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Original Article

Development of an Indirect Enzyme-linked Immunosorbent Assay to Detect Antibodies against Serotype A2013 of Foot and Mouth Disease Virus in Cattle

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ABSTRACT

Foot and mouth disease (FMD) is a contagious animal disease that causes irreparable damage to the economy of countries, including Iran in which this disease is a native one. Among the ways to combat FMD are vaccination and slaughter. Due to the specific situation of Iran, it is not possible to kill infected animals. Therefore, vaccination is the most important way to fight this disease. Serum neutralization test (SNT) and enzyme-linked immunosorbent assay (ELISA) are two main methods to evaluate the safety and calculate antibody titer. In this study, an indirect ELISA test was developed based on the coating of a complete viral particle (140s) which made it possible to determine antibody. In addition, serotype and viral type were determined without the need for time-consuming and complex molecular tasks, including gene expression. Moreover, in case of a new epidemic, a new epidemic condition can be detected using a serum antibody method. However, the coating of the complete viral particle leads to virus purification as well as the conjugated anti-immunoglobulin antibody testing of the same animal. In this study, the SNT was used as a gold standard test to determine the serum antibody level and compare its results with indirect ELISA method to determine the sensitivity and specificity of the indirect ELISA. To measure the anti-virus antibody rate of FMD (type A2013) through receiver operating characteristic analysis with 100% sensitivity and the specificity of 90%, the routine formulas were utilized using 100 % and 82%sensitivity and specificity, respectively. In this study, the cutoff value for the optical density was obtained at 0.3 and there was a significant difference between the vaccinated animals and the unvaccinated ones in terms of antibody level against the A2013 type. This indicates the correctness of the test and the accurate and proportional antibody detection against the understudy viral types of FMD.

Keywords: Antibody, Cattle, Cut off, Enzyme-linked immunosorbent assay, Foot and Mouth disease, Serum neutralization test

Mise au Point d'une Méthode Immuno-enzimatique Indirecte pour la Détection d'Anticorps Dirigés contre le Sérotype A2013 du Virus de la Fièvre Aphteuse chez les Bovins

Résumé: La fièvre aphteuse (FMD) est une maladie animale contagieuse qui cause des dommages économiques souvent irréparables dans de nombreux pays, y compris l'Iran, où cette maladie est endémique. La vaccination

et l'abattage comptent parmi les moyens de lutter contre la fièvre aphteuse. En raison de la situation spécifique de l'Iran, il n'est pas toujours possible de tuer les animaux infectés. La vaccination est donc le moyen le plus important de lutter contre cette maladie. Le test de neutralisation du sérum (SNT) et le dosage immuno-enzimatique (ELISA) sont les deux méthodes principales pour évaluer la sécurité et le titre en anticorps. cette étude, un test ELISA indirect a été développé, basé sur l'enrobage d'une particule virale complète (140), afin de déterminer la présence d'anticorps. En outre, le sérotype et le type viral ont été déterminés sans qu'il soit nécessaire de réaliser des analyses moléculaires longues et complexes, y compris l'expression des gènes. De plus, en cas de nouvelle épidémie, cette méthode sérologique peut contribuer à la détection rapide des animaux affectés. Cependant, l'enrobage de la particule virale complète conduit à la purification du virus ainsi qu'au test d'anticorps anti-immunoglobuline conjugué du même animal. Dans cette étude, le SNT a été utilisé comme test de référence pour déterminer le niveau d'anticorps sériques et ses résultats ont été comprés avec la méthode ELISA indirecte en termes de sensibilité et de spécificité. Pour mesurer le taux d'anticorps anti-virus de la fièvre aphteuse (type A2013) au moyen d'une analyse des caractéristiques de fonctionnement du récepteur avec une sensibilité de 100% et une spécificité de 90%, les formules de routine ont été utilisées en utilisant respectivement une sensibilité et une spécificité de 100%. Dans cette étude, la valeur seuil de la densité optique a été obtenue à 0,3 et il existait une différence significative du niveau d'anticorps contre le type A2013entre les animaux vaccinés et les animaux non vaccinés. Cela indique l'exactitude du test et la détection précise et proportionnelle des anticorps dirigés contre les types de virus de la fièvre aphteuse.

Mots-clés: Anticorps, Bovins, Coupé, Dosage Immuno-enzymatique, Fièvre aphteuse, Test de neutralisation sérique

INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious livestock disease which is one of the main barriers to the provision of the health services and production of livestock, as well as livestock products in a country. Almost all the cloven-hooved animals, including the ruminant species, are the target of this viral disease (Jamal and Belsham 2013). In Iran, this disease is caused by three dominant serotypes of FMD virus, including Asia 1, O, and A. Out of these serotypes, A and O strains have been prevalent for 50 years. Vaccination is a well-established strategy to combat FMD disease. Due to the specific situation in Iran, it is not possible to kill the infected animals; therefore, vaccination becomes the most important technique to fight this disease (Abdollahi and Meshkat 2004). In Iran, the ruminants currently receive vaccination which contains disease-causing microorganism and serotypes. Therefore, it is very important to evaluate the vaccination results as well as determine the immunity level of the vaccinated herd. The methods used to assess the safety and determine the antibody titer existing in the serum are mainly Serum Neutralization Test (SNT) and Enzyme-Linked Immunosorbent Assay (ELISA). In some cases, the diagnosis of the disease is not possible by tissue epithelium or lavender fluid due to the mildness or the aging of the disease. Accordingly, one way of FMD diagnosis is to exhibit the presence of specific antibodies of this disease (Jamal and Belsham 2013). The ELISA is the best and most practical method for the diagnosis of antibodies due to the high sensitivity and specificity of the obtained results accompanied with less time and cost (Yang et al., 2015). In addition, ELISA not only provides the possibility of screening and conducting extensive epidemiological studies (Abdollahi and Meshkat 2004) but also the possibility of determining serotype and subtype. The main objectives regarding the utilization of the ELISA method in this study include the proposal of a simple and fast method with high sensitivity, reliable and consistent results accompanied by a gold standard method and SNT. Moreover, this method is able to detect antibody detection and determine the serotypes and subtypes of viruses. It will further predict and continuously monitor the herd to prevent the development of an exotic virus (Oh et al., 2005).

MATERIAL AND METHODS

Development of foot-and-mouth disease virus, type A2013/IR. A 48-hour cultured monolayer of IBRS-2 cell was utilized to develop the virus. Subsequently, 2 ml of the virus was added with a titer of $10^{6.5}$ to each flask. Cytopathic effect (CPE) was completed after 18 hours, and the viruses were collected. After centrifugation (100g, 15 min), the viruses were initially clarified, the surface fluid layer was collected, and the sediment was removed. In the next step, the viruses were filtered through a 0.45-micron syringe filter for further clarification.

Virus titration. A 96-well microplate was employed to determine the virus titer. For each dilution, the viruses were titrated using the Reed-Muench statistical method.

Inactivation of foot and mouth disease virus antigen, type A_{2013}/IR. The FMD virus antigen was inactivated using the aziridine compound (ethylene amine) with a concentration of 0.01% for 24 h at a temperature of 30 °C.

Safety Test. Safety test was performed to ensure that no live viruses were present in the solution which contained the inactivated viruses using the monolayer of IBRS-2 cell for 3 passages. Complete virus inactivation was confirmed in case of no detected CPE in the sample.

Purification of the inactivated viruses using sucrose cushion. A 20% sucrose cushion was utilized to purify the inactivated FMD viruses and remove the cellular proteins and culture medium. Subsequently, 3 ml of 20% sucrose was prepared and poured into an ultracentrifuge tube (a polycarbonate tube resistant to high-speed centrifugation and pressure). Then, 8 ml of the viruses was gradually added to the tube (A2013). Afterward, the samples were centrifuged for 2 h at 30,000 rpm (150,000 g) using a Beckman L65B centrifuge (rotor SW40) at a temperature of 6 °C under negative pressure conditions.

Injection. In total, 5 ml of the A2013 antigen was subcutaneously injected into a calf at the top of the shoulder. Blood samples were taken for antibody titration 10 days after the booster injection.

Titration of antibody using the SN method. First of all, the serum samples were placed in a water bath at 56°C for 30 min in order to inactivate the complements. Following that, different serum dilutions were prepared in sterile conditions, including 1/8, 1/16, 1/32, 1/64, and 1/128, respectively. The samples were poured into 6 specific wells of the 96-well plate under a biological hood. First, 175 µl of the eagle's minimum essential medium was harvested and poured into the first well (1/8 serum dilution), and 100 µl of the culture medium was added to the next wells up to the sixth well. In the next step, 25 µl of the serum sample was added to the first well resulting in a 1/8 dilution followed by adding 100 µl of the 1/8 dilution to the next well which resulted in a 1/16 dilution. Subsequently, 100 µl of the second well was poured into the third and the same process continued up to the sixth well (i.e., a 1/256 dilution). In this test, 1000 TCID50 of the virus (A2013) was tested for each serum dilution. The plates were placed in an incubator at 37℃ for one hour and six dilutions as well as four repetitions were considered for each serum sample, and each dilution, respectively.

There was repetition for each sample with each well dilution to control the possible effect of serum toxicity in order not to add more virus if any effect was detected. In total, 100 units of virus needed to be placed for each well and each serum dilution. The plates were examined 48-72 h after incubation, and the CPE was found in all the wells using an inverted microscope. After the examination of the plates, the antibody titer was calculated using the Reed and Muench method. A serum titer or dilution that was able to prevent CPE in 50% of the wells would be chosen as the serum titer.

Indirect Enzyme-Linked Immunosorbent Assay. The ELISA was conducted to evaluate the level of FMD antibody titer (type A2013) in the cow to determine antibody titration and screen those samples that only contain sufficient antibodies against the FMD virus.

Determination of the appropriate amount of viral antigen for plate coating. Different dilutions of the virus were poured into all the wells using the checkerboard method in the numerical form from top to bottom (the virus was poured horizontally, and the serum was poured vertically) at 37 ℃ with shaking for 20 min, and then at the same temperature without shaking.

Washing the plate. The plate was washed using PBs (0.002 M) containing Tween-20 (0.05%). Afterward, 200 µl of the washing buffer was added to each well. In the next step, the plate was washed after a few second shaking and 5 min without shaking. This procedure was repeated 3 times.

