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Original Article

Development of an ELISA Test for Detection of Anti FMD Virus Type A87/IRN Antibody Using Irradiated Antigen

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Abstract

Background and Aims: The aim of this study was application of gamma irradiated FMDV antigen for liquid phase blocking sandwich ELISA (B-ELISA) to quantify guinea pig anti FMDV type A87/IRN antibody.

Methods: FMDV type A87/IRN was propagated to a titer of $10^{7.5}$/ml $TCID_{50}$ and then irradiated by gamma irradiator. The irradiated 146 S antigen was purified by sucrose gradient centrifugation after inactivation, and used for inoculation of rabbits and guinea pigs. The anti-FMDV sera were collected from the animals. ELISA test was optimized and the titer of antibodies was compared with that of virus neutralization test (VNT) to determine the correlation between these techniques.

Results: The results of VNT and B-ELISA did not show significant differences ($P>0.05$). The sensitivity and specificity of B-ELISA in comparison to VNT for detection of anti FMDV type A 87/IRN antibody were calculated to be %94.73 and %90.9 respectively.

Conclusion: The ELISA titers in sera collected from animals were higher than those recorded by VNT. These results suggested that, in addition to neutralizing antibodies, the ELISA was measuring other classes of antibodies which did not neutralize FMDV in vitro. Also comparison of the VNT and B- ELISA results by t-test showed that the gamma irradiated inactivated FMDV antigen has unaltered antigenicity.

Keywords: Foot and Mouth Disease Virus; ELISA; antibody; sera

Introduction

Foot and Mouth Disease (FMD) is a highly contagious, vesicular disease of cloven- hoofed animals. It is an economically important disease of domestic livestock which seriously, affects international trade of live animals, and animal products (1).

The intact FMD virions are 146S virus particles, comprising one molecule of ssRNA and 60 copies of each of four polypeptides VP_1, VP_2, VP_3 and VP_4.

The Virus Neutralization Test (VNT) has been routinely used to measure serum antibodies against FMD virus and is known to be a sensitive, and specific test (2). However, a liquid phase blocking sandwich ELISA (B-ELISA) has been documented to be equally sensitive and more rapid and reproducible than the VNT for detection of FMD antibodies (2). The aim of this study was to develop and apply the B-ELISA for detection of antibodies against FMD virus type A87/IRN in serum samples collected from vaccinated and non

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vaccinated guinea pigs and compare of the results as the two tests.

Methods

Virus multiplication and virus titration

FMD virus typeA87/IRN was propagated in BHK21 cell suspension culture (Razi of Vaccine and Serum Reseach Institute, Karaj, Iran). Infected cells were harvested after 36 hr, and clarified by centrifugation (Hettich-32R, Germany) at 1000×g for 15 minutes (2, 3), and the virus titer was determined by the TCID50 method (4).

FMDV Inactivation by gamma irradiation

In this study, a gamma cell instrument (Issledovapel, PX-30 model, Poland) with a dose rate of 0.551 Gy/sec and activity: 3652 Ci was used. Different doses of gamma ray: 10, 20, 25.35,40,45 and 50 kGy were used for the irradiation of virus samples, duration of irradiation was 1.50 h, 3.06 h, 3.82 h, 5.35 h, 6.12 h, 6.87 h and 7.65 h, respectively. For each dose of gamma ray, 10 vials (each vial containing 5 ml of the virus) in frozen state were irradiated.

Safety test and complement fixation test

Infectivity of irradiated virus samples by different doses of gamma ray was determined by cell culture methods. All of the irradiated virus samples were inoculated on IBRS2 cells and the viral titers were obtained by $TCID_{50}$ methods. The antigenecity of irradiated and unirradiated virus samples were determined by complement fixation test (CFT) (5).

Antigen Concentration

Irradiated and Inactivated FMDV 146S antigen was concentrated by saturated ammonium sulphate solution followed by sucrose gradient at 100000×g for 4 hours at 4 ºC. The peak of 146S antigen was collected and read at 259 nm (spectrophotometer- Jenway 1605, England), then the concentration of 146S antigen was calculated (1OD= 132 µg/ml using 1 cm^2 cuvette) (6).

