

Evaluation of specific chicken IgY antibody value developing diagnostic capture antibody ELISA kit against Foot and Mouth disease

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ABSTRACT

The most preferred method for the detection of foot-and-mouth disease (FMD) viral antigen and identification of viral serotype is the enzyme-linked immunosorbent assay (ELISA). Diagnostic tests with high sensitivity are necessary both to distinguish infected vaccinated animals and execute disease control programs for the identification of the carrier animals. The current strategies for the detection of FMD virus are mainly based on the capture antibody (sandwich) ELISA test. The usage of laying pullets as an animal bioreactor for the production of specific egg yolk antibodies (IgY) has increased in recent years due to its high yield, affinity, low price, and quick production turnover. The present study aimed to produce a concentrated and purified IgY polyclonal antibody to design a capture antibody ELISA kit against the FMD virus (FMDV) serotype A. At first, laying hens were immunized with inactivated FMDV serotype virus, and then, on days 14, 21, and 28 following vaccination, the eggs and sera were collected. Afterward, the IgY polyclonal antibodies were extracted and purified from the chicken egg yolk using a polyethylene glycol 6000–ethanol precipitation procedure. Extracts were filtered, purified by ion exchange chromatography, and dialyzed. The purified IgY concentration, estimated by Bradford assay, confirmed its presence by SDS-PAGE and Western blot and also its specific immune reaction by Ouchterlony double immunodiffusion and Dot blot tests. Moreover, for achieving the optimum concentration of antigen/antibody (sera) in sandwich ELISA, a checkerboard titration test was set up based on indirect ELISA results. Eventually, 119 previously confirmed samples (including 80 positive and 39 negative) by both real-time polymerase chain reaction (quantitative PCR, qPCR) and a commercial ELISA kit were used for evaluation of the sensitivity and accuracy of our developed Capture antibody ELISA kit. In this manner, the sensitivity and specificity of our designed kit were 100% and 98%, respectively. Accordingly, the present developed capture ELISA kit based on IgY had high sensitivity and specificity for FMD virus detection and it could be used in the future for both commercial detecting and serotyping applications.

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1. Introduction

Foot and mouth disease (FMD) is a highly contagious viral disease that economically is the most important disease affecting up to 70 species of mammals (1, 2). This disease is caused by FMD virus (FMDV), a single-stranded RNA virus of the Picorna-Viride family which can cause both acute and chronic disease without any symptoms (3). This virus includes seven serotypes, namely O, A, C, Asia 1, Southern African Territory (SAT) 1, 2, and 3, with a wide range of antigenically distinct subtypes from each one of them (4). The FMD is endemic in Iran and the disease control relies on mass vaccination in all susceptible livestock. Iran has faced several outbreaks despite the implementation of the national preventive and control program against FMD (5). The virus neutralization (VN) test is considered to be the gold standard test for FMDV. According to the speed, reproducibility, and efficacy of the VN test, it has been globally and routinely applied instead of other serological tests [6, 7]. However, the enzyme-linked immunosorbent assay (ELISA) test is also applied in order to detect the FMDV as it is less time-consuming than VN and provides faster clinical diagnosis tests. Another method is the real-time polymerase chain reaction (PCR) test which may be a faster diagnostic test for FMDV. However, the ELISA test has the advantages of superior sensitivity and detection of the whole infected particle. Therefore, the production of cost-effective diagnostic ELISA kits with high sensitivity seems to be a crucial diagnostic approach in both detecting and serotyping the virus (6, 7). The tendency of antibodies for antigen binding, as one of the main immunity functions, leads to neutralization and immobilization of the detected antigen. In order to produce polyclonal antibodies against different pathogenic microorganisms, poultry can be an ideal choice. Egg yolk antibody (IgY), has the same function as IgG antibody in the serum, placenta, and colostrum of mammals (8, 9). The characteristics of polyclonal IgY antibody, including affinity to a number of different antigenic epitopes in comparison with a monoclonal antibody, have made it a practical immunological tool regarding passive immunization, diagnostic tests, isolation of proteins, and treatment of diseases (10, 11). Moreover, IgY has been applied in many research studies due to its non-invasive method of preparation and ease of access (12, 13). In addition, IgY is relatively stable and shows consistency under various conditions, such as heat, pressure, different pHs, and proteolytic enzymes (14). Despite IgG, IgY does not bind to proteins G and A, and Fc receptors of

mammals; therefore, false positive reactions in tests are uncommon. Hence, IgY antibodies have been successfully used for scientific, diagnostic, preventive, and therapeutic purposes (15). Due to the physical and chemical characteristics, affinity, the possibility of use in the diagnostic kits, and also the cost-effectiveness of IgY antibody, the present study aimed to produce the concentrated and purified specific polyclonal IgY antibody for development of a capture antibody (sandwich) ELISA kit as a highly sensitive, easy, and cost-effective approach for diagnosing FMDV.