Addition of the serums to the coated plate. Different serum dilutions (1/50-1/2800) were prepared using a diluent buffer (PBS 0.01 M, pH 7.2+5% skimmed milk+0.05% Tween 20) and poured into the specific wells. Table 1 summarizes the content of the wells.

Table 1. The wells in columns 11 and 12 were used blank in

	Column 11	Column 12
A	Coating +[1] , serum - , conjuge	Coating + , serum - , conjuge+
B	Coating - , serum + , conjuge+	Coating - , serum + , conjuge+
C	Coating + , serum + , conjuge-	Coating + , serum + , conjuge-
D	Coating + , serum + , conjuge+	Coating + , serum + , conjuge+

1: with coating, without serum, and with conjugated antibody

RESULTS

In the blank wells or control background, the included modes were as follows: A11, A12 (with coating, without serum, and with conjugated antibody), B11, B12 (without coating, with serum, and with conjugated antibody), C11, C12 (with coating, with serum, and without conjugated antibody, and D11, D12 (with coating, serum, and conjugated antibody). The sample was subject to shaking at 37 °C for 1 h after adding the serum. Afterward, 50 µl of the cow IgG antibody, which had been developed using rabbit and conjugated with a peroxidase enzyme, was added to all the wells with a dilution of 1/40,000 (according to the manufacturer instructions) after washing the plate. Then, it was shaken in the incubator at 37 °C for 1 h. Following that, 50 µl of the chromogen was added to all the wells after washing the plate. When the plate was placed at room temperature in the dark for 15 min, 50 µl of 1.25 molar sulfuric acid was added to all the wells to prevent the sample reaction. Afterward, the optical densities (ODs) were read at 450 nm using an ELISA read.

Determination of the sensitivity and specificity of Indirect Enzyme-Linked Immunosorbent Assay. In the present study, the SNT was considered as the criterion to determine true positive and negative results. The following methods were employed to determine the sensitivity and specificity of the indirect ELISA for measuring FMDV O2010 antibody in cows.

Sensitivity = a / a+c

= a (true positive) / a+c (true positive+false negative)

Specificity = d / b+d

= d (true negative) / b+d (true negative+false positive)

Receiver operating characteristic analysis. Receiver operating characteristic (ROC) analysis was calculated in SPSS software (Version. 22). The maximum OD was considered to be 100 in the positive control. Therefore, the rest of the samples, either positive or negative control, were examined with this OD. It is called a positive percentage. The positive samples containing antibody titer were already specified, plus the negative samples without antibody. All the specifications (positive or negative) need to be inserted when using SPSS. The sensitivity and specificity are determined in the form of a table through the options analysis in the menu bar and selecting the ROC curve.

Determination of the percent error or coefficient of variation. Coefficient of variation (CV %) is measured in two cases as follows:

- Intra-assay coefficient variability: When the samples existing in one plate are tested in duplicate, and the error is calculated within the sample repetitions in the plate.

CV%=Standard deviation (SD) of OD in duplicate sample/Mean of OD in duplicate sample *100

- Inter-assay coefficient variability: When the similar samples existing in more than one plate are tested at the same time. The error that is detected within the sample repetitions is calculated as follows:

CV %= SD of plate means /Means of plate means *100

Cut-off calculation. The cut off value was calculated based on SD and the mean of the negative samples. After determining the positive and negative samples and comparing them with SNTs, 60 negative samples were calculated in this study. The cut off value was obtained using the following formula:

Cut off = Mean +/_ 2SD

It means that serum with an OD bigger than the value of Mean+2SD is considered positive, and the one with an OD smaller than 2SD is negative. If the value obtained is not within the range mentioned, the test needs to be repeated.

Table 2. The amount of virus used in each well

Dilution of the virus in the coating	Virus µg/ml	virus µg/well
1/2	34/25	1/71
1/3	22/83	1/41
1/4	17/25	0/856
1/6	11/41	0/56
1/8	8/56	0/428
1/12	5/70	0/285
1/16	4/28	0/21
1/24	2/85	0/142

Stages of evaluation of stability and durability.

a. **Investigation of the effect of time on the stability of A13 serum samples.** The first step to confirm the readiness of a designed kit to follow the next steps is to obtain repeatable results. For this purpose, we have the same serum(Prepared from the Alborz Veterinary Organization) samples, 1 and 2 serum negative samples, and 3 and 4 serum positive samples. A13 serotype of FMD virus was treated with completely identical conditions for 12 weeks. Moreover, the positive and negative serum samples were treated weekly in two separate plates under completely identical conditions. Subsequently, the serum samples were collected and stored at -20 °C until the next week, it is worth noting that all experiments in this study were carried out only on a laboratory scale. After 12 weeks (approximately 100 days), the results of each plate were analyzed using Excel software to calculate the coefficient of variation.

b. **Identification of the effects of temperature on serum stability during storage.** In order to study the effects of temperature on the storage of serum samples(Prepared from the Alborz Veterinary Organization),

Table 3. Comparison of the antibody titration results using Serum Neutralization Test and indirect Enzyme-Linked Immunosorbent Assay regarding vaccinated animals

