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Development of solid phase competitive enzyme-linked immunosorbent assay to detect foot-and-mouth disease virus A serotype specific antibodies based on immunoglobulin Y antibody

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Abstract

The aim of this study was to using purified chicken antibody (IgY) for developing solid phase competitive (SPC) enzyme-linked immunosorbent assay (ELISA) to detect the foot-and-mouth disease virus (FMDV) A serotype. After immunization of chickens, polyclonal immunoglobulin (IgY) antibodies were extracted and purified from egg yolk and yield was about 5.00 mg mL^{-1} of yolk as well as near 0.40 mg mL^{-1} of specific IgY antibody against FMDV serotype A. Also, optimized sucrose density gradient method produced 228 µg mL^{-1} whole virus which is much higher than that of the conventional method of sucrose density gradient method. The optimum concentration of purified capture IgY and bind type A antigen were 0.50 µg and 0.10 µg *per* well, respectively. The OD values < 0.70 were considered positive, and values ≥ 0.70 were negative for in-house kit base on standard controls. Statistical analysis base on 80 serum samples showed the 96.66% sensitivity, 100% specificity, 100% positive predictive value, 90.90% negative predictive value, 97.50% accuracy, and 98.33% reliability for serum samples for two commercial and in-house kits. The SPCE developed based on IgY antibody is a suitable alternative for the detection of antibodies after vaccination against type A FMDV with high sensitivity and specificity. The present research demonstrated the possibility of commercial development of the SPCE kit using IgY antibodies for the detection of FMDV antibodies in serum samples with adequate sensitivity and accuracy.

Introduction

Foot-and-mouth disease (FMD) as a highly contagious disease is the most economically important disease affecting over 70 species of cloven-hoofed animals. It leads to serious disease epidemic in Middle East countries and also restrictions on international trade in live animals and their products.[1] The causative agent of this disease is the FMD virus (FMDV; Aphtovirus: Picornaviridae), including seven immunologically distinct serotypes (O, A, C, Asia1, and South African Territories 1, 2, and 3) with a wide range of antigenic and epidemiological subtypes within each serotype.[2] Each serotype has several subtypes being immunologically and serologically distinct, and there is no cross-protection between serotypes and even some sub-types whereas protection against one immune serotype does not confer protection against infection with other serotypes or even subtypes.[3] While clinical signs of FMD cannot be distinguished from other diseases, the confirmation of FMD requires laboratory tests. The most common diagnostic methods for FMD are the detection of viral antigens or specific antibodies using enzyme-linked immunosorbent assay (ELISA) and virus isolation methods.[4] Thus, rapid laboratory diagnosis is necessary to confirm clinically suspicious cases. Control of FMD is achieved through surveillance of virus outbreaks and vaccination programs and evaluation of serological responses. There are two main categories of tests for FMD serology, including the tests identifying specific antibodies against non-structural viral proteins, which are conserved among serotypes and induced by live/infectious virus, and the tests identifying specific antibodies against structural proteins (SPs), including potentially protective and serotype-specific surface epitopes induced by vaccines.[5] virus identification and specific antibody responses identification are two used methods for viral infection detection.

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The most common test used for FMD serology is the virus neutralization test (VNT) and liquid-phase blocking ELISA (LPBE), as recommended by the World Organization for Animal Health.[6] Accurate and rapid identification of the disease, determination of circulating virus types, and comparison with each other and with vaccine virus strains can provide control and eradication tools. The VNT as a sensitive, specific, and reliable test is considered.[7] Despite, the VNT is recommended by the International Office of Epizootics as a gold standard serological test for detecting FMD antibodies in animals, but it should be kept in mind that VNT is time-consuming and complicated, and requires cell culture and live virus compared to alternative methods. In addition to other methods, such as multiple ELISA-based methods, including indirect ELISA and blocking assays, competition or sandwich assays for serological monitoring have been widely developed.[8-11]

