Original Article

Study the Profile of Foot-and-mouth Disease Virus Protein by Electrophoresis and Identification of the Immunodominant Proteins

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ABSTRACT

Foot-and-mouth disease is an extremely infectious and occasionally fatal viral disease with a rapid onset and a short course that affects cloven-hoofed animals and results in considerable financial losses. Today, Foot-and-mouth disease is controlled by traditional inactivated vaccines. Due to the short duration of immunity, a study was conducted for proteins of the virus as well as obtaining immunodominant proteins to design more efficient vaccines against Foot-and-mouth disease virus. This research aims to study the profile of Foot-and-mouth disease virus protein by electrophoresis and identification of the immunodominant proteins. The purified Foot-and-mouth disease virus was purchased then the protein concentration of that solution was measured by Lowry method. SDS-PAGE was done to achieve the protein profiles of the virus and immunization of 5 guinea pigs was done, then blood samples were taken for obtaining serum. Finally, serology tests; double immunodiffusion, ELISA, and western blotting were used to evaluate antigen response to antibodies (antigenic immunization). The protein concentration was 3.5 mg/ml. In SDS-PAGE with 10% gel, the protein profile of the virus was observed. After immunization, by conducting double immunodiffusion tests, the sediment lines between the serum antibody and the antigen of the virus were formed. Also, The ELISA test showed that the antibodies were formed against the antigens. In the western blot test, two immunodominant proteins of the FMD virus were obtained. According to the results, the immunodominant proteins of the FMD virus were determined. These proteins can be used in immunological diagnostic methods and also novel vaccines.

Keywords: Double immunodiffusion, Electrophoresis, ELISA, Foot-and-mouth disease, Immunodominant proteins, Lowry method, Western blot

1. Introduction

Foot-and-mouth disease (FMD) is a common viral disease in livestock species such as cattle, buffalo, sheep, goats, and pigs. It is caused by Foot-and-mouth disease virus (FMDV) which is a small single-stranded RNA virus belonging to the family *Picornaviridae*, genus Apthovirus (1, 2). Its symptoms are fever and bullous lesions on the tongue and lips, under the breast, and between the hooves, resulting in weight loss, reduced milk production, and growth delay (1, 2). This disease can bring about economic losses in the livestock industry due to a reduction in animal production (3). Foot and mouth disease is very difficult to control: slaughtering and quarantining of infected animals in areas where the disease does not normally occur or vaccination in endemic areas can be effective. Seven immunologically different serotypes have been identified based on a VP1 coding region sequence (A, O, C, SAT1, SAT2, SAT3, and Asia1) (4, 5), which makes vaccination difficult due to low cross-protection (6). Consequently, Peptide Synthesis vaccination must be considered as a key factor to prevent probable outbreaks in the future but researchers require to identify detailed virus particles precisely to take this measure. FMDV particles form from cleavage of the capsid precursor polypeptide P1, which produces VP1, VP2, VP3, and VP4. VP1, VP2, and VP3 form the outer capsid shell, while VP4 forms the interior surface. V P1 contains the major antigenic domains of the virus, with its G-H loop including a highly conserved Arg-Gly-Asp (RGD) tripeptide at its apex, which can bind to integrins and helps the virus to enter into target cells (7). Therefore, many researchers have used VP1 as a candidate vaccine to prevent FMD.

This research aims to investigate the electrophoretic profile of the foot-and-mouth disease virus protein to identify further immunodominant proteins of the foot-and-mouth disease virus that can be used to develop immunological diagnostic methods and novel vaccines.

2. Materials and Methods

2.1 FMD virus

Inactivated FMD virus serotype O 2016 was purchased from the FMD vaccine production department of Razi Vaccine and Serum Research Institute.

2.2. Protein measurement by Lowry method

To determine the concentration of proteins in FMD virus solution we used Lowry's protein assay method (8). Then absorbance of 0 (Blank), 10, 25, 50, and 100 µl of the standard protein (Bovine serum albumin (BSA) 1mg/ml) and 1:5 and 1:10 of diluted protein solution was determined against a reagent blank at 750 nm in a spectrophotometer serum albumin (BSA) as a standard were determined at 750 nm in a spectrophotometer.