2. Materials and Methods

2.1. Antigen Preparation (Foot and Mouth Disease Virus Culture)

The FMDV serotype A15 (isolate IRN/23/2015) was obtained from the Razi Vaccine and Serum Research Institute of Iran, Department of FMDV. Previously, this antigen had been confirmed as a standard FMDV type A strain using capture ELISA and PCR sequencing methods. The BHK-21 clone 13 cells were seeded in 175 cm² flasks to achieve monolayer cells, including 100 mL RPMI1640 culture medium, 5% fetal bovine serum, and 100 mg/mL penicillin at 37 °C incubator for 48 h. When the cell reached 80% confluence and the supernatant medium was replaced with Earle's Balanced Salt Solution, an amount of 0.2 mL of FMDV type A with titer 10^{6.5} TCID₅₀/mL was added to each flask. After 9 h, all flasks showed complete cytopathic effect. The viral suspension was collected in a volume of 500 mL and centrifuged at 3,500 rpm/4 °C for 15 min. At the next step, the virus was inactivated with binary ethyleneimine 5 mM at 26 °C for 24 h. The remained ethyleneimine in the viral suspension was neutralized by equimolar sodium thiosulfate (5 mM). Polyethylene glycol 6,000 (PEG 6,000) 7.5% w/v was applied to concentrate the inactivated virus. Finally, the prepared antigen was collected in a volume of 8 mL. The concentration of the antigen was determined using the Bradford method.

2.2. Animals Husbandry and Injections

In total, 12 Leghorn laying pullets aged 90-110 days were purchased from Morghak Company (Karaj, Iran), which had received all the ordinary vaccines of poultry. They were randomly divided into test and control groups, each of which included 6 pullets. The

FMDV antigen A was prepared and homogenized by applying ISA71 adjuvant (30% adjuvant and 70% antigen) and was administrated intramuscularly and subcutaneously (0.5 cc) in the test group. The booster doses were injected on weeks 2, 4, and 6 after the first injection. The eggs and sera samples were collected before the first, immediately after the last, and four weeks after the last injections. The collected eggs were disinfected with alcohol and kept at 4 °C until they were transferred to the laboratory. Furthermore, a healthy New Zealand white rabbit (2-3 kg) was obtained from Laboratory Animal Breeding Department and immunized with purified FMDV by application of Freund's adjuvant in doses of 25, 15, and 15 µg on days 0, 14, and 28, respectively. Ten days after the last injection, the blood of the rabbit and the chickens were collected. After being placed in an incubator at 37 °C for 1 h, blood cells and plasma were separated by centrifugation at 2,500 rpm for 5 min.

2.3. IgY Antibody Extraction and Purification

The yolk contents were carefully isolated from the eggs and stored in a special tube. Afterward, it was measured and dissolved in phosphate-buffered saline (PBS) solvent. The IgY was extracted by application of the PEG 6,000-ethanol method according to the provided manual 9 [18]. Besides, Whatman No. 2 filters were used to filter the supernatant liquid. Ethanol was used to remove PEG from the final extracted antibody content. Afterward, dialysis tubing cellulose membrane 12kDa (Sigma-Aldrich Co., Germany) was applied to remove the remaining salts and other precipitants in PBS buffer (pH=7.2) overnight. The extracted content was stored at -20 °C until further use. Finally, the IgY from the yolk and IgG from rabbit sera were isolated and purified through ion exchange chromatography (16). The concentrated chicken and rabbit serums were loaded on the DEAE-sepharose column, the column was washed using phosphate buffer, and the output contents were collected. Therefore, first, the required amount of each of the materials was dissolved in double distilled water; after mixing, the pH was adjusted to 2.7. To prepare PBS containing 1 M KCL, 74 g/l of KCL was dissolved in 0.02 M PBS. Finally, the column was washed with 0.02 M buffer (IgGs and IgYs do not bind to the column) and fractions were collected in 1.5 microtubes. The optical density (OD) of each separated fraction was read at a wavelength of 280 nm. In addition, to check the presence and purity

of the isolated immunoglobulins in the fractions collected from ion exchange chromatography, the SDS-PAGE method and then Western blot were performed.

2.4. Purified Abs Assessment

The Bradford method was used with bovine serum albumin protein to measure the purified protein content of the sample using coomassie blue G-250 at 595 nm wavelength. Based on the initial evaluation, starting from 10 mg/mL, serial dilutions were prepared and then the standard curve was drawn to obtain the amount of protein.

2.4.1. SDS-PAGE and Western Blotting

An amount of 20 µL aliquot of purified antibody was separated on a 12% SDS-PAGE gel. In brief, at first, samples were denatured and reduced in loading buffer without bromophenol blue and heated at 96 °C for 3 min. Afterward, 20 µL aliquot of purified antibody was run on SDS-PAGE gel and the samples were then resolved through 12% Tris-glycine gels and transferred to a nitrocellulose membrane (0.45 µM, GE Healthcare) using a mini-protean tetra cell (BioRad) and then blocked for 1 h. The membrane was washed three times for 15 min and probed with rabbit anti-chicken IgY AG (1/1000 dilution, Sigma-Aldrich, Germany) (1:1000). Membranes were placed in blocking buffer (20mM Tris, 150mM NaCl pH7.6 with 0.1% v/v tween-20 [TBS-T]) for 1 h at room temperature and then washed three times with TBS-T. The DAB substrate was added to the membrane and exposures of the membrane were collected and visualised. The immunological (Ab-anti ab) interaction was visible as brown bands on the nitrocellulose membrane.

