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RESEARCH

Production and characterization of monoclonal antibodies against foot-and-mouth disease virus serotype O and development of a sandwich ELISA for virus antigen detection

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Abstract

Foot-and-mouth disease (FMD) is endemic in India with a majority of outbreaks caused by FMD virus (FMDV) serotype O. In the present study a panel of eight (2F9, 2G10, 3B9, 3H5, 4C8, 4D6, 4G10 and 5B6) mouse monoclonal antibodies (MAbs) were developed against FMDV serotype O Indian vaccine strain, O/IND/R2/75 via hybridoma systems. The MAbs generated were FMDV/O specific without cross-reactivity against FMDV type A and Asia 1. All the MAbs were identified as IgG1/kappa type. Out of eight, three MAbs (3B9, 3H5 and 4G10) demonstrated virus neutralizing activity. The reactivity of all MAbs increased with heat treated (@56^0C) serotype O antigen compared to untreated antigen in sandwich ELISA indicating that their binding epitopes are linear. Six MAbs (except 2F9 and 4D6) reacted with recombinant P1 protein of homologous virus in an indirect ELISA among which only MAb 3B9 bound to VP1. MAb profiling of 37 serotype O field viruses isolated between the years 1962 and 2021 demonstrated antigenic similarity between field isolates and reference vaccine strain. MAbs 5B6 and 4C8 consistently reacted with all 37 isolates. In indirect immunofluorescence assay MAb 5B6 bound well with FMDV/O antigen. Finally, a sandwich ELISA was successfully developed using rabbit polyclonal anti-FMDV/O serum and MAb 5B6 for detection of FMDV/O antigen in clinical samples ($n = 649$). The new assay exhibited 100% and 98.89% diagnostic sensitivity and specificity respectively compared to traditional polyclonal antibody-based sandwich ELISA suggesting that the MAb-based ELISA developed here could be an effective method for detection of FMDV serotype O.

Keywords Foot-and-mouth disease virus · Serotype O · Monoclonal antibody · Sandwich ELISA · Antigen detection

Introduction

Foot-and-mouth disease (FMD) is a highly contagious transboundary disease affecting cloven hoofed animals. FMD is caused by a RNA virus belonging to the genus *Aphthovirus* of the family *Picornaviridae*. The disease severely affects livestock welfare and productivity, imposes international trade restrictions and threatens food security (Knight-Jones and Rushton 2013). FMD virus (FMDV) populations exhibit quasispecies nature due to high mutation rate of the viral genome (Domingo et al. 2003). Globally the virus exists as seven immunologically distinct serotypes: O, A, C, Asia1, SAT1, SAT2 and SAT3. In India, FMD is considered endemic and the disease is reported throughout the year

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in almost all parts of the country with prevalence of three FMDV serotypes viz. O, A and Asia1 (Pattnaik et al. 2012). Culling of animals infected with FMD is not practicable in many countries including India. Hence prophylactic vaccination of susceptible livestock with inactivated FMD vaccine is followed as the primary disease control strategy (Singh et al. 2019). Among the three prevalent serotypes, type O has been dominant over the years and continues to be responsible for more than 90% of FMD outbreaks recorded in India (Subramaniam et al. 2013). FMD cannot be differentiated from other vesicular diseases based on clinical signs only, thus, requires confirmation by laboratory diagnosis. Due to highly contagious nature of FMDV and heavy economic losses that arise from FMD outbreak, diagnosis of the disease at the initial stage of infection is crucial.

Laboratory techniques for detection of FMDV include virus isolation in cell culture, virus antigen detection in sandwich ELISA or viral nucleic acid recognition methods such as RT-PCR/real time RT-PCR (Wong et al. 2020). In comparison to virus isolation and RT-PCR, ELISA requires little technical knowledge or instruments and can be applied for high throughput testing. Immunoassay technologies for FMD diagnosis primarily use FMDV serotype specific polyclonal sera raised in laboratory animals like rabbit and guinea pig (Roeder and Le Blanc Smith 1987). However, polyclonal antibodies have certain inherent limitations such as finite supply, low reproducibility and difficulty in standardization (Veerasami et al. 2008). Alternatively, monoclonal antibodies (MAbs) represent highly specific and homogeneous antibody preparations that can be used as a source of consistent and replenishable reagents for immunoassay (Mahapatra et al. 2008).