Finally, the unknown concentration of protein was calculated by using a standard curve made from dilutions of BSA.cx

2.3. Electrophoresis

Gel electrophoresis was performed with SDS-PAGE method. SDS-PAGE gel was prepared with 4% of stacking gel and 10% resolving gel.

After gel preparation, we loaded our samples and used TM Tricolor protein Ladder, which had specific molecular weights of 11, 17, 20, 25, 35, 48, 63, 75, 100, 135, 180, and 245 kDa. Finally, a current with a constant voltage of 90-110 volts was used. In the end, the gel inside the glass plates was separated and stained with Coomassie Blue staining.

2.4. Determination of molecular weight of protein bands

To calculate the relative movement of the proteins, we used the below formula. Then by making the Rf/MW curve for the standard protein, the molecular weight of the unknown protein bands was determined by replacing the Rf values in the standard curve.

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

2.5. Immunization of guinea pigs and serum collection

5 white male guinea pigs in the weight range 400-500 gr were kept in the Razi Vaccine and Serum Research laboratory, and immunized in 4 stages, first with Freund's complete adjuvant + inactivated FMD virus then in 3 steps Freund's incomplete adjuvant + inactivated FMD virus was injected.

All guinea pigs had specific labels with descriptions of details about animal status, cage number, date of injection, type of injection substance, and physiological reactions or any health problems. After immunization, blood samples were collected to separate the serums and then were kept at -20°C until the test.

2.6. Double immunodiffusion

First, a plate with gel agarose was prepared. After collecting serum from immunized animals, this test was performed to check the immunogenic reaction of antigens with dilutions of 1:2, 1:4, 1:8, and 1:16 of serum. FMD virus antigens were added in the center of the plate and diluted serum samples were added all around the plate (9).

2.7. Western blotting test to check immunogenicity of antigens

After performing the SDS-PAGE test and obtaining the protein bands, to check the immune response Western blot analysis was performed. At first, the protein band was transferred to the nitrocellulose membrane by the Bio-Rad Trans-Blot® Semi-Dry System (half an hour, 10 V). To block the membrane, it was incubated in blocking buffer (3% bovine serum albumin in 0.01 M phosphate-buffered saline) at room temperature for almost 1 hour and a half, and then it was washed by PBS-Tween 0.05% (PBST). The membrane was incubated with guinea pig serum (1:2 diluted) at 4°C, overnight. Then, washing was repeated four times by PBST, and then the membrane was incubated with HRP-labeled anti-guinea pig conjugate (1:10000 diluted) for 1 hour at room temperature, and it was washed again. In the end, 4-CN/HRP was added as substrate.

2.8. ELISA

checkerboard titration for all reagents was carried out for optimizing ELISA. The immunoassay plate was coated with FMDV antigen in coating buffer (Carbonate-bicarbonate buffer 0.05 M, pH 9.6) and incubated at 4°C overnight. On the next day, after washing the plate with PBST (containing 0/05% Tween 20) unbound sites in the wells were blocked by adding 250 µl per well of 5% skim milk to PBST and incubation at 37°C for an hour and a half with intermittent shaking. After three washings by wash buffer, test serum samples, a positive control, and a negative control serum diluted (1:50) in 1% skim milk were dispensed in a well duplicated and incubated for an hour and a quarter. After washing with PBST, rabbit anti-guinea pig horseradish peroxidase-conjugated whole IgG antibodies were added to the wells and incubated for an hour and a quarter at 37ºC. After the last washing, 100µl BM blue Roche substrates were dispensed and the plates were kept for 20 minutes in darkness at room temperature. Color development was terminated by adding 50 µl 0.1 M sulfuric acid in each well. Absorbance was measured at 450nm wavelength in an ELISA reader.

3. Results

3.1. Protein measurement

After measuring the optical absorbance of each solution at 750 nm, the concentration of the available protein (according to Table 1 and Figure 1) was determined to be almost 3.5 ml/mg.

