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Comparative evaluation of liquid-phase blocking ELISA and solid-phase competition ELISA methods for the detection of antibodies to the structural proteins of foot-and-mouth disease types O and A viruses

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Abstract: In this study, we compared 2 methods, liquid-phase blocking ELISA (LPBE) and solid-phase competition ELISA (SPCE), for the detection of antibodies to the structural proteins of foot-and-mouth disease virus (FMDV). These methods were compared using sera collected from cattle (n = 30) without a history of foot-and-mouth disease infection or vaccination, cattle (n = 180) vaccinated with oil-adjuvanted bivalent vaccine, and international reference sera (positive, weak positive, and negative) for FMDV serotypes O and A. The results showed that SPCE had a better specificity (96.67% for serotype O and 100% for serotype A) than LPBE (90% for serotype O and 93.33% for serotype A). Sensitivity of LPBE (97.22% for serotype O and 98.33% for serotype A) was almost equivalent to that of SPCE (98.33% for serotype O and 98.89% for serotype A). It can be concluded that SPCE is more suitable than LPBE for use as a screening test for the detection of antibodies against structural proteins of FMDV.

Key words: Cattle, foot-and-mouth disease, structural protein, liquid-phase blocking ELISA, solid-phase competition ELISA

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that causes international trade restrictions in livestock and their products. Major clinical features of the disease are fever; lameness; vesicles in the mouth, feet, and teats; and high morbidity. Young animals have higher mortality than adults because of myocarditis (1). It has been eradicated from North America and Europe but continues to exist in parts of South America, Africa, the Middle East, Asia, and the Anatolian region of Turkey (2).

The etiological agent, FMD virus (FMDV), is a member of the genus *Aphthovirus* in the family *Picornaviridae*, and it has 7 different serotypes: O, Asia 1, A, C, and South African territories 1 (SAT 1), SAT 2, and SAT 3 (1,3). The genome encodes 4 structural proteins, VP1 to VP4 (4). The G-H loop of VP1 has been identified as the major antigenic site (5). The genome also codes for 8 nonstructural proteins (L^{pro}, 2A, 2B, 2C, 3A, 3B, $3C^{pro}$, and 3D-RNA–dependent RNA polymerase) (1).

FMDV has very high mutation rates because of the lack of a proofreading mechanism of the viral RNA-dependent RNA polymerase (6). The antigenic variants are caused by mutations leading to difficulties in the control of FMD (1). Vaccination is an effective tool for controlling the disease. Inactivated viral strains of FMDV are used as a vaccine (7). Using oil-adjuvanted vaccines has been shown to induce higher levels of antibody than aluminum hydroxide gel-saponin adjuvanted vaccines (8).

Protection against FMD following vaccination is related with the serum neutralizing antibody levels (9). It has been reported that FMD-specific IgM can be detected 2 to 4 days after vaccination (10). Immunity level of the vaccinated cattle population is readily measured by detecting antibodies to the capsid or structural proteins of the virus (11). The internationally accepted tests for determining the FMD antibody status of livestock are the virus neutralization test (VNT), liquid-phase blocking ELISA (LPBE), and solid-phase competition ELISA (SPCE) (12). The VNT is considered to be sensitive, specific and reliable; however, it takes several days (13). ELISA-based methods offer many advantages including high sensitivity, suitability for large-scale screening of field samples, and lack of a requirement for special laboratory conditions, e.g., cell culture or CO_2 environment (13,14). Therefore, these methods have been used to measure antibody levels of vaccinated animals. The aim of this study was to compare the diagnostic value of these ELISA

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methods for the detection of antibodies to the structural proteins of FMDV serotypes O and A.

2. Materials and methods

2.1. Positive sera

A total of 180 cattle (100 Brown Swiss hybrid, 60 Jersey hybrid, and 20 Holstein Friesian hybrid) that had no record of FMD for many years were used for the study. Cattle were vaccinated with oil-adjuvanted bivalent vaccine (containing O_1 Manisa and A_{22} Iraq FMDV strains; payload of antigens of 6 μg and 4 μg, respectively) formulated in a double oil emulsion adjuvant. The same batches of a commercial vaccine were used. Vaccination was carried out by injection of 2-mL volumes subcutaneously. Serum samples were collected 28 days after vaccination. This study was approved by the ethics board of the Faculty of Veterinary Medicine, Selçuk University, Konya, Turkey (Approval No. 2008/081).

