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INDIRECT ELISA FOR DETECTION OF ANTIBODIES TO FMDV NON-STRUCTURAL PROTEINS IN PORCINE SERA

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SUMMARY

An indirect variant of ELISA used for detection of antibodies to nonstructural proteins of the FMD virus in porcine blood sera was developed. The results of the validation showed that the developed method is characterized by high sensitivity, specificity and reproducibility. When testing the blood serum panel obtained from experimentally infected animals, the method allowed to detect antibodies to FMD virus in 7 of 18 sera collected on day 6 post inoculation, in 13 of 19 sera – on day 7 post inoculation, in 16 of 19 sera – on day 8 post inoculation and in all 76 sera obtained on days 9–12 post inoculation. The diagnostic specificity of 3AB-ELISA was 100% when testing 100 knowingly negative blood sera from pigs imported to Russia from Norway. High specificity and sensitivity of the method, established during the development of the method, are confirmed in the course of routine diagnostic tests.

Key words: FMD virus, non-structural proteins, ELISA test.

INTRODUCTION

Foot-and-mouth disease is a highly contagious viral disease affecting domestic and wild cloven-hooved animals including pigs. The disease is classified to transboundary diseases capable of causing epidemics and high economic losses to animal farming.

The FMD agent is non-enveloped RNA virus belonging to *Aphthovirus* genus of *Picornaviridae* family. There are seven virus serotypes: O, A, C, Asia-1, SAT-1, SAT-2 and SAT-3. Many genetic and antigenic virus variants exist within each virus serotype.

FMD is not endemic in the Russian Federation. There is, however, a permanent threat of the disease introduction from Asian countries primarily from China. Therefore, a strategy for FMD prevention and control aimed at prevention the disease occurrence and spread in the country's territory is in place in the Russian Federation. Buffer zone where cattle and small ruminants are vaccinated against FMD has been established in the areas at high risk of the infection introduction and spread. Methods allowing detection of antibodies to FMDV non-structural proteins in animal blood are used to detect infected animals among vaccinated ones. Such antibodies are detected in infected animals but not detected in vaccinated ones when purified vaccines complying the World Organization for Animal Health (OIE) requirements are applied [5, 6].

Earlier, indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to FMDV non-structural proteins in cattle and small ruminant sera was developed and validated by the FGBI "ARRIAH" [2]. This method (3AB-ELISA) has been successfully used for diagnostic and monitoring tests for several years.

Rules for FMD regionalization of the Russian Federation territory came into force in 2017. According to the said rules, animals can be moved out of the zone where anti-FMD vaccination is carried out to the zone without vaccination providing that they lack post-infection serum antibodies to FMDV. Thus, tests of animals for their FMD infection status became obligatory for commercial operations involving animal movements [4].

Since this rule is also compulsory for pigs a test for anti-FMDV antibodies in animal sera has become in-demand for pig farms.

Goal of the study is to develop and to validate 3AB-ELISA intended for detection of antibodies to FMDV non-structural antibodies in porcine sera.

MATERIALS AND METHODS

Antigen. 3AB recombinant protein, derived by expression in *E. coli* was used as an antigen. Expression and purification conditions are described earlier [3].

Animal sera. Reference serum derived from a pig experimentally infected with type A FMD virus was used as positive control; serum from pig lacking antibodies against FMD virus was used as negative control. The said sera were ELISA tested for antibodies to FMDV non-structural proteins with PrioCHECK FMDV NS FMDV antibody test kit, ELISA (Prionics, Switzerland).

Sera from pigs with known infection status were used for determination of working serum dilutions, cut-off value, diagnostic sensitivity and susceptibility of the assay. One hundred and thirty-two sera collected from experimentally FMDV-infected pigs on day 6–12 after infection were used as known positive samples. The said samples were provided by staff-members of the Reference Laboratory for FMD Diagnosis. Sera from pigs imported from Norway to the Russian Federation were used as known negative samples.

Anti-species antibody conjugate. Commercial anti-porcine IgG antibody peroxidase conjugate (Sigma) was used.