2.4.2. Dot Blot test

The Dot Blot test results provide the presence of the purified antibody as well as its activity. For this purpose, the FMDV Type A (5 µg) and controls were fixed as spots on a nitrocellulose paper and the membrane was immersed in 1% bovine serum albumin and shaken in the incubator for 30 min at room temperature. Afterward, it was washed with PBST (PBS, 0.1% (v/v) Tween) for 2 min and then exposed to the diluted primary antibody (IgY). After that, the nitrocellulose membrane was washed in PBST four times, each time for 5 min. In the

following, a secondary antibody from rabbit anti-chicken IgY conjugate (1/1000 dilution, Sigma-Aldrich Co., Germany) was added and incubated for 1 h in a shaking incubator at room temperature. The membrane was washed four times for 5 min in PBST. Diaminobenzidine (DAB) dye according to the provided manual (Sigma-Aldrich, Germany).

2.4.3. Ouchterlony Method (Double Immunodiffusion Test)

A simple Ouchterlony test was used to confirm the presence of a specific IgY polyclonal antibody against FMDV type A (17). The percentage of gel was set up at 0.6 in PBS and also, the NaCl and sodium azide were used at 1.2% and 0.05%, respectively. Moreover, different incubation temperatures of 4 °C, 24 °C, and 37 °C were used to determine the best results.

2.4.4. Checkerboard

After determination of the antigen concentration, amounts of 250 ng, 500 ng, 1 µg, 2 µg, 5 µg, 10 µg, and 20 µg in PBS buffer were added to the wells (100 µL). The plate was covered and kept at 4 °C overnight. After washing and blocking steps, 250 ng, 500 ng, 1 µg, 2 µg, 5 µg, 10 µg, and 20 µg of purified antibody from chicken were diluted in PBS buffer, added to the wells (100 µL), and incubated at 37 °C for 1.5 h. After washing steps, 100 µL of conjugated antibody (rabbit anti-chicken IgY anti-gout [Sigma-Aldrich, Germany]) (1:10000 in PBS) was added to each well and incubated at 37 °C for 1.5 h. Afterward, 100 µL of TMB substrate was added to each well and placed in a dark room for 15 min after washing steps. The reaction was stopped using 50 µL of stopping solution and the optical density was read at 450 nm. Capture ELISA was performed according to the results obtained from the previous step, including the appropriate concentration of the primary antibody (chicken) in a volume of 100 µL was added into the wells, and the plate was incubated at 4 °C overnight. Afterward, the washing and blocking steps were performed. The appropriate concentration of antigen was diluted with PBST in a volume of 100 µL, added to the wells, and incubated at 37 °C for 2 h. After the washing steps, 250 ng, 500 ng, 1 µg, 2 µg, 5 µg, 10 µg, and 20 µg of the purified rabbit secondary antibody were diluted in PBS buffer in a volume of 100 µL and added to the wells and incubated at 37 °C for 1.5 h. Subsequently, 100 µL of (rabbit anti-chicken IgY anti-gout [Sigma-Aldrich, Germany]) (1:5000 and 1:10000 in PBS) was added to each well in two rows and incubated at 37 °C for 1.5 h. The reaction was stopped using 50 µL of stopping solution and the optical density was read at 450 nm. The amount of threshold index was obtained using the following formula;

$$\text{Antibody index} = (\text{negative samples OD} / \text{number of samples mean OD}) + 0.15$$

The cut-off rate according to the amount of antibody index for IgG ELISA tests was obtained at 0.2, and the rates higher than that were considered positive and those lower than that were considered negative.

2.5. Known Positive and Negative Samples

In total, 80 positive tissue samples were collected from the cattle and sheep tongue epithelium which were suspicious of FMDV. The samples had been obtained from herds throughout Iran between 2012 and 2021 and were sent to the FMD Vaccine Production Department of Razi Vaccine and Serum Research Institute, Karaj, Iran. To isolate the virus, epithelial samples were homogenized in a cold porcelain mortar to produce a 10% (W/V) suspension with Eagle's medium. The suspension was clarified by centrifugation at 2,500 rpm for 15 min and the supernatant was collected. Furthermore, 39 negative tissue samples, which had been confirmed by commercial ELISA (IZSLER Co., Italy) and real-time PCR, were obtained.

2.6. Statistics

The absorbance of the extracted optical for positive and negative samples was analyzed using GraphPad Prism8 software, and after drawing the receiver operating characteristic (ROC) curve, the best possible limit with high sensitivity and specificity was determined.

3. Results

3.1. IgY antibody assessment

Results showed that the concentrated and purified IgY antibody/per mL (by PEG-ethanol and Ion exchange chromatography) was 5 mg/mL polyclonal IgY yield from eggs, about 0.30 to 0.35 mg/mL of which was specific polyclonal IgY antibody against FMDV type A.

3.2. IgG Yield After Immunization (From Rabbit and Chicken Serum)

According to the ammonium sulfate method, after completing the dialysis process and ion exchange chromatography, the amounts of purified antibodies were 2.5 and 3 mg/mL for the IgG extracted from the sera of rabbits and chickens, respectively.

3.3. Confirmation of Antibody Tests

The SDS-PAGE test was used and the presence of IgY was confirmed in different dilutions. The heavy chain of the IgY antibody was 65 kDa and the light chain was 27 kDa in the

SDS Page (Fig.1). Western blot test was performed using an anti-Chicken antibody. The appearance of brown spots on nitrocellulose paper confirms the presence of type A specific antibody (Figure 2).