2.2. Negative sera

Sera from cattle (n = 30) with no history of infection or vaccination with FMDV were supplied by Institute for Foot and Mouth Disease, Turkey.

2.3. Test reagents

International reference sera (strong positive antiserum, weak positive antiserum, and negative serum) for FMDV serotype O and serotype A, rabbit anti-FMDV sera (trapping), and guinea pig (detector) antiserum were obtained from the Institute for Animal Health, Pirbright Laboratory, UK. Antigens of the serotype O and serotype A and horseradish peroxidase conjugated rabbit anti-guinea pig immunoglobulin were obtained from the Institute for Foot and Mouth Disease, Turkey.

2.4. Liquid-phase blocking ELISA

The principle of the LPBE assay is liquid-phase blocking of FMDV antigen by specific antibodies in the sera. The LPBE was performed according to the method of Hamblin et al. (14). Briefly, ELISA plates were coated with serotype-specific (serotypes O and A) rabbit anti-FMDV serum and held overnight at 4 °C. In carrier plates, 2-fold series of each test serum were prepared, from 1/16 to 1/128. Control sera (strong and weak positive, and negative) were diluted at 1/16. To each well, addition of the viral antigen increased the final serum dilution to 1/32. The plates were left overnight at 4 °C. Afterwards, rabbit antiserum-coated ELISA plates were washed 3 times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) (pH 7.4), and serum/antigen mixtures were transferred from the carrier plates to the rabbit-serum–coated ELISA plates. Homologous guinea pig antiserum was then added to each well and incubated at 37 °C for 1 h. After incubation, rabbit anti-guinea pig immunoglobulin conjugated to horseradish peroxidase was added to each well. The plates were washed after 1 h of incubation and substrate solution (orthophenylene diamine [OPD] + 0.05% H_2O_2) was added to each well. After 15 min of incubation at room temperature, the reaction was stopped by adding 1.25 M sulfuric acid. The optical density (OD) of each well was read at 492 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), and percentage of inhibition (PI) values were calculated. The sera were considered positive at PI ≥ 50% (14).

2.5. Solid-phase competition ELISA

The SPCE assay is based on competition between antibodies in serum and guinea pig anti-FMDV antiserum for binding to FMDV antigen. The SPCE was carried out as described by Mackay et al. (13). Plates were coated with serotype-specific (serotypes O and A) rabbit anti-FMDV antiserum and incubated at 4 °C for 24 h. FMDV antigen was added to each well of the ELISA plates and the plates were incubated at 37 °C for 1 h. After washing, test sera and control sera (2-fold dilutions of an initial serum dilution of 1:2.5 through 1:20), in blocking buffer (PBST containing 10% normal bovine serum and 5% normal rabbit serum), were added and immediately the addition of serotype-specific guinea pig antiserum immediately followed, giving a final serum dilution of 1/5. Plates were incubated at 37 °C for 1 h. Anti-guinea pig immunoglobulin conjugated with horseradish peroxidase was added to all wells and the plates were incubated at 37 °C for 1 h. After the washing step, substrate/chromogen (OPD + 0.05% H_2O_2) was added to each well. Finally, the reaction was stopped after 15 min by adding 1.25 M sulfuric acid, and OD values at 492 nm were read using a spectrophotometer (Molecular Devices). Sera giving PI values equal to or greater than 60% were considered positive (15).

3. Results

3.1. The specificity and sensitivity of LPBE

The sensitivity and specificity were calculated using standard methods (16,17). Sensitivity was calculated as true positive / true positive + false negative whereas specificity was true negative / true negative + false positive; the results of both calculations were expressed as percentages (16) (Tables 1 and 2).