Animals infected or vaccinated with FMDVs produce antibodies against solid phase or SPs which are mainly serotype-specific. Therefore, different reagents must be used for each of the seven FMDV serotypes and hence, specific assays may be required even for genetically distinct strains within a serotype. In vaccinated cattle with different FMD vaccine formulations, the solid phase competitive ELISA (SPCE) result shows a decrease in optical density (OD) value during the post-vaccination period, due to competition between antibodies in the test serum and enzyme-conjugated antibody for the same antigen. Decreasing in OD value indirectly correlates with the antibody titer in the test serum against that serotype, indicating an increase in antibody titer and the percentage of inhibition (PI), because the antibodies present in the test sera prevent the binding of the conjugate antibody.[12]

The use of new technologies, such as antibody production in egg yolk (immunoglobulin Y [IgY]) with therapeutic and diagnostic purposes is expanding day by day. From the point of view of observing animal rights, chickens are a suitable alternative as antibody producers in high quantities and there is no need to restrain and use invasive methods, such as blood sampling. In many studies, IgY is used due to the richness of its source, phylogenetic distance of this antibody from mammals, cheap price, ease of access, and its relatively high stability compared to the other laboratory animals; so, it has high advantages for ELISA kit development. Also, IgY in chicken egg yolk can be purified in high amounts and easily compared to mammalian immunoglobulin G. On the other hand, IgY is relatively stable in various conditions, such as heat, pressure, acidity, and alkalinity, as well as the effect of proteolytic enzymes. [13]

The control of FMD as an endemic disease in Iran is achieved through mass vaccination in all susceptible animals, but in spite of the implementation of a national prevention and control program, the country has faced multiple outbreaks of this disease.[14] The aim of this study was using purified chicken IgY polyclonal antibody against FMDV serotype A as a capture antibody, and purified inactivated FMDV serotype A as a diagnostic antigen, to develop SPCE and comparison the results with a commercial ELISA kit.

Materials and Methods

Virus, cells, and sample concentration. The FMDV serotype A15 (isolate IRN/23/2015) was obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran. The BHK-21 cells[15] were also obtained from the Razi Vaccine and Serum Research Institute. Then, the cells with 100 mL RPMI1640 culture medium (Biowest Co., Nuaillé, France), 5.00% fetal bovine serum (Gibco, Grand Island, USA), and 100 mg mL^{-1} penicillin (Sigma-Aldrich, Hamburg, Germany) incubated at 37.00 ˚C for 48 hr. When the cells reached 80.00% confluency, the supernatant medium was replaced with Earle's medium (prepared from Razi vaccine and Sera research institute, Karaj, Iran), and 0.20 mL of FMDV type A with titer of $10^{6.5}$ median tissue culture infectious dose *per* mL was added to each flask.[16] After 10 hr, all flasks showed complete cytopathic effect and after three rounds of freeze and thaw, the released viruses were collected as a complete viral suspension in a volume of 1,000 mL. The suspension was centrifuged at 7,000 revolutions *per* minute (rpm) at 8.00 ˚C for 15 min and the supernatant was collected. The inactivation process was performed sequentially, initially with 0.02 M aziridine compound (binary ethylene amine), [17]obtained from Razi vaccine and Sera research institute, Iran), at 30.00 ˚C for 24 hr and then, with 1.50 mM formaldehyde (Merck, Darmstadt, Germany) on a stirrer at 4.00 ˚C overnight. After that, 0.40% chloroform was added. The resulting suspension was filtered through a 0.20 mm filter. In the next step, polyethylene glycol 6,000 (PEG 6,000) (Sigma-Aldrich) 8.50% w/v and 0.60 M NaCl were used to concentrate the inactivated virus. The mixture was shacked at 100 rpm at 4.00 ˚C for 5 hr to overnight. The resulting mixture was then centrifuged at 6,000 rpm at 8.00 ˚C for 15 min. After centrifugation, the supernatant was poured out. The pellet was re-suspended in tris/borate/ethylenediaminetetraacetic acid (EDTA) buffer (Sigma-Aldrich) containing 50.00 mM Tris, 10.00 mM EDTA, and 150mM NaCl based on a three-fold volume of the pellet. Elution was performed, and the suspension containing the virus was shacked at room temperature for 2 hr. Subsequently, suspension was centrifuged at 11,000 *g* at 8.00 ˚C for 40 min and the supernatant was collected.