MAbs against FMDV serotype O has been developed earlier for virus antigen and antibody detection (Chenard et al. 2003; Morioka et al. 2014). However, to confirm the disease in very early stage of infection FMDV antigen-detection ELISA is preferred over FMDV antibody-detection ELISA. In addition, MAbs also represent valuable tools for antigenic analysis of frequently variable viruses like FMDV and to study the nature of antigenic evolution (Seki et al. 2009; Yang et al. 2014). Antigenic profiling ELISA using MAbs is a rapid and sensitive method for characterization of field and vaccine strains. Further, MAb-based system for FMD vaccine matching has advantage over polyclonal antibody-based virus neutralization assay as the former method does not require a live virus (Samuel et al. 1991). However, it requires wider panel of MAbs to match with the polyclonal antibody for recognizing all protective epitopes present on the surface of FMD virus (Yang et al. 2014; Mahapatra et al. 2008). Nonetheless, MAbs have potential implications for monitoring the antigenic changes of field isolates and to characterize any new isolate.

In the present study, we report production and characterization of a panel of MAbs against FMDV serotype O Indian vaccine strain, O/IND/R2/75 and use of these MAbs in antigenic analysis of serotype O field isolates originating from a period of more than 50 years. Further, a MAb-based sandwich ELISA was developed for FMDV/O antigen detection in clinical materials.

Materials and methods

Cell line, viruses and Balb/c mice

The BHK-21 cells used for preparation of FMDV serotype O antigen for immunization of mice and SP2/O-Ag14 mouse myeloma cells for hybridoma production were routinely maintained at ICAR-Directorate of Foot and Mouth Disease-International Center for FMD (ICAR-DFMD-ICFMD) laboratory. Female Balb/c mice of 6–8 weeks old weighing 20–24 g were used for immunization to develop hybridoma clones against FMDV/O/IND/R2/75 (made available from ICAR-DFMD virus repository).

Mice immunization and hybridoma production

Immunization and hybridoma production was performed following the method described by us earlier (Singh et al. 2004). Balb/c mice were immunized with 50 µg of binary ethylene-imine (BEI)-inactivated 146S antigen of FMDV/O/IND/R2/75 mixed with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) followed by three boosters of 50 µg antigen mixed with Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal route at two weeks intervals. Subsequently three immunizations were carried out intravenously without any adjuvant on three consecutive days before the day of fusion. The immunized mouse was anaesthetized, splenocytes were harvested aseptically and the fusion of splenocytes with SP2/O-Ag14 mouse myeloma cells was carried out in 5:1 ratio mediated by polyethylene glycol 1450 (ready-to-use from Sigma-Aldrich). The cell pellet suspended in growth medium containing 1XHAT was seeded in 96-well cell culture plates containing mouse peritoneal macrophages as feeder cells. Once the hybridoma cells had grown and covered approximately one fourth surface of the well, the hybridoma supernatants were screened for their reactivity with type O 146S antigen in sandwich ELISA using plates coated with FMDV type O specific rabbit polyclonal antisera. The cultures (wells) found positive were transferred to 24-well cell culture plates. When the growth of positive hybridomas was completed in 24-well plates the cells were cloned and sub-cloned in 96-well plates in 1 × HT medium.

Screening of hybridomas by sandwich ELISA

Hybridoma cell culture supernatants were screened for the presence of anti-FMDV/O antibody using in-house sandwich ELISA method. To ensure the selection of all positive clones, the screening ELISA utilized the same stock of FMDV type O antigen used for the immunization of mice. Briefly, maxisorp 96 well ELISA plates (Nunc, Denmark) were coated with 50 µl of FMDV type O antiserum (raised in rabbits at ICAR-DFMD) diluted (1:8000) in carbonate-bicarbonate buffer per well and incubated at 4^0C overnight. Plates were washed three times with PBS containing 0.1% Tween-20 (PBST) and 50 µl of serotype O/IND/R2/75 146S antigen per well were dispensed and incubated at 37^0C for 1 h. After washing, 50 µl of hybridoma supernatants diluted (1:2) with blocking buffer (2% LAH, 2% normal rabbit serum and 2% calf serum in PBST) were added and incubated at 37^0C for 1 h. Plates were washed and anti-mouse horseradish peroxidase (HRP) conjugate (Sigma Aldrich) prepared in 1:3000 dilution in blocking buffer were added @50 µl per well and incubated at 37^0C for 1 h. After final washing, 50 µl of substrate solution containing orthophenylenediamine dihydrochloride (Sigma)/hydrogen peroxide was added and allowed to stand at 37^0C for 15 min for color development. The reaction was then stopped using 1 M H_2SO_4. The optical density (OD) values were measured at 492 nm using an ELISA plate reader (Thermoscientific, Switzerland).

Production and characterization of MAbs

Reactive parental hybridomas from the primary screening were selected and subjected to two cycles of single cell cloning by limiting dilution method to confirm the monospecificity of the hybridomas. After two cycles of single cell cloning the positive hybridomas were grown in a T75 flask (Nunc, Denmark) and the supernatant containing MAbs were examined for their reactivity and specificity against FMDV serotypes O, A and Asia 1 using sandwich ELISA by employing respective serotype specific polyclonal antibodies as the capture antibody and corresponding inactivated FMDV serotype(s) as antigen. Stable hybridoma clones secreting antibodies against FMDV/O were selected and further characterized. To determine the MAbs' binding epitopes, their reactivity against in-house recombinant VP1(pETite N-His SUMO-VP1; unpublished data) and P1 capsid protein (Biswal et al. 2015) of homologous virus was examined in an indirect ELISA. The MAbs were further tested for their ability to bind to intact and denatured (heat treated @56^0C for 10 min, 20 min and 1 h) type O antigen in sandwich ELISA.