Animals

Thirty two guinea pigs and two rabbits were provided by the Razi Vaccine and Serum Research Institute. Two of guinea pigs and two of rabbits were used to prepare rabbit and guinea pig anti-FMDV sera. Thirty guinea pigs weighing approximately 250 g each were divided into three groups of ten each, two groups were vaccinated by FMDV type A87/IRN inactivated vaccines by Binary Ethylene Imine (BEI) and gamma irradiation, the third group were kept un-vaccinated as negative control group.

Rabbit and guinea pig anti-FMDV sera

Type-specific antiserum was prepared in rabbits by inoculation of 15 µg of irradiated and inactivated FMDV 146S antigen and Ferunds complete adjuvant in ratio of 1:1 subcutaneously (SC). After 28 days, 15 µg the 146S antigen and Ferunds incomplete adjuvant in ratio of 1:1 were inoculated subcutaneously as booster dose (2, 7). Guinea pig anti FMDV type A87/IRN antiserum was prepared after 2 subcutaneous inoculations; the first inoculation of 10 µg of irradiated and inactivated FMDV 146S antigen and Ferunds complete adjuvant in ratio 1:1 SC and the second inoculation was given after 28 days with 5 µg the 146S antigen incomplete freund's adjuvant. The animals were killed and exsanguinated 12 days after the second inoculation. The sera were aliquoted, inactivated at 56 °C for 30 minutes, and kept at -20 °C (2, 6, 8).

Virus Neutralization Test (VNT)

Neutralization assay was performed as previously described, as the gold standard test (2, 9, 10). Briefly, the sera were diluted in Eagle's maintenance medium (Difco) in 2–fold dilutions, from 1:4 to 1:128. The mixtures of equal volumes of the different sera dilutions and 100 TCID50 FMDV were prepared and incubated at 37 ºC for 1 h. The mixtures were added to the monolayer of BHK21 cells and were grown in flat bottom-96 well microtiter-plates and incubated at 37 ºC for three days in a humidified atmosphere containing 5 % CO2. Antibody titers were expressed as the logarithm of the reciprocal of the final dilution of serum in the virus/serum mixture that neutralized an estimated 100 TCID50 at the 50% endpoint (9, 10).

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B- ELISA

The optimum dilution of the rabbit and guinea pig anti FMDV type A87/IRN anti-sera, were determined by using B- ELISA with chessboard titration. The dilutions of capture rabbit antiserum were tested against dilutions of inactivated viral antigen and guinea pig antiserum to identify the optimum concentration of each reagent for the best discrimination between the positive and negative reference sera. The test was performed as described with some modifications (1). Wells of a flat-bottom 96 well microtiter-plates (F-plate) were coated with 50 ul guinea pig anti-FMD serum diluted (1:16) in carbonate/bicarbonate buffer (pH 9.6, 0.05 M) overnight at 4 °C. After 5 times washing with phosphate-buffered saline solution containing 0.05% Tween-20 (PBSST, 0.01 M, pH 7.4), 50 µl PBSST containing 10% skim milk was added to each well of the F-plate and incubated for 1 hour at 37 °C, washing was repeated again for 3-5 times. Then the F-plate was ready to use. Fifty ul of each test and control (C+ and C-) sera with dilutions of 1:4 till 1:256 were added to the wells of the polypropylene U-bottom micro plate (U-plate) and mixed with 50 µl of a 1/8 of antigen in PBSST- 0.01 M, pH 7.4. The U-plate was incubated at 4 °C overnight. The serum-virus mixtures were transferred to the coated F-plate as duplicate and incubated at 37°C for 1 hour. Then the F-plate was washed as described previously. Fifty micro liters of Rabbit anti-FMDV antiserum as detecting antibody which was diluted in PBSSTM (1:16), was then added to each well of the F-plate, then incubated at 37 °C for 1 hour. An optimal dilution (1:500) of Goat anti-rabbit IgG (Sigma, A 6154) conjugated to horse-radish peroxides in PBSSTM was added, and incubated at 37°C for 1 hour and washed. The final substrate/chromogen solution (4.4 mMH2O2, 3.3 mM OPD and 0.1 M citric acid buffer, pH 5.0) was added. After 15 minutes incubation at room temperature, 1.25 M sulfuric acid was added to stop the enzymatic reaction, and OD of the plate was read at 492 nm by an ELISA reader. In the F-plate, 4 wells were used as blanks and another 4 wells were used for antigen control (CAg), without test sera (10).