3.2. Electrophoresis

In 10% SDS-PAGE gel, a dilution of 20 microliters of FMD along with 15 µl of sample buffer was loaded in one well and 5 µl of marker was loaded in another well. According to Figure 2, the number of visible bands was 8 bands, and according to Tables 2 and 3, the RF and MW of each band were also calculated and their related curves are shown in Figures 3 and 4.

Table 1. Optical absorption of standard protein and diluted samples

Sample	Dilutions	(OD) Optical Density
Standard	Blank	0
	10	0.061
	25	0.185
	50	0.322
	100	0.639
Unknown	1:5	0.446
	1:10	0.220

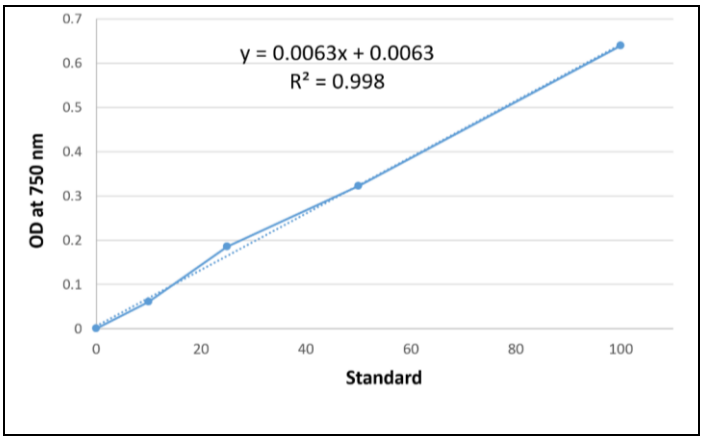

Figure 1. The optical absorbance of standard proteins

3.3. Double immunodiffusion

After taking blood from guinea pigs, and separating sera, a double immunodiffusion test was performed to check the immunogenic reaction of antigens with dilutions of 1:2, 1:4, 1:8, and 1:16 of serum. Deposition lines were shown as a reaction between serum Ab and FMD virus antigen. The animal serum had a positive response up to a titer of 1:16 (Figure 5).

3.4. Elisa

By performing the ELISA test, it was determined that the antibody was formed against the antigen, but there are differences among the 5 guinea pigs' immune responses based on the Ab titer of the serums (Table 4).

3.5. Western blot

In the western blot test, two bands were transferred from 10% gel to nitrocellulose paper, one band was in the range of 60-75 kDa and the other band was in the range of 35-45 kDa, and as a result, these two bands expressed the immunodominant proteins of FMD virus according to figure 6.

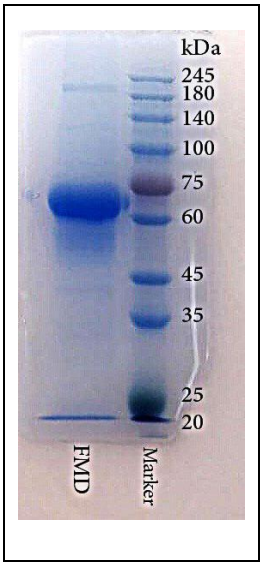

Figure 2. SDS-PAGE electrophoresis gel, 10%

Table 2. RF values for in gel, 10%

standard	245	180	135	100	75	63	48	35	25	20
RF	0.835	0.805	0.761	0.701	0.641	0.552	0.432	0.313	0.149	0.019

Table 3. RF values for serum in gel, 10%

Distance between well to the band	4.7	3.7	3.4	3.1	2.8	2.4	1.2	1.6
RF	0.95	0.75	0.69	0.63	0.57	0.48	0.42	0.32
MW	244	120	98	75	67	58	46	36