The specificity of LPBE was evaluated with sera collected from cattle that had never been infected or vaccinated with FMDV. A total of 30 sera were tested for antibodies to serotype O, and 3 out of 30 sera (90%) gave positive results. In contrast, specificity of the LPBE for serotype A was 93.33% (2/30) when testing the same negative sera (Table 1). Thus, the specificity of LPBE was over 90%.

The sensitivity of LPBE was estimated using positive sera (n = 180) that had originated from vaccinated cattle. Serotype O antibody was detected in 175 (97.22%) and serotype A antibody in 177 (98.33%) of 180 sera (Table 2).

Table 1. Specificities of LPBE and SPCE.

FMDV	Test	No. of sera examined	No. of negative sera	Specificity (%)	95% confidence interval
O_1 Manisa	LPBE	30	27	90.0	73.44%–97.77%
	SPCE	30	29	96.67	82.72%–99.44%
A_{22} Iraq	LPBE	30	28	93.33	77.89%–98.99%
	SPCE	30	30	100.0	88.32%–100.0%

Table 2. Sensitivities of the LPBE and SPCE methods.

FMDV	Test	No. of sera examined	No. of positive sera	Sensitivity (%)	95% confidence interval
O_1 Manisa	LPBE	180	175	97.22	93.63%–99.08%
	SPCE	180	177	98.33	95.20%–99.64%
A_{22} Iraq	LPBE	180	177	98.33	95.20%–99.64%
	SPCE	180	178	98.89	96.04%–99.83%

3.2. The specificity and sensitivity of SPCE

The specificity and sensitivity of SPCE was evaluated using the same sera. Of the 30 negative sera tested by SPCE, 1 was considered positive against serotype O (96.67%) and all negative sera had less than 60% inhibition against serotype A (100%). The specificity of SPCE was over 96% (Table 1).

The sensitivity of SPCE was estimated using positive samples of sera (n = 180). When testing by SPCE, 177 (98.33%) of the 180 sera were positive for serotype O and 178 (98.89%) sera were positive for serotype A (Table 2).

3.3. Examination of reference sera

Reference sera were examined by LPBE and SPCE for screening assays. In LPBE, the strong positive (PI: 86%–95%) and weak positive (PI: 55%–72%) reference sera were found to be positive. The negative results were 0%–30% PI. In SPCE, reference sera produced results within the expected range for the negative (PI: 0%–30%), weak positive (PI: 56%–75%), and strong positive (PI: 83%–96%) samples. LPBE and SPCE scored all negative references as negative (Table 3).

3.4. Repeatability of the assays

Repeatability of LPBE and SPCE was assessed based on the results of the strong positive, weak positive, and negative control sera tested on the same day (within-run repeatability) and on different days over the study period (between-run repeatability). Reference sera showed very consistent results when tested by LPBE and SPCE. PIs for all negative controls were below the cut-off for each serotype (cut-off of 50% PI and 60% PI for LPBE and

Table 3. Results of the international reference sera tested by LPBE and SPCE.

Reference sera		LPBE % inhibition[a]	SPCE % inhibition
O_1 Manisa	Strong positive	91 ± 2.2	91 ± 2.6
	Weak positive	58 ± 2.6	61 ± 4.4
	Negative	7 ± 6.2	13 ± 10.8
A_{22} Iraq	Strong positive	89 ± 2.3	86 ± 3.2
	Weak positive	63 ± 4.5	64 ± 5.8
	Negative	9 ± 6.6	12 ± 7.6

[a]: Values are expressed as the mean percentage inhibition of the sera ± standard deviation.

SPCE, respectively). The variation for all positive and negative controls for each serotype was not significant as the PI obtained for controls was within 2 standard deviations with coefficients of variation of less than 20% (16) (Table 4).

4. Discussion

FMD is endemic in the Anatolian region of Turkey; however, the Thrace region has not had a FMD case since 2001. Most outbreak-associated FMDV serotypes are serotype O or serotype A. Outbreak due to serotype Asia 1 was also reported in 2011. Genetic analysis of FMD viruses demonstrated that the Turkish isolates are closely related to the Middle East isolates. In Turkey, control strategies for FMD are based on vaccination, quarantine, and control of animal movements (2,18,19,20). Postvaccination serosurveillance is an important indicator for the evaluation of preventive vaccination programs. Inactivated FMD vaccines are used in most parts of the world (21). Cattle vaccinated with inactivated vaccines produce antibodies only to the structural proteins (22).