Isotype identification

The isotype of the MAbs were identified by using rapid mouse immunoglobulin isotyping kit (Roche Diagnostic, Mannheim, Germany) as per manufacture's instruction.

Virus neutralization test

Virus neutralization test was performed as described in the manual of diagnostic tests and vaccines for terrestrial animals, **WOAH** (WOAH manual 2022) using BHK-21 cells. Hybridoma supernatants that gave antibody titers of 1/16 or less were considered as negative and those having titers greater than or equal to 1/45 were considered as positive.

Indirect immunofluorescence assay (IFA)

We assessed one selected MAb (5B6) for application in IFA for recognizing viral protein in FMDV/O infected cells. Briefly, BHK-21 cells grown in 24-well plates were infected with FMDV/O/IND/R2/75 at a multiplicity of infection of 1 and incubated for 4 h. The plates were fixed with prechilled acetone and methanol solution (at a ratio of 1:3) for 10 min at 4^0C. After being washed with 1XPBS, the cells were incubated with dilutions of MAb 5B6 (1:20) for 1 h at 37 °C. The wells were washed with PBS and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA) @ 1:500 dilution was added. The mixture was incubated for 1 h at 37 °C. The cells were again washed three times with PBS and observed under the Cellinsight CX7 HCA platform for fluorescence imaging (ThermoFisher scientific, USA).

MAb-profiling of the field virus isolates

A MAb-profiling technique as described by Samuel et al. (1991) was used to assess the reactivity of the MAbs with 37 serotype O field viruses isolated over a period of fifty years (1962–2021). All these viruses were made available from the FMD virus repository, ICAR-DFMD-ICFMD. The reaction profile was divided into four ranges (> 76, 46–75, 20–45 and < 20%) based on the reactivity (OD) values of each virus versus those of the reference virus with the MAb panel, expressed as a percentage.

Development of MAb-based sandwich ELISA for detection of FMDV serotype O antigen

A sandwich ELISA was developed for the detection of FMDV/O antigen using FMV/O specific rabbit polyclonal serum as the coating antibody and MAb 5B6 and HRP-conjugated goat anti-mouse IgG as detection antibody. The protocol was composed of following steps: maxisorp 96 well

flat-bottom ELISA plates were coated with 50 µl/well of rabbit anti-FMDV/O polyclonal antibody diluted (1:8000) in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were washed five times with washing buffer, followed by addition of 50 µl of test antigen and incubated at 37 °C for 1 h. After washing, 50 µl of MAb 5B6 (diluted 1:8) in blocking buffer (2% LAH, 2% normal rabbit serum and 2% calf serum in PBST) was added to wells in duplicate and plates incubated for 1 h at 37 °C. The remainder of the test followed the same steps as described under the heading "screening of hybridomas by sandwich ELISA". Guinea pig hyperimmune sera were added in duplicate as positive control. The 146S antigen of Serotype O FMD vaccine strain O/IND/R2/75 was used as reference antigen in the standardization method.

Traditional polyclonal antibody-based sandwich ELISA

The traditional polyclonal antibody-based sandwich ELISA was carried out as described by Bhattacharya et al (1996). Briefly, 96 well ELISA plates were coated with anti-FMDV/O specific rabbit polyclonal antibody and incubated for overnight at 4 °C. After washing test antigen was added and plates were incubated for 1 h at 37 °C. After washing, 50 µl of guinea pig anti-FMDV/O polyclonal antibody diluted (1:10,000) in blocking buffer were added and plates were incubated at 37^0C for 1 h. Plates were washed, anti-guinea pig HRP conjugate (DAKO) diluted (1:3000) in blocking buffer were added @50 µl per well and incubated at 37^0C for 1 h. The remainder of the test followed the same steps as described under the heading "screening of hybridomas by sandwich ELISA".

Clinical samples

A total of 649 clinical (epithelial tissue, saliva, oral swab and nasal swab) and post mortem samples (liver, spleen, lymph node and heart) collected from suspected cases of FMD from various parts of India (20 Indian states and 2 union territories) were used in the study. These samples were received at ICAR-DFMD from between January to December 2021 for diagnosis of FMD. Samples were kept refrigerated or on ice until received by the laboratory. Swab samples after receipt were centrifuged and the supernatants were collected. For tissue samples a 10% suspension was prepared by grinding aprrox. 0.2 gm of epithelial tissue in sterile mortar and pestle with 1 ml of sterile PBS and 1 ml of chloroform. The suspension was clarified on a centrifuge at 3000 g for 10 min. Once clarified 10% suspensions of tissue samples and supernatants of swab samples suspected to contain FMDV were stored at -80^0C for further analysis. Details of samples are given in Table 1.