Results

FMDV inactivation

According to the Dose/Survival curve for irradiated viral sample, with the equation of Y=-0.17X+7.13, the optimum dose of irradiation to inactivate FMD virus was calculated to be 40-44 kGy. The inactivation methods of *FMD Virus* are: 1) Inactivation by formaldehyde 2) Inactivation by aziridines such as acetylethylenimine, ethylenimine and propylenimine. All of them have some residues in the final products, also some of them are toxic and some of them make allergic responses in the animals and it is possible some viruses to escape during the chemical inactivation process. But, gamma irradiated inactivated FMD virus type A87/IRN does not have any residue, toxic or allergic responses. Also gamma rays have high energy; therefore the viruses cannot escape from inactivation

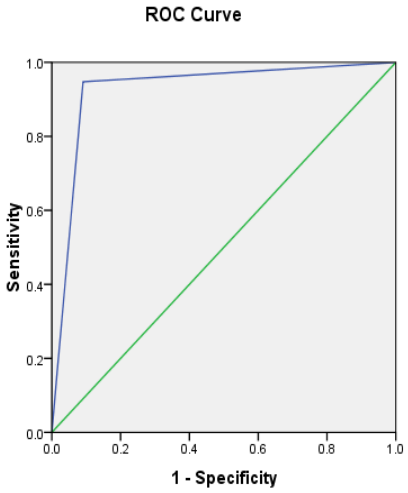

Fig. 1. Diagnostic Gel electrophoresis of the H9 (435bp) RT-PCR products of the H9N2 viruses isolated from live bird markets of Tehran province during 2007**.** M= ladder 100bp (Fermentas), lane 1= (H9 subtype) positive control, lane 2= negative control, lanes 3, 4, 6 and 8= samples contain H9 subtype, lanes 5 and 7= samples do not contain H9 subtype.

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Table 1. The result of antibody detection against FMDV type A87/IRN by VNT as gold standard and B-ELISA titer.

Group	No	B-ELISA		VNT (gold standard)	
		Titration	result	Titration	result
1	1	2.1	+	1.8	+
	2	2.1	+	1.8	+
	3	2.1	+	1.8	+
	4	2.1	+	1.8	+
	*5	0.9	-	1.8	+
	6	2.1	+	1.8	+
	7	2.1	+	1.8	+
	8	2.1	+	1.8	+
	9	2.1	+	1.8	+
	**10	2.1	+	0.9	-
2	11	2.1	+	1.8	+
	12	2.1	+	1.8	+
	13	2.1	+	1.8	+
	14	2.1	+	1.8	+
	15	2.1	+	1.8	+
	16	2.1	+	1.8	+
	17	2.1	+	1.8	+
	18	2.1	+	1.8	+
	19	2.1	+	1.8	+
	20	2.1	+	1.8	+
3	21	0.9	-	0.6	-
	22	0.9	-	0.6	-
	23	0.9	-	0.6	-
	24	0.9	-	0.6	-
	25	0.9	-	0.6	-
	26	0.9	-	0.6	-
	27	0.9	-	0.6	-
	28	0.9	-	0.6	-
	29	0.9	-	0.6	-
	30	0.9	-	0.6	-

Group 1: The guinea pigs which were vaccinated by conventional vaccine.
Group 2: The guinea pigs which were vaccinated by Radio vaccine.
Group 3: The guinea pigs had never been vaccinated or had the disease (control group).
* False negative
** False positive

Complement Fixation Test

The results of CFT for irradiated and non-irradiated samples showed the antigenecity of the irradiated frozen FMD virus was not changed from 0-45 kGy (data not shown).

Concentration of the purified inactivated 146S antigen was obtained; 132×1.36=179.52 μg/ml (11).