	SNT Titer	Indirect ELISA
No	A13	A13
1	1.85	1.109
2	1.55	1.104
3	1.75	0.985
4	2.25	0.963
5	1.96	1.026
6	1.45	0.864
7	2.25	1.115
8	2.05	0.938
9	1.55	0.749
10	1.85	0.824
11	1.55	0.681
12	1.75	0.759
13	2.25	0.911
14	1.85	0.658
15	1.65	0.518
16	1.95	0.483
17	1.85	0.721
18	1.65	0.491
19	1.95	0.792
20	1.65	0.837
21	1.95	0.641
22	1.75	0.67
23	1.65	0.836
24	1.85	0.925
25	1.65	0.738
26	1.95	0.916
27	1.65	0.818

c. Then, 2 flasks from each positive and negative serum samples were stored at two different temperatures (refrigerator temperature 2-8 °C, and environment temperature15-25 °C) and at 37 °C (incubator) as well as -50 ° C (freezer) in two periods of 24 and 48 h. After the storage time has elapsed, the relevant samples were evaluated for antibody response to FMD. The original substances of each flask (stored at -20 °C) was assessed through the same test and also under repeatability conditions aimed to reduce scattering of points in reference to time.

d. **Cross-reaction test.** This test was conducted to ensure that there was no cross-reaction occurring between FMD virus A13 and the antiserums A15 and O 2010, the Newborn Bovine Serum (NBS) collected from the calves in the slaughterhouse, NBS treated with polyethylene glycol (PEG) 6000, the normal horse serum, and the negative control serum available in the commercial kit LBPE. One µg of the inactivated, purified virus (A13) was coated in each well of the plate in the above-mentioned conditions.

DISCUSSION

Live virus titration. The titer of the virus A13 developed in the BHK cell was obtained at 105.8.

Safety test results. According to the results, no CPE was detected after performing 3 passages with the virus inactivated by ethylenediamine on the IBRS-2 (BA) cell. In addition, the developed A13 viruses were effectively inactivated and no live virus was found in the viral suspension.

Mass measurement of the inactivated virus (140S). The sucrose cushion method was employed for purification using ultracentrifuge. The measurement results were obtained using the formula A = Σ bc. The OD that was read after the purification at a wavelength of 280 nanometers is as follows: A280=0.531

The obtained values represent the amount of the concentrated virus (µg/µl).

$$A280=0.531 \quad A_{13-C}=\frac{A}{\sum b}=60.4/20=\ 3.02\ \mu g/ml$$

Table 4. Comparison of the antibody titration results using Serum Neutralization Test and indirect Enzyme-linked Immunosorbent Assay regarding the unvaccinated animal

	SNT Titer	Indirect ELISA
No	**A13**	**A13**
30	0.6	0.135
32	0.9	0.225
27	1.05	0.296
25	0.9	0.489
34	1.25	0.325
35	0.9	0.209
21	0.9	0.186
23	0.6	0.196
28	0.6	0.236
29	0.6	0.266
22	0.9	0.373
24	0.6	0.56
27	0.9	0.285
33	0.9	0.199
31	0.6	0.157
26	0.9	0.22
32	0.9	0.256
34	1.05	0.396
25	0.6	0.265
SVC[1]	0.9	0.201
NBS[2]	1.55	1.187
C-kit[3]	0.6	0.141

[1] Serum vaccinated cattle
[1] Normal bovine serum
[1] Kit negative control

Serum titration results using serum neutralization test. The SNT was considered a gold standard test in the present study. The test samples included positive control samples developed through the injection of viral antigen to the calf, positive or vaccinated samples containing an antibody protective titer, and negative or unvaccinated samples as the negative control containing no antibody protective titer.

Antibody protective titer against the A13 positive control serum. The cow antiserum protective effect against the A13 type virus was titrated using SNT and Reed and Muench method. The obtained titer was 2.18 and the equivalent dilution of which was 1/151.

Titration of negative cattle serums based on the SN method. The titer of all the negative (20) serums that were tested using the SNT was smaller than or equal to 0.9 indicating that there was no antibody amount that could be considered protective. The obtained result is consistent with the findings of indirect ELISA.

The most appropriate amount of virus for coating. Table 2 illustrates the utilization of different virus dilutions to determine the best coating level in the test. The ELISA plates were coated with different dilution. Selected amounts for coating the ELISA plate wells are listed in the table below. In addition, Figure 1 depicts the amount of the 140S particles of FMDV type A_{13} in each ml of the purified virus solution. The results obtained in columns (Table 1) 11 and 12 of the plate were monitored as the controls. In case of the lack of any of the three factors, including coating, serum, and conjugated antibody, the OD was low. However, when all three elements were present, OD levels were high, indicating the binding through conjugated antibody.

Table 5. a. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the means of two consecutive ordered observed test values.