The internationally accepted methods for the determination of antibody response after vaccination are the VNT, LPBE, and SPCE. The reference method for the detection of antibodies against structural proteins of the FMDV is the VNT; however, it takes 2–3 days to complete, requires cell culture and live virus, and must be performed in high-security conditions. These special requirements of the VNT make it not suitable for large-scale serological surveillance. Therefore, LPBE has been applied as a routine screening method for FMDV in many laboratories. It is easier to perform and its results are consistent with those of the VNT (12,14). However, it has several drawbacks, including lack of stability of inactivated antigens and false positive reactions occurring at a rate of 4% up to 18% (22,23). For these reasons, SPCE has been developed for the detection of antibodies against FMDV. One of the advantages of SPCE is its highly purified and adequately stable 146S preparations of virus used as antigen (13).

The aim of this study was to determine diagnostic values of LPBE and SPCE. Using the same set of cattle sera, we compared LPBE and SPCE for their specificity and sensitivity. We determined that specificities of LPBE for serotype O and serotype A were lower than those achieved with SPCE (Table 1). Serotype O and A LPBE gave a specificity of 90% and 93.33%, respectively, at a cut-off of 50 PI (14). A cut-off value of 60 PI (15) was used for serotypes O and A SPCE, which gave a specificity of 96.67% and 100%, respectively. Mackay et al. (13) and Niedbalski (24) also obtained similar results, and they reported that specificity of SPCE was considerably higher than that of LPBE. In another study, Paiba et al. (15) reported that specificity of SPCE for serotype O at a cut-off point of 60 PI was 99.44% for cattle sera, 99.50% for sheep sera, and 100% for pig sera.

The sensitivity of SPCE determined by testing positive sera was slightly higher than that of LPBE (Table 2). Similar results were obtained in other studies (13,25,26). Martinez and Quintero (27) reported that sensitivity of LPBE for serotype O_1 Cruzeiro was 96%. Brocchi et al. (25) found that diagnostic sensitivity of SPEC was 99.7%. Niedbalski (24) reported that sensitivity of LPBE and SPCE was 99.1% and 99.4%, respectively.

Table 4. Repeatability data for strong and weak positives and negative reference sera.

Test	Within-run						Between-run					
	C ++[a]		C +[b]		C - [c]		C ++		C +		C -	
	O_1	A_{22}	O_1	A_{22}	O_1	A_{22}	O_1	A_{22}	O_1	A_{22}	O_1	A_{22}
	89.3[d]	86.0	57.6	57.5	0.6[g]	0.4	92.7	89.2	57.7	59.9	0.9	0.6
LPBE	1.2[e]	2.2	2.8	4.0	0.07	0.05	1.6	3.1	2.5	2.3	0.1	0.08
	1.3%[f]	2.5%	4.9%	7.0%	12.5%	12.0%	1.7%	3.5%	4.4%	3.9%	16%	13.2%
	92.4	88.6	62.0	64.2	1.2	1.06	89.7	84	60.1	62.0	0.9	1.05
SPCE	3.3	2.7	3.7	5.6	0.1	0.2	1.6	1.9	5.1	4.7	0.2	0.1
	3.5%	3.1%	5.9%	8.8%	12.3%	18.5%	1.7%	2.3%	8.5%	7.7%	24.2%	16.2%

[a]: Strong positive control serum.
[b]: Weak positive control serum.
[c]: Negative control serum.
[d]: Results are expressed as the percentage inhibition.
[e]: Standard deviation.
[f]: Coefficient of variation.
[g]: Optical density of the negative control serum.