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Table 2. Positive and Negative samples of VNT and ELISA test.

VNT / ELISA	Positive	Negative	Total
Positive	18	1	19
Negative	1	10	11
Total	19	11	30

Titration of vaccinated guinea pig sera against inactivated FMDV antigen by VNT and B-ELISA

The anti-sera titration of 3 groups of vaccinated (by routine vaccine and radio-vaccine) and unvaccinated guinea pigs by VNT and B-ELISA are shown in table 1. End-point titers were expressed as the $\log_{10}$ reciprocal of the final dilution of serum in the serum/virus mixtures giving 50% of the absorbance recorded in the virus control (10).

The optimal cut-off value for the optical density of an ELISA can be determined by Receiver Operating Characteristic (ROC) analysis of sensitivity and specificity at different cut-off values (12). In the present study the smallest cut-off value is the minimum observed test value minus 1, and the largest cut-off value is the maximum observed test value plus 1, but the best cut-off value was obtained 0.5 because the sensitivity and specificity were more than 90%. In order to determine the appropriate ELISA cut-off point that would serve as the threshold between positive and negative samples, 30 serum samples from three groups of guinea pigs were analyzed. The optimal cut-off point chosen was at 50% of the reactivity of the high positive control serum sample (by VNT) which resulted in a specificity and sensitivity of 90.9% 94.73%, respectively.

Statistical analysis

Statistical analysis for VNT and B-ELISA was done by t-test, the results of VNT and B-ELISA did not have a significant difference ($P>0.05$). The sensitivity and specificity of B-ELISA in comparison to VNT for detection of antibody against FMDV type A87/IRN were calculated by ROC curve (figure 1). The Area under the ROC curve was 0.928 and the sensitivity and specificity are %94.73 and %90.9 respectively.

Sensitivity = [real positive / (false negative + real positive)] ×100

Sensitivity = 18/ (1+18) ×100= 94.73

Specificity = [real negative / (false positive + real negative)] × 100

Specificity = 10/ (1+10) ×100= 90/9

Discussion

Some researchers have used the VNT to study the relationship between antibody titer and protection in FMD vaccinated animals. However, the results have not always been consistent. Have et al in 1983 reported a correlation between VNT and protection in cattle with each vaccine studied, but concluded that the antibody titer which corresponded to protection depended on the batch and strain of FMD vaccine used (7). Black et al in 1984, however, reported a correlation between VNT antibody titer and protection in vaccinated pigs and suggested that a group mean serum antibody titer may provide the basis for a revised oil-emulsion vaccine potency-testing procedure. Hamblin et al in 1987 reported, VNT test is generally considered to be sensitive and specific, but the wide variation in antibody titers recorded previously in replicate tests, suggests that the assay is not always reliable (13). This variation is probably due to differences in the sensitivity of the tissue culture cells, which ultimately affect the dose of virus used in the test. In a previous publication, Hamblin et al in 1986 showed that ELISA and VNT titers in the sera of animals which had received a single vaccination against FMD or had recovered from clinical FMD were generally in agreement (14). The liquid-phase blocking ELISA has been successfully used in several laboratories for the quantification of antibodies to FMDV and many countries now accept its use as a replacement for the virus neutralization test (15). Morioka et al (16) reported monoclonal antibody-based sandwich direct ELISA (MSD-ELISA) methods that could detect foot-and-mouth disease virus (FMDV) antigens, both multiserotype (MSD-ELISA/MS) (for O, A, C

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and Asia1) and single-serotypes (MSD-ELISA/SS) (for O, A and Asia1 specifically).
In this study the titration of sera from 3 groups of guinea pigs which were either vaccinated or unvaccinated were measured by VNT and B-ELISA. The ELISA titers in sera collected from animals were higher than those recorded by VNT. These results suggested that, in addition to neutralizing antibodies, the ELISA was measuring other classes of antibodies which did not neutralize FMDV in vitro. Also in this study, gamma irradiated inactivated FMDV antigen was used for preparation of antibody against FMDV, and compared the results of VNT and B- ELISA by t- test and showed that the gamma irradiated inactivated FMDV antigen has unaltered antigenicity.

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