Enzyme-linked immunosorbent assay (ELISA). 3AB-ELISA development was the subject of the study and described

Table 1
Effect of blocking solution formulation on mean OD values of control sera
n = 4

Blocking solution	Values for antigen-containing wells			Values for antigen-free wells	
	OD, CS+	OD, CS-	S/N	OD, CS+	OD, CS-
Milk concentration:					
1%	1.19 ± 0.09	0.21 ± 0.01	5.63 ± 0.05	0.07 ± 0.01	0.07 ± 0,01
3%	1.09 ± 0.08	0.16 ± 0.01	**6.90 ± 0.07**	0.07 ± 0.01	0.07 ± 0.01
10%	1.00 ± 0.08	0.15 ± 0.01	**6.55 ± 0.06**	0.07 ± 0.01	0.07 ± 0.01
Bovine serum albumin concentration:					
1%	1.42 ± 0.09	0.35 ± 0,01	4.04 ± 0.08	0.12 ± 0.01	0.10 ± 0.01
3%	1.31 ± 0.05	0.38 ± 0.02	3.44 ± 0.06	0.12 ± 0.01	0.09 ± 0.01
10%	1.29 ± 0.09	0.34 ± 0.01	3.79 ± 0.07	0.10 ± 0.01	0.09 ± 0.01
Normal equine serum concentration:					
1%	0.98 ± 0.08	0.24 ± 0.01	4.14 ± 0.08	0.09 ± 0.01	0.08 ± 0.01
3%	1.22 ± 0.04	0.29 ± 0.01	4.17 ± 0.05	0.09 ± 0.01	0.08 ± 0.01
10%	0.96 ± 0.05	0.19 ± 0.01	5.00 ± 0.06	0.08 ± 0.01	0.07 ± 0.01

in Results and Discussion. ELISA using PrioCHECK FMDV NS FMDV antibody test kit, ELISA (Prionics, Switzerland) was carried out in accordance with its manufacturer's instruction.

Statistical processing of ELISA results was performed in accordance with the OIE recommendations [7].

RESULTS AND DISCUSSION

Optimization of reagents and assay procedure. Working dilutions of antigen and anti-species conjugate, optimal formulation of blocking solution, temperature and time periods for each ELISA stage as well as admissible optical density (OD) values of control sera were determined.

Working antigen and anti-species conjugate dilutions were determined with chessboard titration using positive and negative sera. The antigen and conjugate dilutions giving the following OD values of positive control serum and negative control serum were used as working dilutions: 1.0–1.1, and 0.08–0.19, respectively. Working antigen dilution was 2 μg/ml and working anti-porcine IgG conjugate dilution was 1:20,000.

Tests of 1%, 3% and 10% skimmed milk and 10% equine serum based on TBS buffer containing 0.01% Twin-20 were performed to select blocking solution. The 3% and 10% skimmed milk was found to be the most optimal as a component of blocking buffer and for dilution of samples (Table 1).

Forty-seven porcine sera with different antibody levels were tested by serial dilution method at four different dilutions: 1:20, 1:40, 1:80, 1:160 to determine optimal working serum dilution. S/P ratio was determined for each serum dilution:

$$S/P\ ratio = (OD - NC_x)/(PC_x - NC_x),$$

where OD – mean optical density of tested serum;
NC_x – mean OD of negative control serum;
PC_x – mean OD of positive control serum.

Then, lg S/P and lg T were calculated for each dilution. The obtained data were processed using Statistica computer programme. At the end, correlation coefficient was determined (Table 2).

Table 2
Correlation coefficient for different serum dilutions

Dilution	Correlation coefficient
1:20	0.80186
1:40	0.82486
1:80	0.87514
1:160	0.81618

Table 2 shows that the highest correlation coefficient, R = 0.87, when standard error was the lowest, 0.66646, was estimated when the sera were diluted at 1:80. Therefore, the said dilution was selected as a working one.

Linear regression equation for 1:80 serum dilution was as follows:

$$lg\ T = 2.9941 + 1.3653 \times lg\ S/P,$$

where 2.9941 and 1.3653 – A and B coefficients, respectively.

Scatterplot of lg S/P versus lg T for 1:80 working serum dilution is given in Figure.

The following 3FD-ELISA procedure was established based on the results of all parameters' optimization. Recombinant protein (100 μl) at its working dilution made with 0.05 M carbonate-bicarbonate buffer (pH 9.6) was added to each well of the plate and the plate was incubated for 16–18 hours at 4 °C. Then, 100 μl of blocking solution (PBS, 10% skimmed milk, pH 7.4) were added to the plate wells and the plate was incubated for 1 hour at 37 °C. After the plate washing with PBS-T tested sera diluted 1:80 with blocking buffer (PBS, 3% skimmed milk, pH 7.4) were added to wells (100 μl per well) and the plates were incubated in temperature-controlled shaker at 37 °C for one hour. The plates were washed again and the conjugate at its working dilution was added, the plates were incubated under the same conditions. ABTS substrate was added after washing and the reaction was stopped by adding 1% sodium dodecyl sulphate solution in 10–15 minutes; the results were recorded with spectrophotometer at wavelength of 405 nm.