Evaluation of MAb-based sandwich ELISA

The newly developed MAb-based sandwich ELISA was evaluated by testing 649 field samples collected from FMD suspected animals and the diagnostic test parameters were compared to traditional polyclonal antibody-based sandwich ELISA (Bhattacharya et al. 1996). Samples showing OD values of 0.1 or more were judged as FMD positive in both the ELISAs. The polyclonal antibody-based sandwich ELISA has been recommended by **WOAH** as the preferred procedure for detection of FMD viral antigen and identification of viral serotypes (Ferris and Donaldson 1992). Therefore this test was taken as "gold standard" for comparison of diagnostic sensitivity and specificity of the newly developed MAb-based ELISA.

Statistical analysis

The test results (OD values) obtained by the newly developed MAb-based sandwich ELISA and the reference polyclonal antibody-based sandwich ELISA for FMDV/O antigen detection in clinical samples ($n = 649$) were compared

Table 1 Details of clinical samples used in the study

Species Specimen	Cattle	Buffalo	Sheep	Goat	Pig	Mithun	Indian gaur	Total
Epithelial tissue	352	10	13	4	10	6	1	396
Oral swab/ Saliva	127	–	7	2	3	–	–	(139)
Nasal swab	7	–	–	20	1	–	–	28
Vesicular fluid	5	–	–	–	4	–	–	9
Heart muscle	20	–	–	2	5	–	–	27
Liver	28	–	–	3	–	–	–	31
Lymph node	2	–	–	–	–	–	–	2
Lungs	6	–	–	1	–	–	–	7
spleen	7	1	–	–	2	–	–	10
Total	554	11	20	32	25	6	1	649

by paired t test. The diagnostic sensitivity and specificity of the MAb-based sandwich ELISA were calculated by using the formula as follows: Sensitivity = TP/ (TP + FN) × 100 where TP = True positive, FN = False Negative and Specificity = TN/ (TN + FP) × 100 where TN = True Negative, FP = False Positive. Graphpad prism software version 5 (GraphPad Software, Inc., San Diego, CA) was used for the statistical analysis of the data.

Results

Production and characterization of MAbs

During preliminary screening twenty one parental hybridomas (data not shown) showing positive reactivity against type O 146S antigen were subjected to two cycles of single cell cloning by limiting dilution method. In the second single cell cloning all the wells in a micro-titration plate with growing hybridoma cells gave a positive reactivity with serotype O 146S antigen in sandwich ELISA which ensured the monoclonality of the selected hybridomas. After two cycles of single cell cloning eight stable hybridoma clones (5B6, 4C8, 4D6, 4G10, 3H5, 3B9, 2F9 and 2G10) secreting antibodies specific to FMDV/O were identified. The MAbs were then subjected to further characterization. The specificity of the MAbs to the homologous virus type O and heterologous virus types, A and Asia 1 were tested in sandwich ELISA using respective serotype specific rabbit polyclonal coating antibody and FMDV serotype specific 146S antigen. Results showed that the MAbs were highly specific for FMDV serotype O without any cross–reactivity against FMDV serotype A and Asia1 (Fig. 1). All the MAbs were characterized as IgG1/k type (Table 2) using IsoStrip mouse monoclonal antibody isotyping kit. Efficacy of MAbs to neutralize homologous virus, when tested in a virus neutralization test, revealed that three MAbs (3H5, 4G10 and 3B9) were capable of neutralizing the reference vaccine virus (O/IND/R2/75) while other five MAbs (5B6, 4C8, 4D6, 2F9 and 2G10) failed to neutralize (Table 2). The reactivity of the MAbs was further tested against intact and denatured type O antigen (heat treated @56^{0}C for 10 min, 20 min and 1 h) (Table 2; Fig. 2). It was observed that the reactivity (OD values) of all MAbs increased with heat-treated type O antigen in comparison to untreated antigen. Maximum OD values were recorded at 20 min of heat treatment.