Coordinates of the Curve

Test Result Variable(s):	Percent positive	
Positive if greater than or equal to [a]	Sensitivity	Specificity (-1)
6.4377	1.000	1.000
7.8891	1.000	.900
8.6629	1.000	.800
9.8667	1.000	.700
11.7799	1.000	.600
14.0903	1.000	.500
16.3904	1.000	.400
17.4463	1.000	.300
17.5795	1.000	.200
18.1040	1.000	.100
19.1960	1.000	.000
38.9710	.900	.000
65.2496	.800	.000
72.7369	.700	.000
73.9425	.600	.000
78.9129	.500	.000
83.1218	.400	.000
85.2157	.300	.000
90.3765	.200	.000
94.7124	.100	.000
96.8545	.000	.000

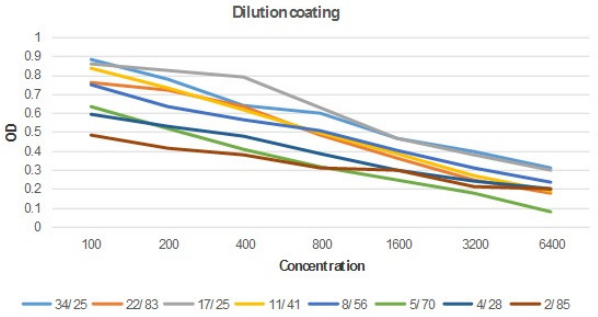


Figure 1. The illustration of different coating levels for different virus dilutions containing sufficient antibody. According to the Figure, the best concentration level of the purified virus is 17-23 µg/ml or 0.8-1.14 in each well to coat the bottom of the plate for conducting the indirect ELISA.

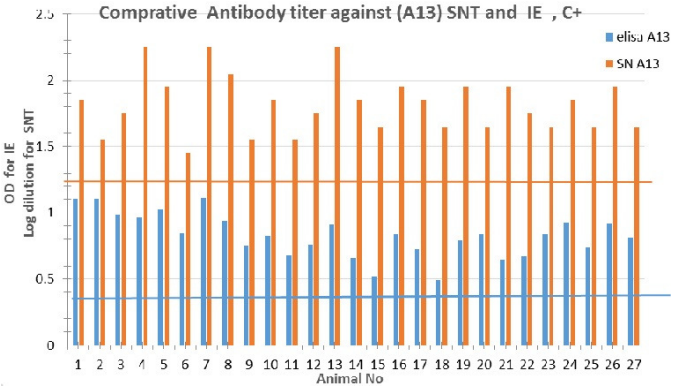


Figure 2. Comparison of the Serum Neutralization Test with Enzyme-Linked Immunosorbent Assay antibody titer against A13 in vaccinated animals.

Comparison of the antibody titration results using Serum Neutralization Test and Enzyme-Linked Immunosorbent Assay. Tables 3 and 4 show the vaccinated and unvaccinated animals.

Cut-off calculation. According to the SNT results, there were no antibodies in the negative cow serum samples. The amount of cut off that is necessary for A13 antiserum titration was obtained at 0.20-0.24 OD. In the test, the cutoff value was considered 0.3. If the ODs was bigger than this value, it indicated protective

antibodies; otherwise, the smaller ODs represented non-protective antibodies.

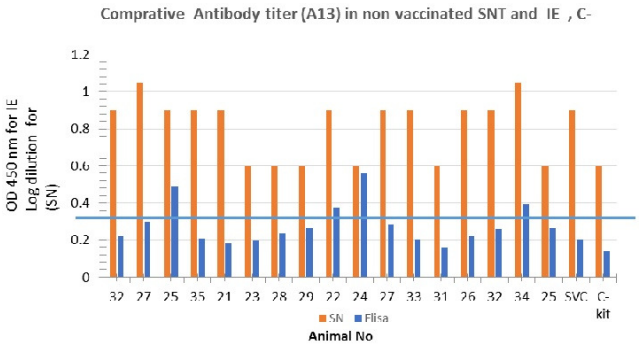


Figure 3. Comparison of the Serum Neutralization Test with Enzyme-Linked Immunosorbent Assay antibody titer against A13 in unvaccinated animals.

In this case, the protective antibody in the indirect ELISA was titrated according to the comparison with the SNT with a dilution of 1/800. In other words, a serum titer of equal to or bigger than 1/800 in the indirect ELISA indicated the minimum protective effect of the antibody, and the smaller values indicated an ineffective antibody titer. The value was considered 1/16 in the SNT.

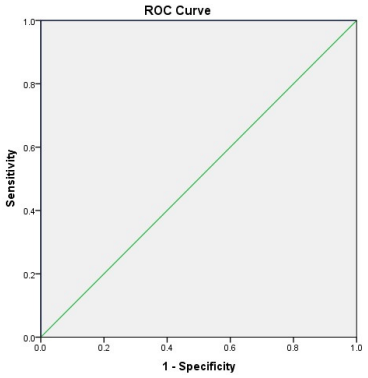
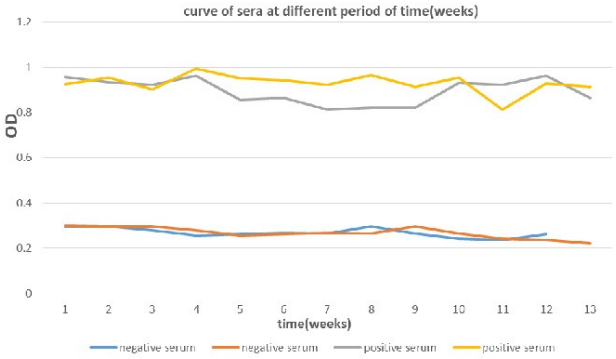


Figure 4. Sensitivity and specificity of the indirect Enzyme-Linked Immunosorbent Assay with Roc curve.