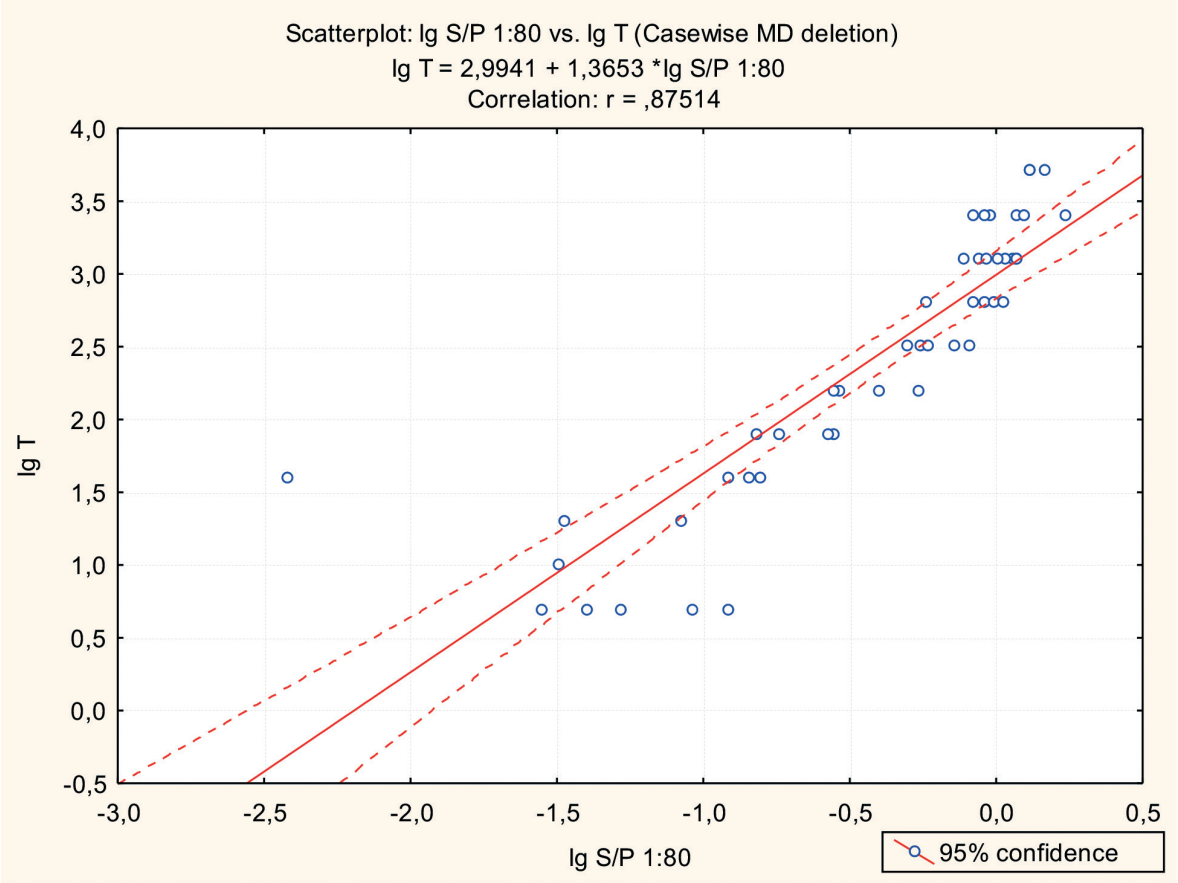

Fig. Diagram of lg ELISA antibody titre dependence from lg S/P determined in 1:80 working serum dilution

Assessment of the reaction result repeatability. To test the reaction results for their repeatability positive porcine serum at its working dilution was added to the plate wells and OD value was measured in each well. Coefficient of variation was calculated according to the following formula:

$$C = (\delta/\bar{x}) \times 100\%,$$

where δ – standard deviation;
$\bar{x}$ – arithmetical mean.

Coefficient of variation was 8.6% when positive sera were tested on one plate that was indicative of good repeatability (Table 3).

Determination of 3AB-ELISA analytical specificity. To determine analytical specificity 10 heterologous porcine sera containing antibodies against porcine reproductive respiratory syndrome virus, classical swine fever virus, porcine circovirus type 2, Aujeszky's disease virus were tested. Antigen activity when it reacted with heterologous sera was not higher than background level induced when it reacted with non-immune serum.

Determination of cut-off value for 3AB-ELISA. A total of 92 known negative porcine sera were tested. Cut-off value was determined by calculation of mean OD for negative sera plus three standard deviations. Mean negative serum OD value with three standard deviations was 0.392. When porcine sera were tested, cut-off value corresponded to percentage of positives (PP) equal to 30%. Porcine sera with PP < 30% were considered negative, porcine sera with PP ≥ 30% – positive.