3.4. Validation of Specific Antibody Function

A dot blot test was performed to confirm the specific immuno-reaction of IgY antibody with FMD virus (Fig.3). The appearance of brown spots on nitrocellulose paper confirmed the specific interaction of concentrated and purified IgY antibody (in dilution of ~1:1000) from egg yolk and serum with FMD virus type A.

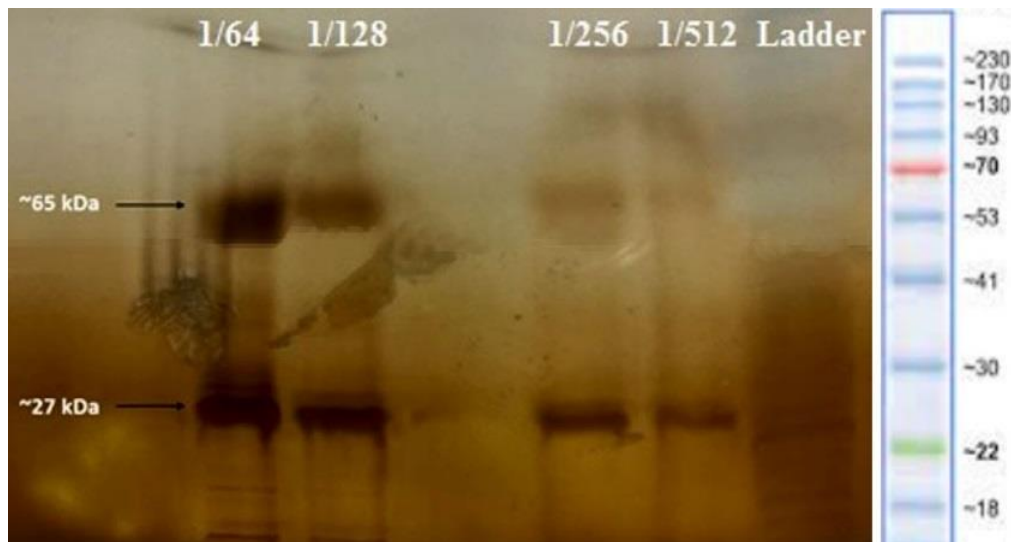


Figure 1. Results of SDS-PAGE assay for extracted IgY stained with silver nitrate.

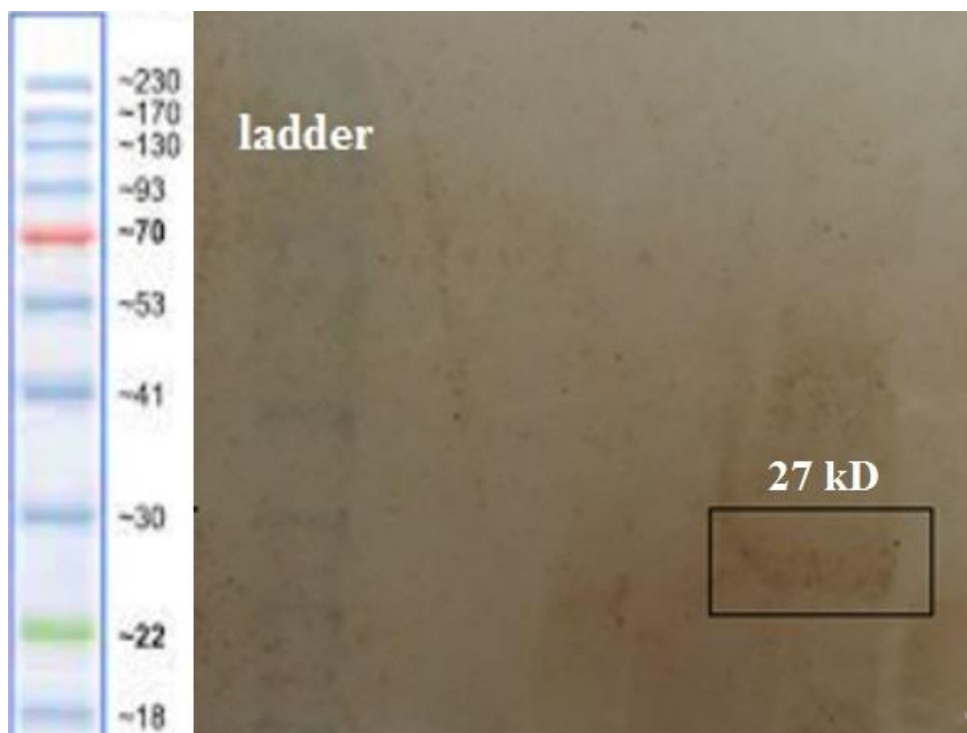


Figure 2. results of Western Blot assay using Anti-chicken IgY (IgG) (whole molecule) alkaline phosphatase antibody produced in rabbit to detect of chicken IgY. Dab dilution was 1:2000 (Size of the band ~ 27 kD). It seems that conjugate ab mainly react with light chain of IgY antibody