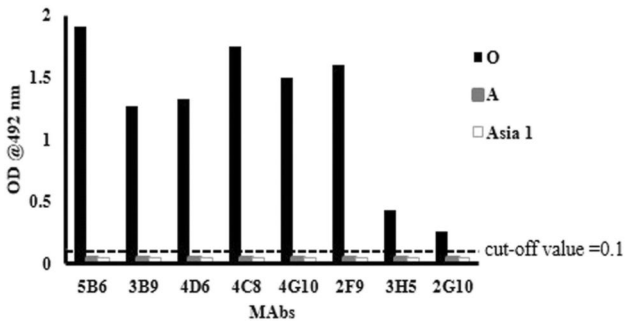


Fig. 1 Production of FMDV/O specific MAbs; Reactivity of MAbs against FMDV type O, A and Asia1 in sandwich ELISA

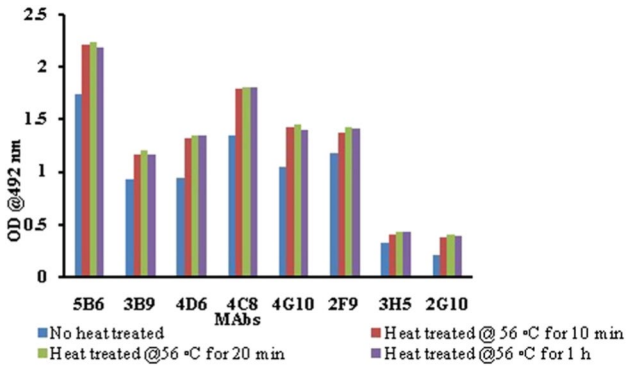


Fig. 2 Reactivity of anti-FMDV/O MAbs against native and heat treated (@56^0 C for 10 min, 20 min and 1 h) FMDV type O antigen in sandwich ELISA

Table 2 Characteristics of anti-FMDV/O MAbs

MAb	Isotype	Virus neutralization ability (neutralization titre)	Reactivity to heat treated FMDV/O antigen	Reactivity to FMDV/O recombinant P1/VP1 protein
4C8	IgG1/k	–	+	P1
5B6	IgG1/k	–	+	P1
4G10	IgG1/k	+(1/45)	+	P1
3B9	IgG1/k	+(1/64)	+	P1 and VP1
3H5	IgG1/k	+(1/64)	+	P1
2G10	IgG1/k	–	+	P1
4D6	IgG1/k	–	+	–
2F9	IgG1/k	–	+	–

IFA

We performed an IFA to assess the binding of MAb 5B6 with FMDV/O/IND/R2/75. The results showed that the MAb 5B6 worked well with FMDV/O antigen in infected BHK-21 cells (Fig. 3).

MAb-profiling of FMDV type O field isolates

A total of 37 FMDV serotype O field viruses isolated between 1962 and 2021 were subjected to MAb-profiling with the panel of eight MAbs as described earlier. Out of eight, two MAbs namely 5B6 and 4C8 consistently reacted with all the 37 isolates and the reactivity values were in the range of 44–100% with a mean reactivity of 69% and 71% respectively (Table 3). Remaining six MAbs (4D6, 4G10, 3B9, 3H5, 2F9 and 2G10) showed a varying degree of reactivity (14–100%) with the field isolates (Table 3). MAbs 2F9, 3B9 and 3H5 exhibited homogenous affinity (> 76% reactivity) with 62%, 72% and 78% of the isolates respectively while MAbs 4G10, 4D6 and 2G10 showed homogenous affinity towards 29%, 40% and 45% of the isolates respectively. However, reduced affinity (< 45% reactivity) was observed for these six MAbs towards isolates of 2008, 2011, 2017 and 2021 (Table 3).

Development and evaluation of MAb-based sandwich ELISA

A MAb namely 5B6 was selected for development of a MAb-based sandwich ELISA for FMDV/O antigen detection. The main criteria for the selection of MAb 5B6 was the consistent reactivity of the MAb with type O field isolates of more than five decades in ELISA and greater readability (OD@492) in the assay. FMDV/O in samples was captured by anti-FMDV/O rabbit polyclonal antibody followed by detection with MAb 5B6 and HRP-conjugated goat anti-mouse antibody. Conditions for the MAb-based sandwich ELISA were first optimized by performing a checkerboard titration using different dilutions of polyclonal antibody, MAb and HRP-conjugated goat anti-mouse antibody. Optimal conditions for the assay included 1:8000 dilution of polyclonal capture antibody, 1:8 dilution of MAB 5B6 and 1:3000 dilution of the HRP-conjugated anti-mouse antibody. For evaluation of the diagnostic efficacy of the developed MAb-based sandwich ELISA a total of 649 clinical materials (Table 1) received from geographically distinct states of India during suspected FMD outbreak investigations in 2021 were tested by the new assay and the test results were validated by using conventional polyclonal antibody-based sandwich ELISA as the reference method. The MAb-based sandwich ELISA was able to detect all 198 samples found positive by the polyclonal ELISA method (diagnostic sensitivity 198/198 or 100%; Table 4). Moreover, the MAb-based ELISA showed better ($p < 0.001$) sensitivity (more than two fold higher OD values) for each of the clinical samples tested than the polyclonal antibody-based sandwich ELISA (Fig. 4). Similarly, out of 451 samples declared negative in the polyclonal ELISA, 446 samples were found negative in the MAb-based ELISA (diagnostic specificity 446/451 or 98.89%; Table 4). Further, the MAb-based ELISA had comparatively low backgrounds (OD of 0.04) than the polyclonal ELISA (OD of 0.05) for the FMD negative samples.