Virus purification using the sucrose density gradient method. The obtained solution was filtered by 0.20 mm filter, and absorbance was measured at 254 nm using Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The obtained

number was multiplied by 126.7 to obtain the virus concentration *per* mL. Next, using the sucrose gradient method,[18] in a polycarbonate tube containing 2.00 mL of 20.00% sucrose (Sigma-Aldrich), ultra-centrifugation was performed using a L65B centrifuge (Beckman Brea, USA) at 30,000 rpm at 6.00 ˚C for 3 hr. Then, the supernatant was removed, and the pellet of each tube was re-suspended in 1.00 mL of phosphate-buffered saline (PBS) containing 0.50% sucrose (pH: 7.80). Then, absorbance was measured at 254 nm, and the virus concentration was obtained as μg mL^{-1}. In the control group, all steps were the same, except the inactivation which was performed only with binary ethylenimine (BEI), and the buffers used exclusively consisted of regular PBS.

Cultivation of pullets and immunization. A total of 12 Leghorn pullet breeders, aged 90 to 110 days, were purchased from Morghak Co. (Karaj, Iran), which had received all the common poultry vaccines. They were randomly divided into an experimental group and a control group, with six pullets in each group. The FMDV antigen A was prepared using Montanide ISA 71 VG adjuvant (Seppic, Paris, France) including 30.00% adjuvant and 70.00% antigen, and administered to the experimental group *via* intra-muscular and subcutaneous routes (0.50 mL). Booster doses were administered at weeks two, four, and six after the initial injection. Egg samples and sera were collected before the first injection, after the final injection, and four weeks after the final injection. The collected eggs were decontaminated with alcohol and stored at 4.00 ˚C until transferred to the laboratory.

Antibody concentration and purification. The yolk contents were carefully separated from the egg whites and collected in a specialized tube. The amount of yolk was measured and dissolved in PBS. The IgY was extracted using the PEG 6,000-ethanol method according to the Polson *et al.* method.[13] Then, Whatman No. 2 filters were used to filter the liquid. Ethanol was used to remove PEG 6,000 from the final extracted antibody. Dialysis was performed using a 12.00-kDa cellulose dialysis bag (Sigma-Aldrich Co., Germany) in PBS (pH: 7.20) overnight to remove residual salts. The extracted content was stored at –20.00 ˚C for future use. Finally, IgY was separated from the yolk and rabbit immunoglobulin G, and purified using DEAE Sepharose ion exchange chromatography (Sigma-Aldrich).[19] The OD of the fractions at a wavelength of 280 nm was measured. The separated immunoglobulin fractions were confirmed for the presence and quantitative and qualitative purities using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blot, dot blot, and double immune diffusion test.[20]

Checkerboard assay. To determine the concentrations of antigen and IgY antibody for designing the in-house ELISA kit, different dilutions of antigen and antibody were evaluated using the checkerboard assay.[20] The cut-off value in the present study was determined based on the agreement of some empirical considerations and the PI where the percentage of specificity and sensitivity reached the maximum value through receiver operating characteristic curve analysis.

Preparation of positive and negative reference sera. Bovine reference sera were obtained from immunized cows with type A and serum neutralization (SN) titers above 10^2 log using the commercial SPCE kit type A (Izsler, Brescia, Italy) at dilutions of 1/10 and 1/30, with a PI above 70.00% and OD below 0.25. Additionally, indirect ELISA showed an OD above 1.40. For preparing the negative reference serum, blood samples were taken from calves under three months of age (unvaccinated), and the SN titer of the sera was considered below $10^{0.9}$ log. Negative samples also had a PI below 50.00% in SPCE at a dilution of 1/10. Furthermore, the negative samples had OD below 0.20 in the indirect ELISA.