Figure 2 illustrates the antibody titer tested using the SNT method in comparison with the indirect ELISA test for both vaccinated and unvaccinated animals. In the figure, the blue horizontal line represents the obtained cut off value of OD using indirect ELISA (in a level of 0.3), the red line represents the obtained cut off value of OD using SNT (with a dilution logarithm of 1/2). The lines indicate the amount of antibody in the serum. According to Figure .2, there is a similar level of protective antibody titer in both tests.

This means that ODs of indirect ELISA was bigger than 0.3 when the SNT antibody was within the acceptable protective range in the case of vaccinated animals (i.e., a dilution of 1/2). The OD read in an indirect ELISA test was smaller than 0.3. However, according to Figure .3, the amount of antibody titers in non-vaccinated animals or negative controls is slightly different. Consequently, positive results in the direct ELISA (False positives) were present in 4 out of 19 tested sera which were negative in the SNT (positive-negative). Moreover, the results showed an OD of over 0.3, which affects the extent of the test feature leading to reach 82%.



The Sensitivity and specificity of the indirect Enzyme-Linked Immunosorbent Assay

Method 1: According to the formula mentioned in section 2 for calculating sensitivity and specificity and given the fact that the SNT was regarded as a gold standard test, the sensitivity and specificity were obtained as follows:

The number of true positive cases based on the SNT results (in the case of A13 virus): 21

The number of true negative cases based on the SNT results (in the case of A13 virus): 19

The number of false negative cases based on IE results (in the case of A13 virus): 0

The number of false positive cases based on IE results (in the case of A13 virus): 4

Method 2: In this method, the sensitivity and specificity were obtained at 100% and 90%, respectively (Figure 3 and Table 5).

Table 6. Raw data related to optical densities/ coefficient of variation (%) results by analyzing sera lots at different periods of time (weeks) for the evaluation of the 3-months stability

Time(weeks)	Sera[1]	1	2	3	4
	Plate 1	0.301	0.366	0.956	0.925
0	Plate 2	0.298	0.351	0.933	0.955
	Plate 1	0.296	0.355	0.922	0.903
1	Plate 2	0.28	0.352	0.963	0.996
	Plate 1	0.256	0.299	0.856	0.952
2	Plate 2	0.263	0.296	0.865	0.942
	Plate 1	0.269	0.282	0.812	0.923
3	Plate 2	0.266	0.274	0.823	0.966
	Plate 1	0.296	0.252	0.823	0.915
4	Plate 2	0.266	0.248	0.932	0.953
	Plate 1	0.242	0.286	0.922	0.812
5	Plate 2	0.236	0.289	0.963	0.927
	Plate 1	0.263	0.269	0.912	0.904
6	Plate 2	0.222	0.259	0.921	0.93
	Plate 1	0.226	0.288	0.955	0.966
7	Plate 2	0.236	0.269	0.912	0.985
	Plate 1	0.239	0.286	0.963	0.908
8	Plate 2	0.355	0.266	0.891	0.945
	Plate 1	0.359	0.263	0.855	0.963
9	Plate 2	0.299	0.252	0.905	0.923
	Plate 1	0.293	0.212	0.912	0.912
10	Plate 2	0.215	0.358	0.93	0.932
	Plate 2	0.218	0.3	0.955	0.962
11	Plate 1	0.286	0.296	0.922	0.933
	Plate 2	0.273	0.258	0.937	0.966
12	Plate 1	0.288	0.269	0.952	0.925
Mean		0.270808	0.290654	0.916423	0.927577
Variation coefficient (%)		13.74503	14.57772	4.762848	4.37100

1 and 2 serum negative samples and 3 and 4 serum positive samples

Percent error calculation. Percent error was calculated in two ways as follows: Firstly, it was calculated for a plate and samples in duplicate fashion, and the result was called intra-assay error. In this study, the intra-assay error was obtained at 6.65%, which is an acceptable error since it was smaller than 10%. Similarly, the intra-assay error of 3.25% was also an acceptable rate of error. Another percent error was called inter-assay error which was calculated regarding the plates containing similar samples. According to the results in this study, the inter-assay error was obtained at 3.6% and 6.11% for plates with similar negative samples (5 plates) and positive samples, respectively. An acceptable inter-assay error is smaller than 15%.

Stages of evaluation of stability and durability:

a. **The effect of time on the stability of A13 serum samples.** Figure 5 and Table 6 illustrate the results of the repeatability and stability of the durability of the used sera over a period of twelve weeks. The CV% (coefficient of variation) of less than 15% can be indicative of the proper repeatability of the kit. It should be noted that Figure 5 also reveals the gap between the two positive and negative sera samples and their separation from each other which is clearly visible on the diagram.

b. **Identification of the effects of temperature on serum stability during storage**. Table 7 shows the results of stored serum samples at different temperatures and reference temperatures (stored at -20 °C), which were examined under the same conditions. The data indicate that there is no significant difference between the results of storage at different temperatures. Moreover, the distribution is the same and the used serums are appropriately stable.