Discussion

Early diagnosis of FMD is critical to limit propagation and transmission of FMDV and consequently minimize the economic losses. In FMD endemic settings where multiple serotypes are prevalent and vaccination is practiced as a disease control strategy, identification of FMDV serotype(s) involved in outbreak is critical for the proper implementation of the vaccination-based control program (Subramaniam et al. 2013). FMDV type O is one of the most prevalent serotypes worldwide as well as the major cause of FMD outbreaks in India. In the present study, a panel of eight MAbs was developed against FMDV serotype O Indian vaccine strain, O/

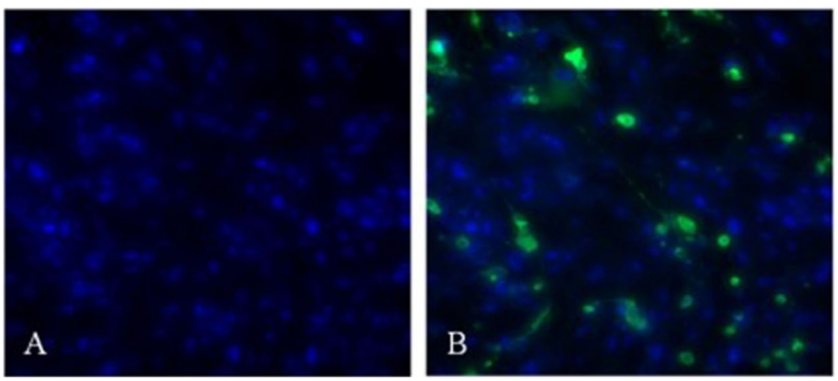
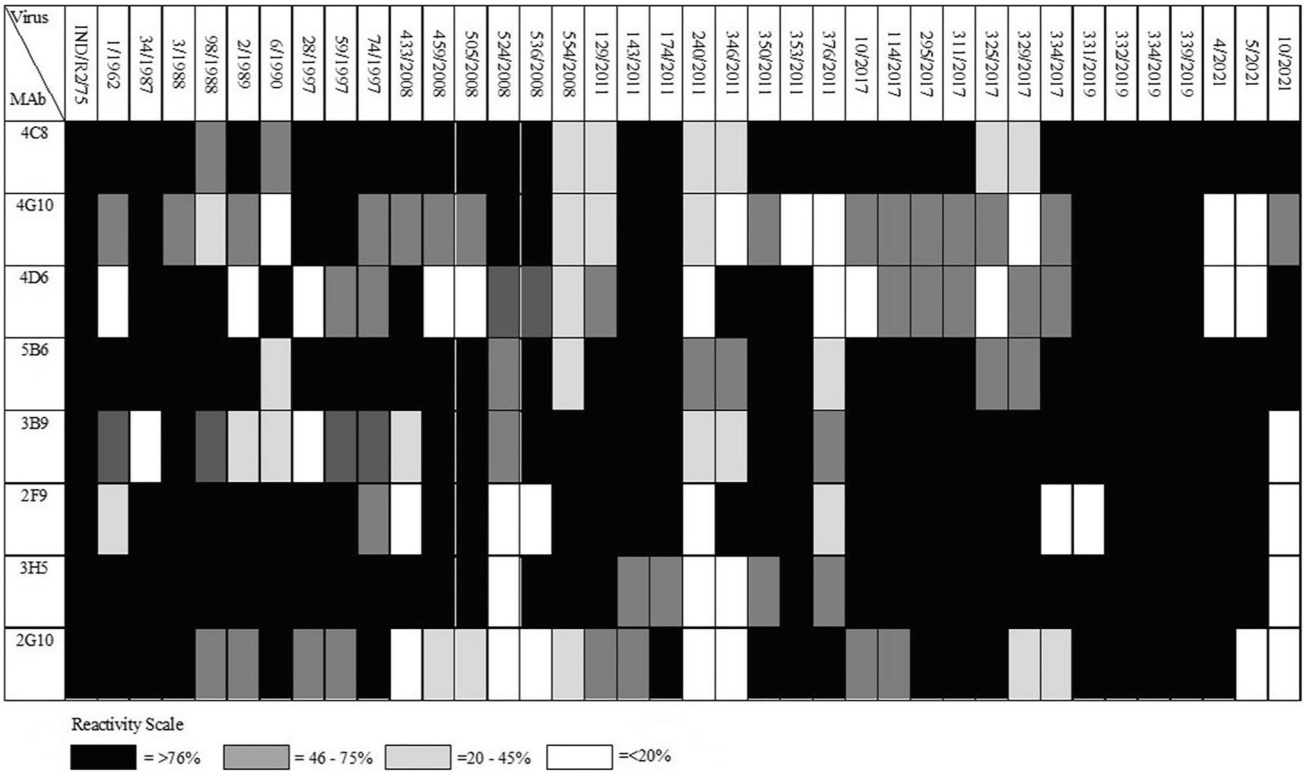