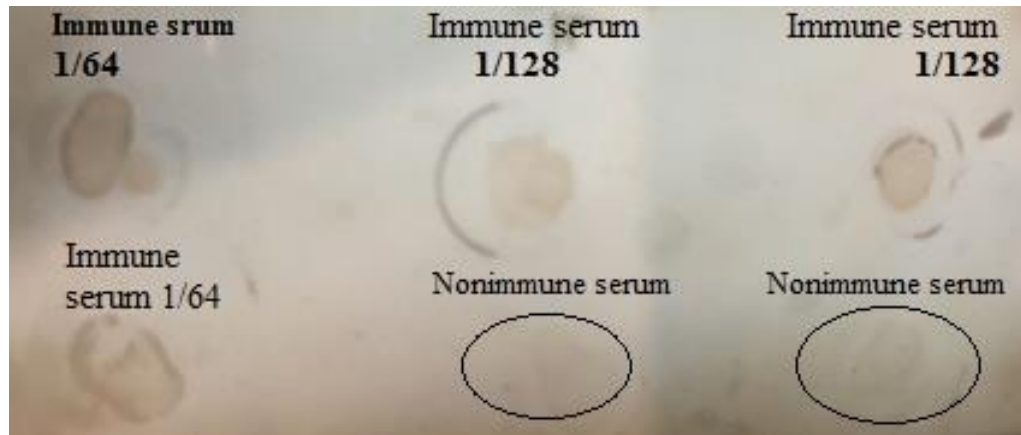


Figure 3. Result of dot blot test for confirmation of immunization and the reaction of virus with different dilutions of Sera. All the upper rows indicated positive sera and the lower row indicated negative sera except one against FMDV type A antigen.

3.4.1. Double Immunodiffusion Test

The Ouchterlony specimens test for FMDV type A was performed at dilutions of 1:2 and 1:4 together with a virus with a 10^6 TCID₅₀. The sediment line is clearly shown in figure 4.

3.5. Commercial ELISA Test Results

As described in the method and material section, 119 previously prepared samples which were delivered to the Reference Laboratory of FMD of Razi vaccine and sera research institute were screened with the commercial ELISA kit. A number of 80 samples of serotype A were detected by the ELISA method (80 positive, 39 negative).

The OD values above 0.1 were considered positive and those below 0.1 were considered negative. Table 1 summarizes the samples and their corresponding ODs which are reported as serotype A (FMD positive) by commercial ELISA Kit. These samples are used for comparative evaluation of the sensitivity and specificity of our ELISA kits.

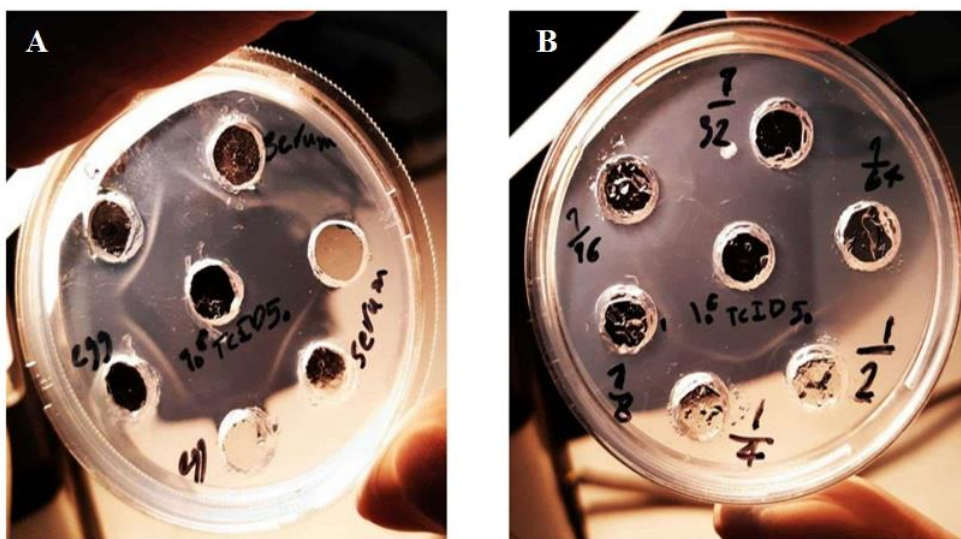


Figure 4. Ouchterlony specimens test. A: Without dilution for antibodies in egg and serum against FMDV type A 10^6 TCID₅₀; B: diluted for egg antibodies in 1:2 and 1:4 against FMDV type A 10^6 TCID₅₀ the well in the center of plate is FMDV type A, and other wells are the antibodies of eggs and sera.

and the statistical formula $\text{Cut-off} = X + 2SD$ was applied. This limit was obtained with a probability of 0.2. The X represented the average OD of the samples and SD was

Table 1. Results of some commercial ELISA kit results for suspected samples for FMDV type A. Then, these positive and negative samples direct for using in our developed ELISA kit.