Solid phase competitive ELISA. The appropriate concentration of primary antibody (chicken) in a volume of 100 mL was added to the wells, and the plate was incubated at 4.00 ˚C overnight. Then, wells were washed by PBS three times with a volume of 200 mL *per* well. Next, the appropriate concentration of antibody was diluted in PBS containing Tween 20 (Sigma-Aldrich) in a volume of 100 mL and added to the wells. The plate was then incubated at 37.00 ˚C for 1 hr. After blocking step, washing was performed three times, the bovine serum diluted in diluent buffer containing PBS and 0.03% Tween (with a ratio of 1/50) was added to the wells, and incubation was carried out at 37.00 ˚C for 45 min without washing. After that, 100mL of type-specific FMDV serotype A monoclonal antibody conjugate was added to each well, and incubation was performed at 37.00 ˚C for 30 min. Subsequently, washing was carried out three times. Then, 100 mL of 3,3',5,5'-tetramethylbenzidine (prepared from Razi Vaccine and Sera Research Institute, Arak, Iran) substrate was added to the assay content, and incubation was performed for 20 min at dark room temperature. Afterwards, stop solution with a concentration of 0.60 normal H_2SO_4 was added to the wells of the kits. The results were read at a wavelength of 450 nm using a spectrophotometer (Bio-Rad, Hercules, USA). In the evaluated plate, four and two wells were used for the negative and positive control standard, respectively, and 80 serum samples from vaccinated cattle out of 240 samples were evaluated with titers of 1/10 and 1/30, as well as a negative control. These samples were evaluated with the available kit. Therefore, each sample was tested in three ELISA titers using both the commercial kit (Breccia Institute) and the in-house kit (prepared in Razi Vaccine and Sera Research Institute). Additionally, two wells were considered as blank, and the results were repeated three times. The OD was read at 450 nm and converted to the PI which is directly proportional to the antibody level in the test serum. In the commercial kit, a PI of 70.00% or higher

at a 1/10 dilution was considered as a positive antibody serum in the cut-off. The PI for reference and test sera was calculated according to the following formula:[21]

$$PI = 100 - \frac{OD_{450}\ test\ serum}{OD_{450}\ maximum\ (or\ reference\ OD)} \times 100$$

where, reference or maximum OD is the mean OD of four wells processed with negative control.

In the in-house kit, a PI equal or higher than 50.00% at a 1/10 dilution was considered positive, while values lower than this were considered negative. These criteria were used for screening serum samples. The cut-off at 50.00% PI has been shown to have a 99.60 - 100% specificity for non-serotype A SPCEs.[7] The cut-off value for SPCE is usually estimated based on the mean PI + 3 standard deviation (11). Furthermore, the second criterion (1/30) determining the level of antibody was considered positive when the PI (level of antibody) was equal or higher than 50.00%. Strongly, positive sera (80.00% ≤ PI) were observed in both dilutions of 1/30 and 1/10, and sera with 80.00% ≤ PI in the dilution of 1/10 but 50.00% ≥ PI in the dilution of 1/30 were considered weak positives.

Criteria for test validity. These criteria were based on negative control samples. In the spectrophotometer, the OD was ≤ 1.00% for commercial kits and ≤ 0.70 for in-house kits. In the commercial kit, the serum in positive control wells had a PI of 90.00% or higher, and for serotype A, a PI higher than 50.00% in the dilution of 1/30 and 1/10 was considered based on the kit protocol.

Repeatability test. Six serum samples were tested in three repetitions to evaluate the repeatability of the ELISA kit designed under optical parameters. Coefficient of variation (CV) of inter- and intra-assay was also calculated using the ELISA kit.