Fig. 3 IFA (**A**) Normal BHK-21 cells as a negative control, (**B**) BHK-21 cells infected with FMDV/O/IND/R2/75 and stained with MAb 5B6

Table 3 Reactivity profiles of type O field viruses (isolated between 1962 and 2021) with the anti-FMDV/O MAb panel



IND/R2/75 and characterized using various ELISAs, isotype analysis, virus neutralization and immunofluorescence assay technique. All eight MAbs generated were type O specific as they reacted only with FMDV serotype O without cross-reacting with type A or Asia 1. This could be due to the fact that antigens from other serotypes were not applied for initial screening. The MAbs were characterized as IgG1/k type, the predominant antibody isotype present in the mouse serum. When tested against intact vs. heat treated (@56^0C for 10 min, 20 min and 1 h) type O antigen in sandwich ELISA the binding reactivity of the MAbs increased with heat treated antigen as compared to untreated antigen. The increased OD values probably points to the contribution of serotype O internal antigens following heat treatment in addition to the exposed epitopes. Similar observation has been reported in a recent study by Ludi et al. (2022). Since all the MAbs exhibited higher reactivity against heat treated type O antigen it is assumed that their epitopes are linear in nature. More reactivity observed after 20 min of heat treatment possibly implies higher dissociation of 146S antigens by this time point. To determine the binding epitopes of the MAbs, their reactivity was assessed against recombinant P1 and VP1 protein of homologous vaccine strain in an indirect ELISA. Out of eight MAbs, six reacted with the capsid precursor P1 protein while MAbs 2F9 and 4D6 failed to do so, even though they demonstrated positive reactivity with native serotype O antigen in sandwich ELISA. It is believed that the recombinant capsid precursor P1 is structurally and antigenically similar to native virus capsid (Goodwin et al. 2009) however; we assume that the subtle difference between the structures of unprocessed capsid protein and 146S virion could be the reason behind the non-reactivity of these two MAbs with P1. Out of the six P1 reacting MAbs only MAb 3B9 bound to VP1. Since MAb 3B9 also showed virus neutralizing activity it was predicted to be directed against antigenic site 1 of FMDV/O. To our limitation, the protein specificity of other MAbs could not be ascertained due to

Table 4 A two-way contingency table representing the results obtained by MAb-based sandwich ELISA and the gold standard polyclonal antibody-based sandwich ELISA for FMDV/O antigen detection in clinical samples ($n=649$)

MAb-based sandwich ELISA	Polyclonal antibody-based sandwich ELISA		
	Positive	Negative	Total
Positive	198	5	203
Negative	0	446	446
Total	198	451	649

Relative diagnostic sensitivity of MAb-based sandwich ELISA; 198/198 = 100%

Relative diagnostic specificity of MAb-based sandwich ELISA; 446/451 = 98.89%

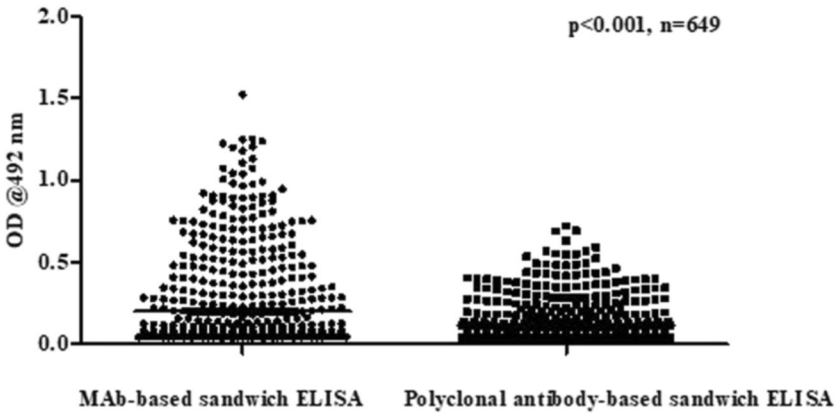


Fig. 4 Comparison of MAb-based sandwich ELISA and polyclonal antibody-based sandwich ELISA in detecting FMDV type O antigen in clinical samples ($n=649$)

unavailability of specific capsid proteins such as VP2 and VP3 which needs further studies.