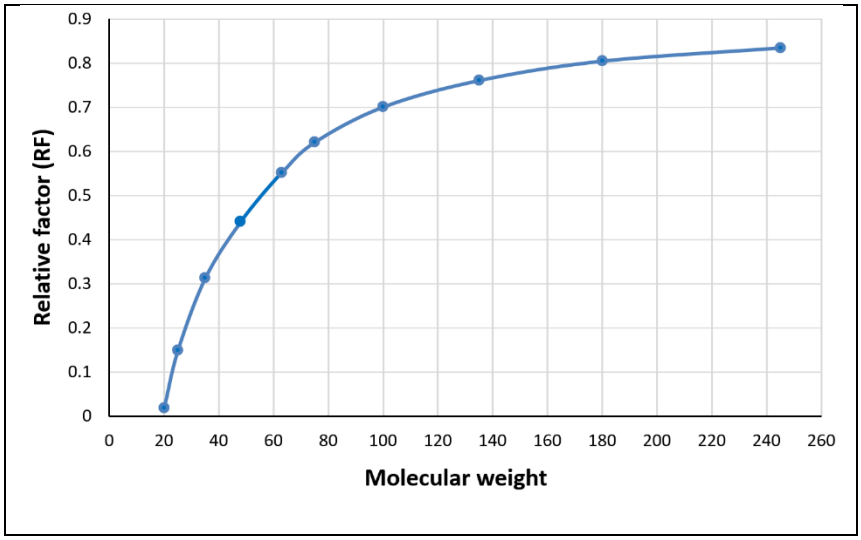

Figure 3. The curve of RF and molecular weight for the marker in gel, 10%

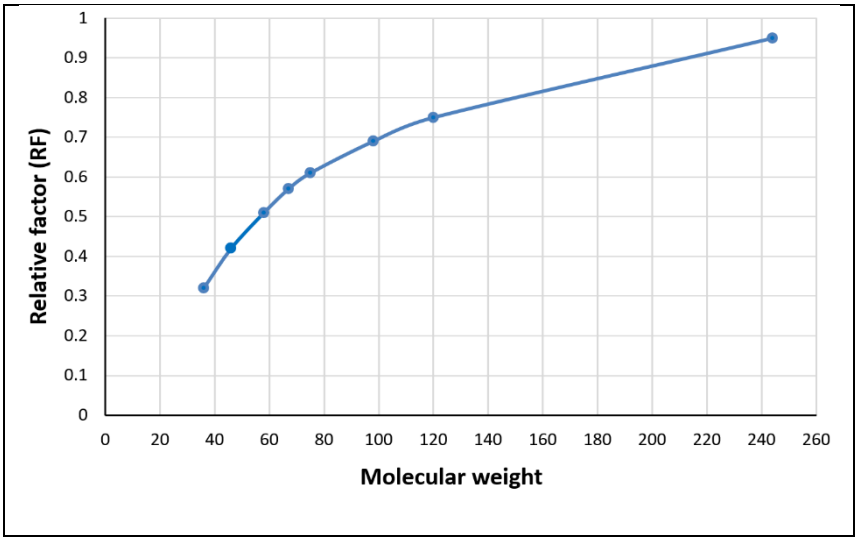

Figure 4. The curve of RF and molecular weight for serum in gel, 10%

4. Discussion

To control and reduce FMD, vaccination is a key factor, which requires improvement of the detailed knowledge about its structure. Most of the FMD vaccine produced these days consists of inactivated purified FMD virus, and use Montanide ISA 201 or 206 as an adjuvant (10). High replication rate, extreme transmissibility to many species, and antigenic diversity made FMD virus, a difficult pathogen to defeat, so these vaccines need to update and improve (11). Other vaccines platforms are shown effective in investigations, such as peptide vaccines (12), Recombinant VLP vaccines (13), Viral vector

Figure 5. Double immunodiffusion results of guinea pigs serum against FMDV

Table 4. Elisa results

Serum number Serum dilutions	1	2	3	4	5
1:100	3.5	3.65	3.75	3.67	3.66
1:200	2.95	2.8	2.9	3	3
1:600	2.05	2.4	2.6	2.9	3
1:800	1.11	1.7	1.7	2.3	2.9
1:1000	1.02	1.4	1.4	2	2.8

