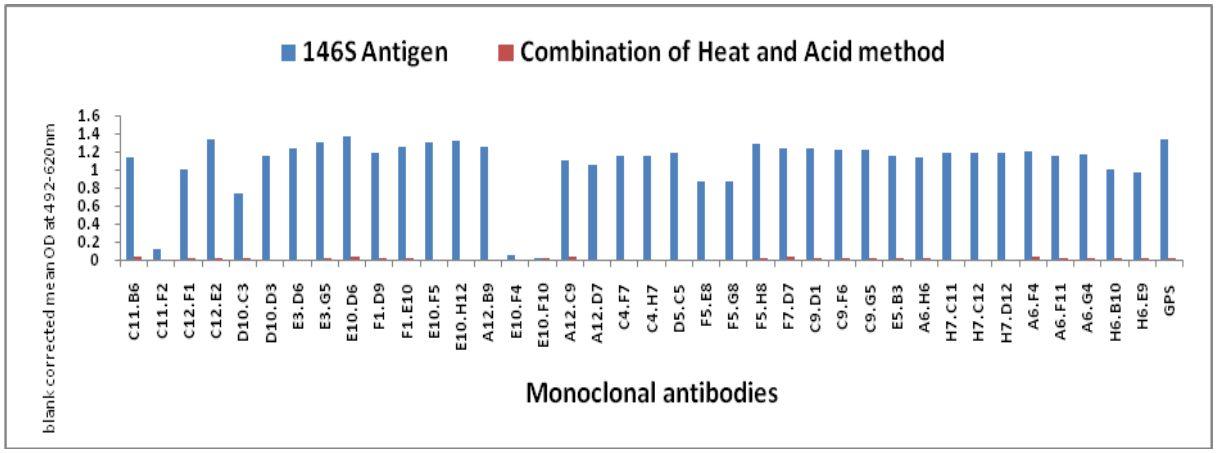


Fig.4B Reactivity of monoclonal antibodies with 146S antigen and 12S antigens prepared by combination of heat and acid method



The pKa of histidine residues is 6.8, hence the pH below 6.8 or mild heating enhances the electrostatic repulsion between protonated imidazole side chains. The critical residue histidine was identified at positions VP3-141 and VP3-144 which are oriented at inter protomeric junction in juxtaposition to aspartic acid and glutamic acid on the counter protomer (Twomy *et al.,* 1995). These two residues were identified as potential destabilizers as the numbers of histidine, lysine and arginine residues are greatly exceeding the number of negatively charged aspartic acid and glutamic acid residues on its counter part of the protomer. Later these residues were identified as highly conserved among all the serotypes of FMDV.

In the present study different methods for conversion of 146S antigen into 12S subunits were compared simultaneously by use of monoclonal antibodies. The strong acid method involves the use of 10%v/v of 1N HCl for conversion of 146S into 12S. Barnett *et al.,* (1989) used strong acid method for preparation of 12S subunits and applied these antigens for characterizing the monoclonal antibodies as either 146 specific or 12S specific or both. In their experiment characterization of mAbs, they reported that some of the mAbs did not recognize the 12 subunits in sandwich ELISA while poor reactivity with subunits in case of competition ELISA.

In a different study, out of 12 monoclonal antibodies raised against Asia 1, ten were characterized as 146S specific and two were characterized as poorly reactive to 12S subunits based on the antigens prepared by strong acid method (Sameer *et al.,* 2008). In both of these studies, either absence of reactivity of mAbs or poor reactivity of mAbs with 12S subunits is possibly may due to complete denaturation of the antigens by the action of 1N HCl. The feasibility of HCl method for preparation of 12S subunits was assessed in the present study. It was revealed that the pH of strong acid is 4.0, at which it causes complete denaturation of proteins (Fig. 4A and 4B). Instead of using the acid at pH below 4.0, slight increase in the pH to 4.5 to 5 resulted in slight increased reactivity of mAbs with 12S subunits but not on par with that of 146S antigen (Fig. 3A and 3B). This variation in reactivity of mAbs with antigens treated with HCl under different pH conditions, clearly evidenced that the pH along with strong acidic nature of HCl has drastic effect on antigenicity of 12S subunits. This makes strong acid method is not an appropriate method of conversion of 146S to 12S.

On contrary to strong acid method, the weak acid method was used by many authors for characterization of monoclonal antibodies to FMDV (Crowther *et al.,* 1982; Smit saart *et al.,* 1990; Steve *et al.,* 1986; Rao *et al.,* 1994; Yang *et al.,* 2007). The weak acid 0.2M NaH_2PO_4 pH4.4 was used for preparation of 12S subunits from 146S antigen of serotype A.

This 12S subunits were used for production of a monoclonal antibody that specifically recognizing non neutralizing linear epitope which is conserved among all serotypes (Yang *et al.,* 2007). These earlier reports provided the evidence for feasibility of NaH_2PO_4 for effective conversion of 146S antigen into 12S subunits. Moreover, our present study also revealed that NaH_2PO_4 at pH 4.5 can be successfully used for effective conversion of 146S antigen into 12S subunits.

The heat method involves mild heating of 146S antigen at 56^0C for 1hour, which results in complete conversion of 146S antigen into 12S subunits. The Graph 3a & 3b showed reactivity of all mAbs with 146S and 12S subunits prepared by heat method. The results indicated that mAb binding epitopes of all

these mAbs are commonly represented in both 146S and 12S subunits. Harmsen *et al.*, (2011) successfully used the heat method for preparation of 12S antigen standards for quantifying the antigen concentration in the vaccine. They developed an ELISA with variable heavy chain domains of llamas that specifically recognized either 146S or 12S particles of Asia-1 serotype (Harmsen *et al.*, 2015). The 146S antigen concentration and 12S antigen concentration in the vaccine samples were estimated in ELISA by the use of 12S antigen standards prepared by heat method and applied in vaccine degradation studies.

In conclusion, all these reports suggested the heat method and weak acid method at pH4.5 are best methods for conversion of 146S antigen into 12S and retains the antigenicity of subunits. However, among these two methods heat method is the best one, because in this method no need to add any reagents to the antigen sample and overcome problems associated with pH.

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