	1	2	3	4	5	6	7	8	9	10	CP	CN
1	0.086	0.088	0.077	0.065	0.068	0.075	0.076	0.087	0.063	0.021	2.558	0.088
2	1.652	1.419	1.894	0.593	0.063	0.106	0.112	0.046	1.431	0.163	1.403	0.096
3	0.143	0.196	2.809	2.108	0.064	0.477	0.197	0.102	2.705	0.129	2.672	0.076
4	0.021	0.083	0.080	0.064	0.065	0.073	0.076	0.092	0.062	0.054	1.591	0.074
5	0.093	0.082	0.077	0.069	0.062	0.068	0.069	0.099	0.060	0.026	1.383	0.078
6	2.158	2.006	2.788	1.746	0.098	0.196	0.219	0.170	2.259	0.210	2.101	0.021

3.6. Assessment of the Purified Abs Function (Specific Immunological Reactions)

According to the results of indirect ELISA optimization and a prepared checkerboard titration of antigen, purified IgY antibody from chicken serum, and secondary antibody at different concentrations, the optimum concentrations were determined at 0.5 $\mu\text{g}/\text{well}$ for the antigen, 0.1 $\mu\text{g}/\text{well}$ for purified polyclonal IgY antibody, and dilution of 1:10000 for conjugated goat anti-chicken. The initial concentration of the antigen column (column 1) was 10 $\mu\text{g}/\text{mL}$ and the positive serum was considered with an initial dilution of 1:100. It should be mentioned that both were diluted by two-fold serial dilution. The data was entered into Excel software and the desired curve was drawn. According to the results, the 1:640 dilution of the coated antigen, which was about 440 ng to a maximum of 500 ng/mL, was the premium one for coating and generating a kit from the stock virus. In order to optimize the sandwich ELISA and determine the appropriate concentration of detection Ab or the secondary Ab, the checkerboard with different concentrations of purified IgG polyclonal antibody from rabbit and conjugated Anti-Rabbit Ab were used. Accordingly, the concentration of polyclonal IgG as 0.2 $\mu\text{g}/\text{well}$ and the dilution of conjugated anti-rabbit Ab as 1:10000 was determined as the optimum concentrations.

3.7. Evaluation of Confirmed Positive Samples of Livestock Using ELISA Sandwich Technique

The appropriate dilution of FMD strain A from the positive samples was assessed for application in the sandwich ELISA technique. The results indicated 1:100 dilutions according to the viral OD above 0.2 in the commercial kit (IZSLER, Italy). In order to determine the cut-off rate, 16 confirmed positive samples were tested

the standard deviation. The optical absorbance of 0.21 was considered as the cut-off for the ELISA test with antibodies produced against virus antigens to detect type A of FMDV. According to the obtained results from the previous stage and the estimation of appropriate concentrations and dilutions of antigens and antibodies, the designed system was implemented on the samples which had been confirmed to be positive or negative. In total, 119 samples, including 80 positive and 39 negative ones, were used for comparative evaluation. Regarding FMDV samples, 100% of 80 positive samples were positive for the presence of FMDV antibodies using the designed test; therefore, the sensitivity of the test was 100%. In order to comparatively and specifically detect and identify the types of FMDV, it is necessary to have a quantifiable kit and threshold limit. The testing of 39 previously confirmed negative samples (with the commercial ELISA and real-time test) resulted in 38 negative samples. Therefore, the specificity of the designed kit was assessed as 98%. The sensitivity and specificity of the designed ELISA kit are summarized in table 2.

Table 2. Statistical parameters obtained from the results of capture antibody ELISA.

Statistical parameters	ELISA
Sensitivity	100%
Specificity	98%
Positive predictive value	97%
Negative predictive value	97%
Accuracy	99%
Validity	100%

3-7. Comparison Between the Commercial ELISA and the Present ELISA Kit

The obtained results of the ELISA test (figure 5) were in accordance with the area under the ROC curve (AUC). According to table 2, the present designed test determined 80 out of 80 positive samples using the commercial kit. Moreover, the designed kit resulted in 100% positive samples. Therefore, the positive predictive value of this test was estimated at 97%. Moreover, the designed kit identified 38 out of 39 negative samples, while the commercial kit identified 39 negative samples. Therefore, the negative predictive value in this test was calculated at 97%. Furthermore, the accuracy and reliability of the present test were estimated at 99% according to the 0.998 AUC.

4. Discussion

In the present study, according to the need for a fast-reliable ELISA test to identify FMDV and the powerful infrastructure of Razi Vaccine and Serum Research Institute, we designed an indirect capture ELISA kit to detect type A FMD virus applying polyclonal IgY

antibody. The results of comparative analysis with the available commercial kit showed great sensitivity and specificity (100% and 98%, respectively) for the present designed kit. The rapid and accurate procedure can be very important for FMD control and indication of the requirement for specific Abs to detect different serotypes through diagnosis kits. The production of specific IgY Abs with the potency to identify different serotypes contributes to early diagnosis and disease control. The yolk of vaccinated eggs is an excellent source for the production of specific polyclonal IgY and has many advantages in comparison with specific IgG obtained from animal sera. In a study performed by Ephrem et al. [21], laying hens were immunized by inactive local FMDV serotypes (18).