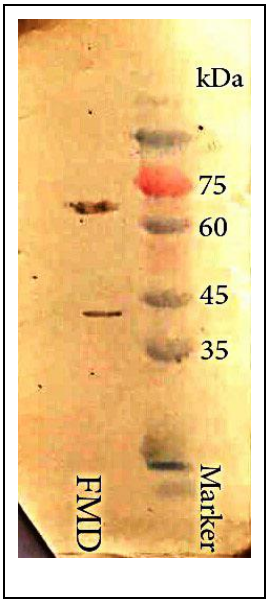

Figure 6. Western blot for 10% gel

vaccines (14, 15), DNA vaccines(16-18), and Modified live attenuated vaccines (19, 20). To develop novel vaccines, immunodominant Ag should be identified which are recognized by B-cells and T-cells of the host. However, recognition of T-cell epitopes is difficult because of that FMDV has a high number of sequence variations in its capsid proteins, which is shown by the existence of seven different serotypes and a large number of subtypes(21, 22). Today, many studies have been conducted in the field

of FMD proteins and their efficiency to make new vaccines. In some cases, by examining FMD immunodominant proteins, new approaches were found to design novel vaccines (23-25).

In a study conducted by Morgan and Moore (24) swines were vaccinated with a single dose of foot-and-mouth disease (FMD) virus immunodominant protein 1 (VP1) peptide ($VP1_{200-213}$ and $VP1_{141-160}$) expressed in Escherichia coli, it caused the induction of neutralizing antibody response in the vaccinated animals (24). In the present study, the dominant FMD protein induced antibody response in guinea pigs. Electrophoresis results of Vande Woude and Bachrach (26) showed that 75S empty capsids contained 25,000, 37,500, and 50,000-dalton molecular weight zones, which confirm the current study results. In the present study, two molecular weights of immunodominant proteins were determined, one of them was in the range of 60-75 kDa and the other was in the range of 35-45 kDa. Also, the difference between guinea pigs in antibody response tests, double immunodiffusion, and ELISA, might be due to the distinct immune response in each animal. Determining Ag that is recognized by antibodies in a way that there are low cross-reactions can help to develop serologic assays, so further studies and purification of our protein band could be useful in designing new serologic methods.

Su, Duan (25) shows that the immunodominant proteins can stimulate the immune response and the most important immune responses resulted from the vaccination of that designed immunodominant proteins by expressing the heterology of the immunodominant proteins of foot-and-mouth disease virus and examining the immune response after that in mice. In the current research, by examining the electrophoretic profile of foot and mouth disease and obtaining immunodominant proteins, and conducting an ELISA test, it was found that these proteins can be used to induce immune responses and design a vaccine. In another study, by examining the proteins of foot-and-mouth disease proteins to create immune responses in mice, the specific antigen against the serum antibody was detected. Cellular immune responses to VP1 and 3D protein were confirmed. The results showed that all the groups immunized by the examined proteins caused cellular immune reactions, which shows that both Ags effectively caused immune responses (23). It should be noted that the use of foot-and-mouth disease proteins to prepare new vaccines seems to be necessary, especially in endemic zones of disease, so in this regard, the current research was conducted and we found the electrophoretic profile of FMD which cause immune responses.

Finally, by identifying the dominant immune proteins, they can be used to make a novel vaccine. In such a way that by determining the molecular weight of the immunodominant proteins, which are in the ranges of 60-75 kDa and 35-45 kDa, these proteins can be purified and separated by different methods, and new vaccines can be prepared with appropriate adjuvants.

Authors' Contribution

Study concept and design: F. G. and R. M.
Acquisition of data: F. G. and M.H
Analysis and interpretation of data: F. G. and M.H.
Drafting of the manuscript: F. G. F. and R.M.
Critical revision of the manuscript for important intellectual content: R. M.
Statistical analysis: F. G. and M.H.
Administrative, technical, and material support: F. G. and R. M

Ethics

This study was approved by the Ethics Committee of Razi vaccine and serum research institute. Code of ethics : RVSRI.REC.98.013

Conflict of Interest

The authors declared that they have no conflict of interest associated with this study.

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