Statistical analyses. Microsoft Office Excel (version 16.0; Microsoft Corporation, Redmond, USA) and SPSS Software (version 16.0; SPSS Inc., Chicago, USA) were used for data analysis. After examining the results obtained from titration, appropriate concentrations and dilutions of coated IgY antibody, antigen, serum, and serotype-specific monoclonal antibody horseradish peroxidase-conjugated against FMDV type A were determined for the ELISA. The evaluation of the IgY capture kit for the diagnosis of FMD was performed using the ELISA on 80 selected positive and negative serum samples out of a total of 240 samples. Afterwards, the sensitivity, specificity, accuracy, and validity of the in-house SPCE with the specific IgY-attached killed FMD antigen type A were determined in comparison with the commercial kit (Izsler) for assessing herd titers after vaccination using computational and statistical methods. This commercial kit and the SN test were considered as a golden standard for comparative studies. The parameters examined for the in-house SPCE kit are listed in Table 1.

Results

Inactivation, concentration, and purification of the virus. The pellet of the antigen of serotype A with median tissue culture infectious dose of 10^6 *per* mL, was observed in a 20.00% sucrose gradient (Fig. 1). After reconstitution in 10.00 mL of PBS, the absorbance at 254 nm was 1.80. The virus concentration was determined to be 228 µg mL^{-1}, indicating acceptable and suitable recovery and purification. In the common method, the yield was 1.60 mg L^{-1}. Figure 1 shows the steps and the pellet obtained after ultra-centrifugation. The OD in the elution and separation stages from PEG 6,000 was 2.10, indicating the loss of some antigens in the virus purification process. Results of the safety test showed the absence of live virus 48 hr after deactivation in the viral samples. Inactivation with formalin along with BEI increases the thermostability of the virus antigen in the kit and on the other hand, protects the virus from structural changes and damage during the steps.

Concentration and purification of IgY antibodies by the PEG-ethanol followed ion exchange chromatography. Findings showed the purified IgY antibodies obtained from the PEG-ethanol and ion exchange chromatography methods were about 5.50 mg mL^{-1} from the yolk extraction of eggs, which ~ 0.40 mg mL^{-1} of those were specific polyclonal IgY antibodies against FMDV type A. The SDS-PAGE test was used and the presence of IgY was confirmed in different dilutions. The heavy chain of the IgY antibody was 65.00 kDa and the light chain was 27.00 kDa in the SDS-PAGE. 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.[20]

Table 1. Parameters examined for the in-house solid phase competitive enzyme-linked immunosorbent assay kit.

Parameters	Formula
Sensitivity	(False negative + true positive) / true positive × 100
Specificity	(False positive + true negative) / true negative × 100
Positive predictive	(False positive + true positive) / true positive × 100
Negative predictive	(False negative + true negative) / true negative × 100
Accuracy	Total /(true positive + true negative) × 100
Validity	(sensitivity + specificity) / 2.00 × 100
Concordance	(Number of positive results with two tests + number of negative results with two tests) / total × 100 Concordance = C/SA × 100 C is the total number of subjects in concordance. SA is the total number of subjects assessed.