It is well known that there is no immunological cross-protection between FMDV serotypes and even intraserotype protection is sometimes limited (Jamal and Belsham 2013). Hence, monitoring the FMDV field isolates for their antigenic relatedness to the in-use vaccine strain is essential for better control of the disease (Mahapatra and Parida 2018). The reactivity profiles of the MAb panel developed in the present study were investigated against 37 type O field isolates in reference to the current type O Indian vaccine strain (O/IND/R2/75). The result exhibited that all virus isolates starting from the oldest isolate (of the year 1962) available to the latest type O isolate (of the year 2021) reacted with two MAbs namely 4C8 and 5B6 indicating that the binding epitopes of these two MAbs are conserved within the serotype. The remaining six MAbs showed a varied reactivity pattern with the virus isolates. They showed homologous affinity towards most of the field isolates while reduced affinity of the MAbs were observed for few isolates. This may be attributed to mutation of their antigenic epitopes. Moreover, it was interesting to find that the viruses exhibiting low reactivity with the MAb panel were mostly isolated during the year 2008, 2011, 2017 and 2021 years that witnessed countrywide increased number of FMD outbreaks (Subramaniam et al. 2013; ICAR-DFMD, Annual report 2021). Cyclic pattern and surge in FMD incidence in every 3–4 years is almost always observed to be associated with emergence of genetic variants which could be the reason behind the low reactivity of the MAbs with the isolates. Further analysis of these viruses based on lineage differentiation may provide more detailed information. Nevertheless, barring the aforesaid isolates there were not much antigenic variation between the reference virus and the reported field viruses indicating adequate antigenic coverage by the current type O vaccine strain, O/IND/R2/75. An earlier study by Tosh et al. (1995) also reported similar findings that the serotype O field isolates were serologically related to the corresponding Indian vaccine virus strain. However, to specify the minor antigenic differences between the isolates, the binding epitopes of all the MAbs needs further characterization. In the present study, the reactivity of MAb 5B6 with FMDV/O/IND/R2/75 was also checked by IFA. Results exhibited that MAb 5B6 can be successfully used as a primary antibody to detect FMDV serotype O in infected BHK-21 cells and thereby validated the broad application of this MAb.

Antigen detection sandwich ELISA is an important assay for detection and serotyping of FMDV (Roeder and Le Blanc Smith 1987; Morioka et al. 2014). In the present study, a sandwich ELISA was developed using type O specific polyclonal antibody and MAb 5B6. The new assay was evaluated by analyzing varied clinical samples collected during suspected FMD outbreaks from a broad host range which included cattle/buffalo/sheep/goat/pig/mithun/yak. These samples were from varied tissue/clinical materials such as epithelial tissue, oral swabs, nasal swabs, vesicular fluid, heart muscle, liver, lungs and spleen etc. which are the most likely clinical samples from field for FMD diagnosis. The MAb-based sandwich ELISA showed 100% diagnostic sensitivity and greater absorbance (OD) values for the samples tested than the conventional polyclonal sandwich ELISA. These results corroborated with the findings of an earlier study by Morioka et al. (2014) for FMDV antigen detection. Relative diagnostic specificity of the new MAb-based ELISA was found as 98.89%. Indeed, out of five clinical samples declared FMD negative by the polyclonal antibody-based sandwich ELISA, four samples were detected as FMD positive in the MAb-based sandwich ELISA. These four samples were subsequently confirmed positive (data not shown) by the in-house FMDV serotype differentiating multiplex-PCR assay (Giridharan et al. 2005). This data highlights that the new MAb-based ELISA could be used to rule out the false negative results of traditional polyclonal antibody-based sandwich ELISA and thereby improve the

reduced sensitivity issue reported for the latter (Reid et al. 1998; Mohapatra et al. 2007) for FMDV antigen detection. These approaches may be more important for high throughput screening of samples from reduced FMD prevalence area, intended for creation of disease free zones (DFZs). Lastly, use of wide range of field samples (species as well as type of specimen) proved the robustness of our MAb-based sandwich ELISA for detection of serotype O antigen in any kind of clinical materials/post-mortem samples intended for FMD diagnosis/virus detection.

Conclusion

In a nutshell, a panel of MAbs were generated against FMDV/O/IND/R2/75 and characterized for their binding affinity and specificity, isotype analysis and virus neutralizing ability. Use of the developed MAb panel in antigenic analysis of serotype O field isolates revealed broad antigenic coverage by the current Indian type O vaccine strain IND/R2/75. A MAb-based sandwich ELISA was successfully developed for detection of FMDV serotype O antigen in clinical materials. The new assay demonstrated excellent sensitivity and specificity compared to the conventional polyclonal antibody-based sandwich ELISA and could be used an alternate method for detection and serotyping of FMDV/O. Besides, these MAbs may be useful for pen-side detection of FMDV type O after further characterization.

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Data availability Data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval All experimental procedures were approved by the Institute Animal Ethics Committee of ICAR-DFMD-ICFMD and performed according to the guidelines (Approved number: 01/2018).

Competing interests The authors declare that they have no competing interests.

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