International reference sera were tested by LPBE and SPCE methods and the results were compared. All strong and weak positive reference sera were detected as positive by the LPBE and SPCE methods (Table 3). The repeatability of LPBE and SPCE were assessed by calculating the coefficients of variation (Table 4). The coefficients of variation calculated were less than 10% for reference sera, considered acceptable for practical use (16). Additionally, PIs obtained were consistently within the range of 2 standard deviations, which means that the variation between tests was within the normal range of acceptability. Weak positive sera showed very consistent results when tested using LPBE and SPCE. It has been suggested that weak positive reference serum is the minimum standard for the serologic assays used for herd-based serosurveillance (24). Therefore, these data suggest that LPBE and SPCE can be useful to assess of the herd immunity levels induced by vaccination.

The possibility of using LPBE and SPCE to measure an antibody response was also investigated by testing 180 sera from vaccinated cattle. The results demonstrated that SPCE antibody titers for FMDV serotypes O and A were all similar to or higher than LPBE antibody titers. The highest titers obtained by SPCE for samples of serotypes O and A were more than 2 times higher than those measured by LPBE (Figure). These results suggested that the SPCE can detect lower amounts of FMDV serotype O- and serotype A-specific antibodies than the LPBE.

In conclusion, this study has demonstrated that SPCE for FMDV serotypes O and A as both a screening and a titration assay was sensitive and specific. Additional advantages of SPCE are that the assay is easier to perform and is carried out with inactivated virus antigen, unlike the VNT. SPCE is more suitable than LPBE for the evaluation of vaccination programs as well as import/export testing in support of international trade, because of its high specificity and sensitivity and its low variation in results.

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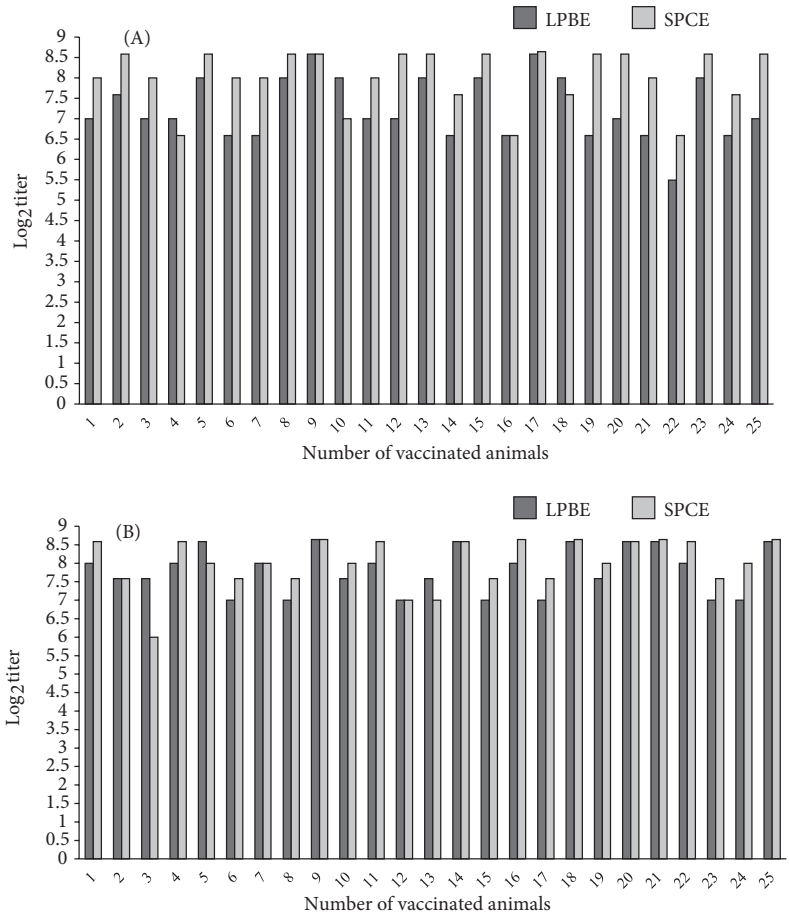

Figure. Comparison of antibody titers in animals vaccinated (n = 25) with types O_1 Manisa and A_{22} Iraq as measured by LPBE and SPCE. A: O_1 Manisa, B: A_{22} Iraq.

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