c. **Cross-reaction test.** The results of the cross-sectional test indicate that the A13 coated plate is high and A15 is relatively high since it has the same type as the A13; however, it has a different subtype. This can be differentiated heterologous and homologous virus. The high level of anti-serum in the cattle draws our attention because it comes from the slaughterhouse and showed a high antiserum level when it was untreated. However, the normal antisera of the cow with Polyethylene Glycol is due to the antibody sedimentation of the lower header and this method can be a way of controlling PEG, which is used to cope with the virus. This study was conducted on an experimental scale to give the students this opportunity to utilize it in their thesis. However, it takes several years for a kit to be employed commercially since it requires the examinations in terms of stability and durability. In this study, the SNT test was used as a gold standard test to determine the rate of antibody level in serum and it was

compared with an indirect ELISA method (Malekdar and Mahravani, 2017). The SNT method provides the possibility of comparing the antibody's protection rate by the titer of serum. Many experts, including Jin ski oh et al. emphasized the utilization of this method and compared SNT with ELISA methods in one of their studies (Oh et al., 2005). According to the results obtained from the abovementioned study, SNT and ELISA were compatible with each other and there were no significant differences between SNT and ELISA. In the same line, other studies have utilized indirect ELISA. For instance, Jitendra employed this method to detect antibodies against the 2B protein of FMD disease for differential diagnosis of vaccine-derived antibodies or antibodies derived from the disease (Jitendra et al., 2013). In the present study, it was the first time that an indirect ELISA was utilized to measure the antibody against the structural proteins of the A13 FMD virus. Moreover, the pure and inactive full virus was used in this regard (Malekdar and Mahravani, 2017). In this study, due to the need to coat the full virus, the FMD virus was made pure to facilitate its separation from other cellular proteins and culture media. Furthermore, in a study conducted by Shojaee and Zibaei (2017), the virus was purified by a sucrose cushion. Due to the heavier bone virus, the cellular proteins and the resulting viscosity medium passed through a concentration of 20% sucrose and precipitated at the bottom of the tube. Bachrach et al. (1975) employed this method to purify fever virus. By coating complete viral particle, there is no need to take any necessary molecular action, such as gene expression, and producing a specific protein. Accordingly, the test speed becomes much higher and in the event of the occurrence of a new epidemic, it is possible that by using complete viral particle, new epidemic status and the new viruses emerging from the antibody test can be quickly determined (Emam, 1996). Some limitations, including the generated virus, require purification. The anti-immunoglobulin conjugate antibody of the same animal is needed for all animals. The buffer used for coating was bicarbonate-carbonate buffer with amino acid pH= 9.2, and its neutral protein was negatively charged. Because of the positive charge of the plate, the Maxisorp can easily attach to it and establish a solid bond (Dagenais et al., 1994). The amount of coated virus by considering the specificity of Maxisorp Plate was 1600 to 650 ng immunoglobulin G per Cm^2, and the area of each well was 0.3 cm in a square (Fabrizio and Marradi, 2013). The IgG coating capacity in each well was between 180 and 195 ng. This study determined the amount of 1 microgram per well or 20 micrograms per ml. Due to the limited capacity of coating in each well, a large amount of these viruses will be washed out from the well. The sensitivity and the attribute value were obtained at 100% and 82%, respectively, using the usual formulas that are mentioned in the procedure (Malekdar and Mahravani, 2017). This means that all the samples that were positive in the SNT have antibody titer at a protective level and were diagnosed positive based on an indirect ELISA. According to Figures 3 and 4, the samples of sera were negative in the SNT (real negative) and showed positive results in the indirect ELISA (false positive). Moreover, 18% of errors were detected in the negative samples and the specificity decreased to 82%. In the second method, which was calculated by Rock analysis in SPSS software (Tekleghiorghis and Weerdmeester 2014), the sensitivity was 100% and the attribute value was calculated as 90%. In similar studies conducted by Jitendra, 6% of samples from the regions where the disease was not observed had antibody titers that were considered as false positive with a sensitivity and specificity of 95.3% and 94.3%, respectively. In addition, the CV% error was also within an acceptable range (Jitendra et al., 2013). Ming Yang utilized the competitive ELISA to detect anti-antibody ant protein B3 of the FMD virus. The measured sensitivity in the abovementioned study was obtained at 99.4% and the attribute value was 96.4%. Based on the results of this study, it is difficult to detect non-structural proteins in the vaccinated population (Ming et al., 2015). In this study, a significant difference was observed between

vaccinated and unvaccinated animals regarding OD cut off=0.3. In terms of the antibody titer rate, the last dilution where titer was equal or higher than 0.3, was calculated at 1.008 dilutions. This means that the serum titer is equal to or greater than 1/8 in the direct ELISA indicating the presence of a minimum and appropriate antibody level (Selim, Abouzeid et al. 2010). The amount below this dilution indicates the absence of an appropriate antibody titer. This rate was obtained at 1.16. In SNT that indicates the correctness of the test and the accurate and appropriate diagnosis of antibodies against the types of FMD virus. According