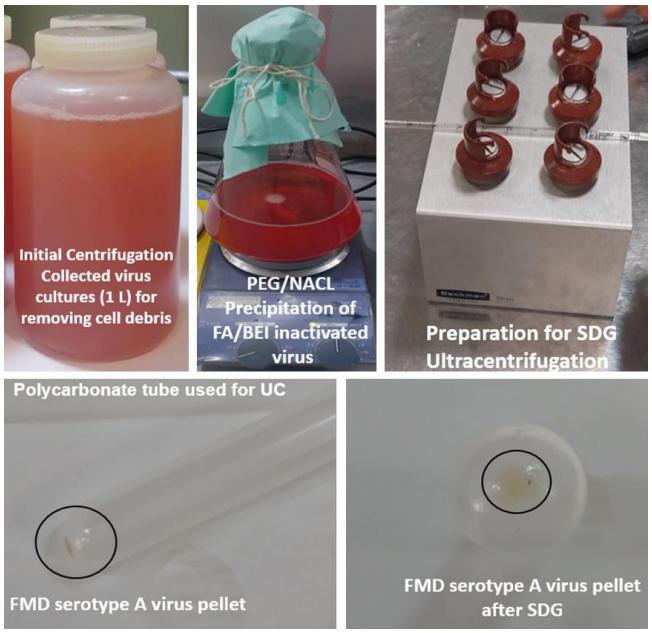

Fig. 1. Optimized concentration and purification steps for foot-and-mouth disease (FMD) virus serotype A. The cultivation of BHK-clone 21 intestinal cells and FMD virus serotype A, collection in 1-liter plastic bottles for centrifugation, precipitation with polyethylene glycol (PEG)/NACL, re-suspension and elution or separation of the virus from PEG with Tris/borate/EDTA buffer, centrifugation again to remove PEG sediment, subsequent deactivation of the virus with binary ethylenimine (BEI) and then formalin, chloroform addition, sucrose density gradient (SDG) with 20.00% sucrose in ultracentrifugation, removal of the supernatant and re-suspension in distribution buffer, spectrophotometry at 254 nm, and storage for subsequent use in the enzyme-linked immunosorbent assay kit.

Enzyme-linked immunosorbent assay. Based on the results of indirect ELISA, capture antibody ELISA, A-type antigen titration, and IgY antibody titration in the checkerboard and optimization results, the appropriate concentration of purified capture IgY antibody coating was determined to be 0.50 µg *per* well, and the optimal concentration for type A antigen binding was 0.10 µg *per* well.[20] Serum dilutions from vaccinated animals were also considered at 1/10 and 1/30. Out of all the available serum samples (240 samples) evaluated with commercial SPCE, 80 samples (with negative results of less than 70.00% PI at 1/10 dilution and less than 50.00% PI at 1/30 dilution, and positive with equal to or above 70.00% PI at 1/10 dilution and equal to or above 50.00% PI at 1/30 dilution, which had been previously tested for anti-A type antibodies using commercial ELISA kit) were selected. The OD values < 0.70 were considered positive, and values ≥ 0.70 were negative for in-house kit positive and negative controls as the test validity indicators. A sample of the results for high-titer samples compared to the indirect ELISA is shown in Figure 2. Samples reported as positive at 1/90 dilutions were considered strong serum titer samples.