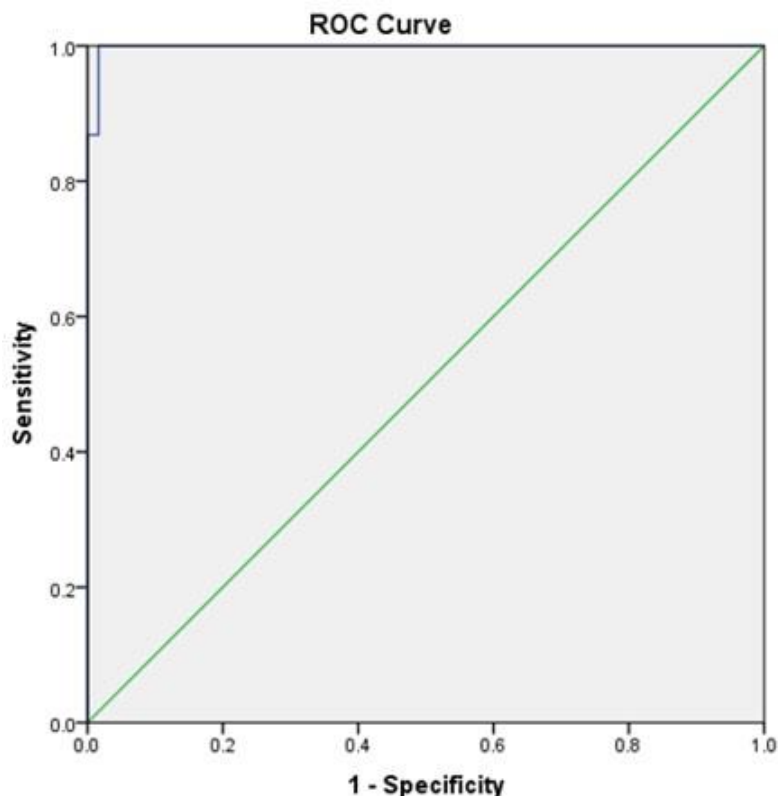


Figure 5. ROC curve for the analyzed data obtained from ELISA results for livestock samples

In line with the present study, polyclonal IgY antibodies have been produced using the polyethylene glycol-ethanol precipitation method. The presence of polyclonal IgY antibodies against selected serotypes of FMDVs was detected by ELISA kit. The titer of IgY antibodies obtained from egg and serum was separately measured by the VN test method of three serotypes O, A, and SAT-2 on days 14, 21, and 28 after immunization, respectively. The results showed that the IgY titer obtained from egg yolk had significant differences from the IgY titer obtained from serum. Similar to the findings of the present study, Ephrem et al. [21] concluded that egg yolk antibody production (IgY) can be the best alternative to other laboratory animals [21]. Moreover, the purified antibody from yolk eggs in our study was 5 mg/mL, in comparison with rabbit sera IgG (2.5 mg/mL) and chicken sera IgG (3 mg/mL). In another study, anti-FMDV IgY immunoglobulins for O, A, and SAT2 serotypes were prepared in egg yolk by immunization of the laying hens with a local FMD vaccine (19). Antibody titers against three serotypes (O, A, and SAT2) were measured and then the obtained IgY was conjugated to horse radish peroxidase. The results showed a sensitivity of 85% in the detection of the virus in infected samples, which indicated the success in preparing anti-FMD immunoglobulins conjugated with horseradish peroxidase enzyme for use in diagnosis. Hereby, the designed ELISA test reached 100% sensitivity, compared to the available commercial test. In another study, chicken monoclonal and IgY antibodies were prepared against FMDV, in which the a/m antibodies were used for serotyping of 30 tongue epithelial samples and 60 tissue culture fluids. The obtained results were compared with those of the usual ELISA antigen detection method. Findings showed that monoclonal antibodies and chicken IgY can be replaced by conventional polyclonal antibodies for the common FMDV serotypes (20). Although the usefulness of monoclonal abs and chicken IgY in achieving sufficient sensitivity and specificity of the ELISA has been emphasized, our designed ELISA method based on IgY polyclonal Ab showed high sensitivity and specificity. The OIE Terrestrial Manual recommends ELISA as the most preferred method for the detection of FMD viral antigen and the identification of viral serotypes. Moreover, OIE ascertains that sero-immunological tests of FMD are used for four main purposes, such as to certify and monitor animals prior to import or export, confirm suspected cases of FMD, prove absence (or presence) of infection, and evaluate the efficacy and potency of

commercial vaccination regimes [24]. The present study mainly aimed to detect the FMD virus by IgY antibody. However, in order to identify the types of FMDV, it is necessary to have a quantifiable commercial monoclonal or polyspecific against seven types of FMD virus. Based on the affected species and the geographical origin of suspected samples, it may be suitable to simultaneously test for swine vesicular disease virus or vesicular stomatitis virus. Ideally, a complete differential diagnosis should be undertaken in all vesicular conditions [24]. In conclusion, the findings indicated that preparation of FMDV IgY from yolk could be considered to have diagnostic benefits in FMDV type A detection. The present designed ELISA method based on IgY polyclonal Ab showed high sensitivity and specificity indicating high accuracy and reliability. Therefore, the results of the present study could provide a promising future for the development of commercially available capture antibody (sandwich) ELISA to detect FMD virus.

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Authors' Contribution

Study concept and design: Z.I., MM.R., B.H., N.H. and M.A.

Acquisition of data: Z.I. and MM.R.

Analysis and interpretation of data: Z.I. and MM.R.

Drafting of the manuscript: Z.I.

Critical revision of the manuscript for important intellectual content: Z.I., MM.R., B.H., N.H. and M.A.

Statistical analysis: Z.I. and MM.R.

Administrative, technical, and material support: Z.I., MM.R., B.H., N.H. and M.A.

Ethics

The procedures were approved by the ethics committee of the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Conflict of Interest

The authors declare that there is no conflict of interest.

Grant Support

Not applicable.