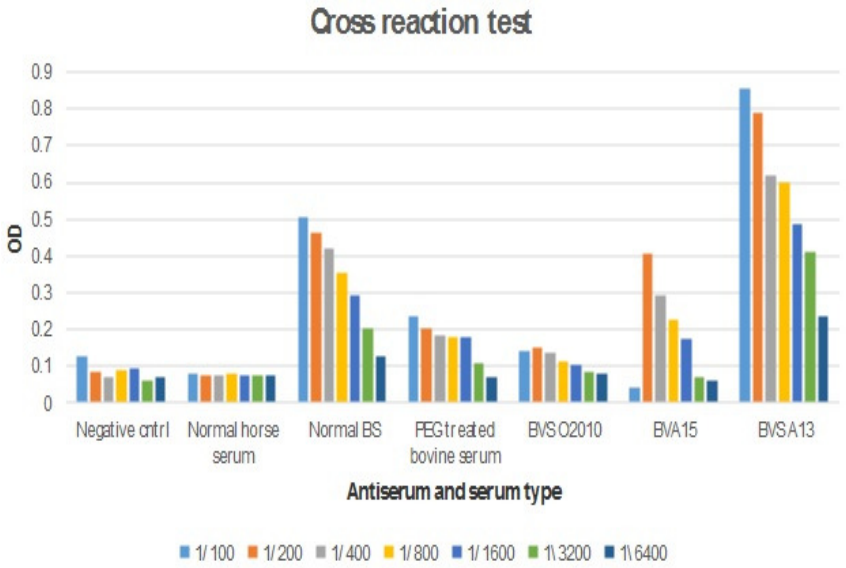


Figure 6. The results of the optical density read at a wavelength of 450 nm in different wells

Table 7. Results expressed by optical density ratio in Enzyme-Linked Immunosorbent Assay by calculating the means of the sample values detected in the reference sera and the test samples

Sera[1]	Refrigerator (2-8 °C) 24 hours	Refrigerator (2-8 °C) 48 hours	Incubator (37 °C) 24 hours	Incubator (37 °C) 48 hours	Environment (15 °C-25 °C) 24 hours	Environment (15 °C-25 °C) 48 hours	Freezer (-50 °C) 24 hours	Freezer (-50 °C) 48 hours	Reference sera (-20 °C)
1(flask1)	0.302	0.266	0.479*	0.24	0.327	0.298	0.333	0.298	
1(flask2)	0.302	0.266	0.29	0.253	0.333	0.285	0.347	0.284	0.308
2(flask 1)	0.28	0.26	0.298	0.215	0.325	0.258	0.325	0.296	
2(flask 2)	0.284	0.264	0.226	0.283	0.236	0.552*	0.325	0.256	0.265
3(flask 1)	0.953	0.947	0.951	0.933	0.952	0.963	0.632*	0.942	
3(flask 2)	0.95	0.94	0.963	0.856	0.943	0.963	0.963	0.963	0.908
4(flask 1)	0.999	0.995	0.932	0.966	0.962	0.961	0.958	0.968	
4(flask 2)	0.986	0.982	0.966	0.963	0.985	0.982	0.963	0.984	0.982

*Outliers detected in one of the triplicate reactions with 99% confidence.

Table 8. Cross-reaction test results for different serums and antiserum

Serum or antiserum name	Plate Row	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
BVS A13	A1 - A7	0.856	0.789	0.62	0.602	0.489	0.412	0.234
BVS A15	B1 - B7	0.443	0.407	0.293	0.228	0.174	0.071	0.063
BVS O2010	C1 - C7	0.161	0.137	0.125	0.116	0.085	0.072	0.051
PEG treated bovine serum	E1 - E7	0.237	0.201	0.186	0.178	0.178	0.107	0.068
Normal BS	F1 - F7	0.506	0.462	0.419	0.353	0.295	0.201	0.127
Normal horse serum	G1 - G7	0.08	0.077	0.075	0.078	0.076	0.077	0.075
Negative control	H1 - H7	0.128	0.085	0.071	0.089	0.092	0.063	0.068

to the results, serums containing appropriate antibody in this SNT and indirect ELISA were compatible with each other. In order to express the accuracy and/or the ability to repeat the immunoassay test results in sciences and social sciences and its behavior (Mannonen et al., 2006), two types of error measurements have been proposed as follows:

1. Intra-assay Coefficient Variability (CV)
2. Inter-assay Coefficient Variability (CV)

A CV is a dimensionless number, which is a standard deviation for measuring what is distributed in the mediums (Schultheiss et al., 2009). The method of calculating each error is explained. According to Mannonen et al. (2006), the error rates for the intra-assay and inter-assay are up to 10% and 15%, respectively (Mannonen et al., 2000). In the test designed in this study, the intra- and inter-assay error rates are 6.65% and 3.6%, respectively, and both are within the allowed range. In the event that these errors are higher than the limit, the piping is considered to be inappropriate and non-uniform (Hemmings, 2009). The measurement of antibodies against structural proteins by indirect ELISA method, with less time and cost, as well as the higher speed with good sensitivity and specificity will be proper. Indirect ELISA test using full antigen of FMD virus in determining the antibody titer with the SNT is comparably replaceable and its results are reliable.

Given that among the studies presented by the case-investigators, none actually talked about the use of a direct coating of complete virus particle for measuring antibody against FMD virus. However, this study is the first to address this issue which is considered as the unique point of this research (Jitendra et al., 2013).

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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