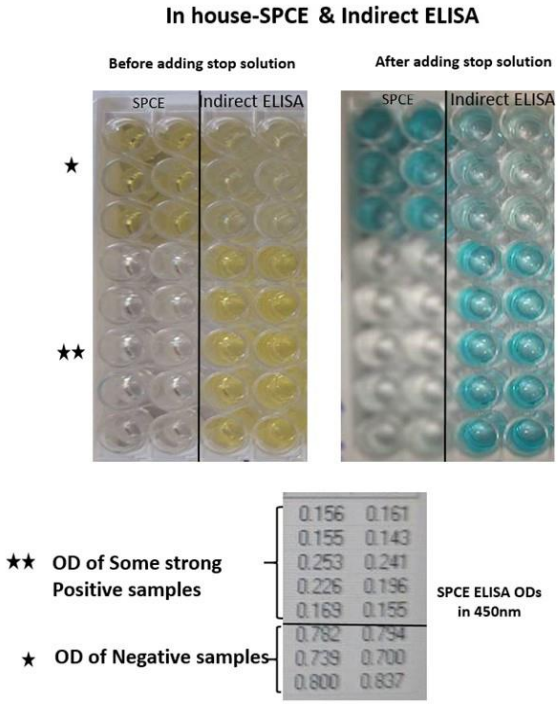

Fig. 2. Two columns of plates were used for samples with high serum titer (percentage of inhibition [PI] over 80.00%) at dilutions of 1/10 and 1/30 using the in-house solid phase competitive enzyme-linked immunosorbent assay (SPCE) kit. The same samples were also tested with the indirect enzyme-linked immunosorbent assay (ELISA) using negative control samples. Wells that did not show color in the SPCE were considered positive (left side of the images), and optical density (OD) values above 0.25 indicated high titers for these samples. As observed, negative samples showed a PI of less than 70.00% at a dilution of 1/10 (OD: approximately above 0.70) and exhibited color change. In the indirect ELISA, wells showed color (right side of the images) indicated positive samples, while those that did not show color were negative. The positive control (bovine reference serum) showed a PI of 95.00% at a dilution of 1/10 and 90.00% at a dilution of 1/30. Furthermore, OD values above 1.60 were reported for negative samples.

Also, the results showed similarities in positive cases between the commercial and in-house kits at medium and high titers at dilutions of 1/10 and 1/30, and differences in positive cases of the kits at dilution of 1/10; whereas, PI range was from 50.00 to 70.00%. In the in-house kit, true negative samples (using the commercial kit) were reported as negative at all dilutions, but 2 weak positive samples were reported as negative (Table 2). The commercial kit considered a cut-off of 70.00% PI at a dilution of 1/10, while the in-house kit considered 50.00% PI. Similarly, at a dilution of 1/30, the cut-off was 50.00% PI.

Serological tests comparison. Statistical analysis showed the 96.66% sensitivity, 100% specificity, 100% positive predictive value, 90.90% negative predictive value, 97.50% accuracy, and 98.33% reliability for serum samples for two commercial and in-house kits (Table 3).

Table 2. Comparison of overall test results based on the presence of specific immunoglobulin G antibodies in vaccinated cattle sera against foot-and-mouth disease using a commercial kit (Izsler) and the designed kit based on Capture IgY antibody and also native whole antigen.

Parameters	PET 1/10 (Commercial/In-house kit)	PET 1/30 (Commercial kit)	PET 1/90 (Commercial/In-house kit)	NET (Commercial /in-house kit)	Total
Positive test	20/18	20/20	20/20	20/20	-
Negative test	2	0	0	0	-
Total	20	20	20	20	80

PET: Positive standard test; NET: Negative standard test.

Table 3. Comparison of various statistical parameters for different enzyme-linked immunosorbent assays of the in-house kit compared to the commercial foot-and-mouth disease virus type A kit (Izsler) using 40 vaccinated cattle serum samples.

Statistical parameters	Results (%)
Sensitivity	96.66
Specificity	100
Positive predictive value	100
Negative predictive value	90.90
Accuracy	97.50
Validity	98.33

Comparison of coincidence rates. The designed kit showed a positive coincidence rate of 96.66% and a negative coincidence rate of 90.90% compared to the commercial kit. The overall coincidence rate was 97.50%.

Repeatability test. The CV for intra- and inter-assay repeatability tests was less than 6.00% and 10.00%, respectively, indicating high repeatability and low CV.

Discussion

Foot-and-mouth disease is a highly contagious, economically important viral disease. Control of the disease depends on vaccination, rapid diagnosis, strict care and transportation of animals, quarantine of infected animals, imposing restrictions on buying and selling animals, and sometimes exterminating millions of livestock.[22] The VNT is considered a gold standard method for detecting neutralizing antibodies against FMDV. However, VNT requires facilities with high limitations to handle live viruses and is not suitable for large-scale serological surveillance.[23] Another method is LPBE using polyclonal anti-sera produced in laboratory animals, such as rabbits and guinea pigs. This method has been recognized by the International Office of Epizootics as a suitable serological measurement for routine evaluation of serum titers after FMD vaccination.[24] It is showed that the results of LPBE (using SPCE) were consistent with VNT and LPBE, although VNT and LPBE could often detect antibody responses in some animals a few days earlier than SPCE for serotypes SAT 2, SAT 3, A, and C.[23,25]