References

- Hunter A. O.I.E. Manual of Standards for Diagnostic Tests and Vaccines, List A and B diseases of mammals, birds and bees, 3rd edn. Trop Anim Health Prod. 1998;30(3):158-158.
- Al-Salihi KA. The epidemiology of foot-and-mouth disease outbreaks and its history in Iraq. Vet World. 2019;12(5):706-12.
- Grubman MJ, Baxt B. Foot-and-mouth disease. Clin Microbiol Rev. 2004;17(2):465-93.
- Lloyd-Jones K, Mahapatra M, Upadhyaya S, Paton DJ, Babu A, Hutchings G, Parida S. Genetic and antigenic characterization of serotype O FMD viruses from East Africa for the selection of suitable vaccine strain. Vaccine. 2017;35(49 Pt B):6842-9.
- Ilbeigi K, Bokaie S, Aghasharif S, Soares Magalhães RJ, Rashtibaf M. Risk factors for recurrence of FMD outbreaks in Iran: a case-control study in a highly endemic area. BMC Vet Res. 2018;14(1):253.
- Paton DJ, Sumption KJ, Charleston B. Options for control of foot-and-mouth disease: knowledge, capability and policy. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2009;364(1530):2657-67.
- Kabelo T, Fana E, Lebani K. Assessment of the sensitivity of primary cells and cell lines to the Southern African Territories (SAT) serotypes in the diagnosis of foot-and-mouth disease virus. Heliyon. 2020;6(5):e03905.
- Morioka K, Fukai K, Yoshida K, Yamazoe R, Onozato H, Ohashi S, T, et al. Foot-and-mouth disease virus antigen detection enzyme-linked immunosorbent assay using multiserotype-reactive monoclonal antibodies. J Clin Microbiol. 2009;47(11):3663-8.
- Ma LN, Zhang J, Chen HT, Zhou JH, Ding YZ, Liu YS. An overview on ELISA techniques for FMD. Virol J. 2011;8:419.
- Xu Y, Li X, Jin L, Zhen Y, Lu Y, Li S, You J, Wang L. Application of chicken egg yolk immunoglobulins in the control of terrestrial and aquatic animal diseases: a review. Biotechnol Adv. 2011;29(6):860-8.
- Pereira EP, Van Tilburg MF, Florean EO, Guedes MI. Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. Int Immunopharmacol. 2019;73:293-303.
- Li X, Wang L, Zhen Y, Li S, Xu Y. Chicken egg yolk antibodies (IgY) as non-antibiotic production enhancers for use in swine production: a review. J Anim Sci Biotechnol. 2015;6(1):40.
- Lee L, Samardzic K, Wallach M, Frumkin LR, Mochly-Rosen D. Immunoglobulin Y for Potential Diagnostic and Therapeutic Applications in Infectious Diseases. Front Immunol. 2021;12.
- Thomsen K, Christophersen L, Jensen PØ, Bjarnsholt T, Moser C, Højby N. Anti-Pseudomonas aeruginosa IgY antibodies promote bacterial opsonization and augment the phagocytic activity of polymorphonuclear neutrophils. Hum Vaccines Immunother. 2016;12(7):1690-9.
- Yokoyama H, Peralta RC, Sendo S, Ikemori Y, Kodama Y. Detection of passage and absorption of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. Am J Vet Res. 1993;54(6):867-72.
- Rahman S, Van Nguyen S, Icatlo Jr FC, Umeda K, Kodama Y. Oral passive IgY-based immunotherapeutics: a novel solution for prevention and treatment of alimentary tract diseases. Hum Vaccines Immunother. 2013;9(5):1039-48.
- Sunwoo HH, Lee EN, Menninen K, Suresh MR, Sim JS. Growth Inhibitory Effect of Chicken Egg Yolk Antibody (IgY) on Escherichia coli O157:H7. J Food Sci. 2002;67(4):1486-94.
- Polson A, Coetzer T, Kruger J, Von Maltzahn E, Van der Merwe KJ. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immun invest. 1985;14(4):323-327.
- Tompkins ER, Mayer SW. Ion Exchange as a Separations Method. III. Equilibrium Studies of the Reactions of Rare Earth Complexes with Synthetic Ion Exchange Resins.1. Journal of the American Chemical Society. 1947;69(11):2859-65.
- Mujtaba MG, Baliban T, Bhagu J, Herrera M. A Laboratory Exercise Simulating Antibody and Antigen Reactions of the Ouchterlony Double Immunodiffusion Assay Using Inorganic Salts. Journal of Microbiology & Biology Education. 2021;22(2):e00103-21.
- Ephrem S, Tesfaye S, Tadesse F, Bilata T, Paeshuysse J. Production of Polyclonal Antibodies (IgY) Against Foot-and-Mouth Disease (Serotype: O, A and Sat-2) in Ethiopia by Using Layer Hens. Adv Dairy Sci Res. 2023;1(1):23-9.
- Ibrahim EE, El Helw HA, Shafik NG, Mekhail MA. Conjugation of foot and mouth disease IgY in chicken egg yolk with horse radish peroxidase for typing of foot and mouth disease virus. Journal of Applied Veterinary Sciences. 2017;2(1):35-42.
- Veerasami M, Singanallur NB, Thirumeni N, Rana SK, Shanmugham R, Ponselaran S, et al. Serotyping of foot-and-mouth disease virus by antigen capture-ELISA using monoclonal antibodies and chicken IgY. New Microbiologica. 2008;31(4):549-554.
- OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2022.