Determination of 3AB-ELISA diagnostic specificity. 3AB-ELISA diagnostic specificity was determined by testing 100 known negative sera collected from pigs imported to the RF from Norway. No false-positive results were obtained. Thus, 3AB-ELISA specificity was 100% when the said panel of sera was tested.

Determination of the 3AB-ELISA diagnostic sensitivity. To test 3AB-ELISA for its diagnostic sensitivity sera from pigs experimentally infected with FMD virus were used: four pigs were infected with type A FMD virus, 11 pigs were infected with type O FMDV, 4 pigs were infected with type Asia-1 FMDV. Blood samples were collected before infection and on day 6–12 after infection.

All sera collected before infection were seronegative when tested with 3AB-ELISA. Anti-FMDV antibodies were detected in 7 out of 18 sera on day 6 after infection,

Table 3
Test of 3AB-ELISA for repeatability

Serum characteristics	Minimal OD value (x_{min})	Maximum OD value (x_{max})	Mean OD value ($\bar{x}$)	Standard deviation (δ)	Coefficient of variation (c),%
Positive serum	0.881	1.239	1.019	0.088	8.6

Table 4
Results of 3AB-ELISA tests of sera collected from pigs experimentally infected with FMD virus

Days after infection	3AB-ELISA results (positive/tested)			
	type A	type O	type Asia-1	Total
0	0/4	0/11	0/4	0/19
6	2/4	4/10	1/4	7/18
7	3/4	6/11	4/4	13/19
8	4/4	8/11	4/4	16/19
9	4/4	11/11	4/4	19/19
10	4/4	11/11	4/4	19/19
11	4/4	11/11	4/4	19/19
12	4/4	11/11	4/4	19/19

in 13 out of 19 sera – on day 7 after infection, in 16 out of 19 sera – on day 8 after infection and in all 76 sera collected on day 9–12 after infection (Table 4).

Sera collected on day 6–8 after infection were included in the panel for testing the method for its ability to detect antibodies at early stage after infection as a proxy of its analytical sensitivity. Such "early" sera are not taken into account when diagnostic sensitivity was determined. It could be argued that diagnostic sensitivity of the method was 100% when sera from experimentally infected pigs were used since all sera collected on day 9–12 after infection were tested positive with 3AB-ELISA.

Assessment of 3AB-ELISA for its use in routine diagnostic testing. According to the international requirements, validation of a diagnostic method should not be limited to a series of experiments; it should be performed continuously as long as it is used during tests.

In 2014–2017 3AB-ELISA was used for testing field porcine sera submitted from the RF farms. The scheme used for these tests was the same as that one used for tests of sera from cattle and small ruminants [2]. At first, samples were tested with 3-AB ELISA served as a screening test-system. Then, all sera tested positive were additionally examined with PrioCHECK FMDV NS FMDV antibody test kit, ELISA used as a confirmation test-system (Prionics, Switzerland).

Several type O FMD outbreaks were reported in pigs in the Primorsky Krai, Russian Federation, in 2014 [1]. Besides aphthae materials, porcine sera were submitted to the FGBI "ARRIAH" from one farm for FMD diagnosis confirmation. Antibodies to FMDV non-structural proteins were detected when the samples were tested with 3AB-ELISA. The same samples were also tested positive when they were examined with the confirmation test-system. Thus, the serological methods confirmed that pigs had been affected with FMD on this farm.

In 2015–2017, 4,833 porcine sera from various FMD-free RF regions were tested for antibodies to FMDV non-structural proteins: 4,802 samples were tested negative with 3AB-ELISA and 31 samples were tested positive with 3AB-ELISA. Positive results were not confirmed when the said samples were retested with PrioCHECK FMDV NS FMDV antibody test kit, ELISA (Prionics, Switzerland) and consequently they were interpreted as false-positives. Thus, diagnostic specificity of 3AB-ELISA used for routine testing was 99.4%. It is close to the diagnostic specificity (100%) determined at the stage of the method development.

Thus, 3AB-ELISA characteristics (high sensitivity and specificity) determined at the stage of the method development were confirmed during routine diagnostic testing.

CONCLUSION

3AB-ELISA for the detection of antibodies to FMDV non-structural proteins in porcine sera was developed. Its validation has showed that 3AB-ELISA is capable of detecting antibodies to FMD virus in sera of infected animals with high specificity and sensitivity. 3AB-ELISA high specificity and sensitivity were confirmed by routine diagnostic test results.

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