However, there are several limits for widespread use of these assays due to the limited availability of polyclonal anti-sera because of the need to generate appropriate anti-sera for circulating strains, and also their low sensitivity.[23]

In this study, it was attempted to develop and evaluate the SPCE kit for serological surveillance of type A FMDV, focusing on screening and titration of antibodies after vaccination, assessing immunity against different serotypes, and investigating the import/export of livestock. In this way, chicken-derived purified polyclonal antibodies were used as capture antibodies to bind the killed FMDV antigen to the surface of a 96-well ELISA plate for subsequent competitive evaluation of herd immunity. Compared to the monoclonal antibodies, polyclonal antibodies demonstrated a stronger affinity by recognizing different epitopes on the viral capsid antigens of type A FMDV, providing higher sensitivity and better performance for the SPCE kit for FMDV diagnosis.

Various studies have reported the quantitative and qualitative diagnostic values, as well as the characteristics and sensitivity of SPCEs for different serotypes of FMDV.[25] The SPCE kit does not experience changes in OD or lack of response due to the genetic changes in type A SPC ELISA, and its sensitivity and accuracy remain unaffected compared to the other types of ELISA and SN tests because the conjugated monoclonal antibody targets conserved regions of FMD virus.[11,26] Additionally, the current kit can be used for sheep and goats, and also cattle, which is a disadvantage of indirect kits for FMD. Furthermore, the results indicate a very low background in the competitive well, where the conjugate does not bind to the antibody but specifically attaches to the virus. This suggests that if the virus is not properly purified, some serum antibodies against non-viral elements produced in animal vaccination can mistakenly bind to it, resulting in background noise caused by the conjugate binding to these antibodies.[26]

In the present study, the characteristics and sensitivity of the SPCE method for FMDV type A were about 99.04 and 100%, respectively. A significant correlation ($r = 0.9334$; $p < 0.0001$) was observed between SPCE and VNT titers, indicating that SPCE can identify neutralizing antibodies against FMDV type A and also can be used for evaluating protective immunity.[23] In a study conducted in Africa, sensitivities of 97.30% and 100% were reported for the developed SPCE in goat serum samples.[27] It has also been reported that SPCE has sensitivity and specificity of 100% and 99.00%, respectively, against sheep experimentally exposed to type O virus.[11] Furthermore, the development of SPCE kit based on virus-like particles (VLPs) has been highly successful.[28] Additionally, pure FMD-VLPs of type O were tested in different batches, and the CV for inter- and intra-assay was less than 7.00% and 14.00%, respectively, indicating high repeatability and low CV.[28] These results

were consistent with the present study, demonstrating more acceptable results. Şevik and Öztürk have compared different methods for assessing herd titers in Türkiye and showed that SPCE has better performance (100% for type A) compared to the LPBE (93.33% for type A).[29] They also reported that the sensitivity of LPBE (98.33% for type A) was almost equivalent to SPCE (98.89% for type A). It can be concluded that SPCE is more suitable as a screening test for the detection of antibodies against SPs of FMDV compared to the LPBE.[29] Ran *et al.* have indicated that the concordance rates of in-house SPCE kit based on VLP antigen with commercial kits (VDPro® FMDV Type O ELISA Kit and PrioCHECK® FMDV Type O Antibody Test Kit) were 97.80% and 98.20%, respectively. Repeatability values based on CVs within and between runs were 7.00% and 14.00%, respectively.[28] Furthermore, SPCE has shown high concordance rates (kappa = 0.925) compared to the VNT and LPBE for testing clinical serum samples and serial serological monitoring. Therefore, SPCE is an alternative method for the detection of antibodies after vaccination against type A FMDV with high sensitivity and specificity.[30]

The present research demonstrated the possibility of commercial development of the SPCE kit using IgY antibodies for the detection of FMDV antibodies in serum samples with adequate sensitivity and accuracy, promising commercial utilization and kit development for diagnostic and quality control purposes.

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Conflict of interest

The authors declare no competing interest.

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