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DEVELOPMENT AND BASIC FEATURES OF INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES AGAINST PORCINE EPIDEMIC DIARRHEA

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SUMMARY

Spread of porcine epidemic diarrhea and its increased threat for the pig industry necessitate development of advanced techniques for the disease diagnosis. Use of recombinant antigens of the disease agent seems prospective. The recombinant antigen-based indirect enzyme-linked immunosorbent assay has been developed for the detection of antibodies against porcine epidemic diarrhea virus. The research performed allowed for determination of all necessary test conditions. Basic features of the developed method were determined during its validation. The test precision was above 90% pursuant to its repeatability and reproducibility, diagnostic specificity of the test amounted to 99.47%, and its sensitivity as compared to commercial ID Screen PEDV Indirect (IDvet, France) was 92% with very good compatibility of the two tests (*k*-criterion – 0.88) – 92%. The test-system demonstrates high stability upon the change of the key test component.

Key words: porcine epidemic diarrhea, antibodies, enzyme-linked immunosorbent assay.

INTRODUCTION

Porcine epidemic diarrhea (PED) is an acute intestinal disease characterized by vomiting and watery diarrhea. The disease is caused by RNA-virus of *Coronaviridae* family [1, 3].

Pigs of all age groups are susceptible to the disease. The acute disease is reported at the initial introduction of the infection onto the farm. PED symptoms resemble typical outbreak of porcine transmissible gastroenteritis (TGE). Mortality of 1–14-day-old suckling piglets due to dehydration can reach 90% [2, 13].

The disease causes gross economic damage to pig industry due to high mortality of piglets and decreased weight gain of fattening pigs resulting from the loss of appetite after recovery [3].

PED has been known since 1970-s and for a long time it mostly circulated in Europe and some Asian countries. In 2013, PED was first introduced into the USA, where it inflicted significant damage to the pig industry. The disease soon spread to Canada and Mexico. Large-scale PED epidemics have been recently reported in China, South Korea and Japan [11].

Global spread of PED and its increased threat to the pig industry actualize the issue of the development of up-to-date disease diagnostic tools.

Such serological methods as neutralization test, immunohistochemistry, immunofluorescence, enzyme-linked immunosorbent assay (ELISA) are used for the detection of PED virus antibodies. The majority of these methods are based on the use of native virus antigen [5, 7, 9]. Recombinant antigens of PED agent are promising for their use in serological tests.

The basic PED structural proteins include Spike protein (S), nucleocapsid protein (N) and membrane protein (M). The proteins were demonstrated to contain antigenic determinants and they can be used as antigens in immunological tests [8, 12, 13]. In various PEDV isolates, the N protein is the most conservative in its amino acid composition [11]. The FGBI "ARRIAH" produced the PEDV recombinant nucleocapsid protein and practiced conditions for its synthesis in *E. coli* [4].

The work was aimed at the development of recombinant antigen-based indirect ELISA and determination of the basic test properties.

MATERIALS AND METHODS

Antigen. Previously obtained PEDV recombinant protein was used as an ELISA antigen [4].

Blood sera. Pig sera collected on PED infected and free farms were used. Pig sera collected from pigs vaccinated against porcine reproductive and respiratory syndrome (PRRS), transmissible gastroenteritis (TGE), etc. were used as heterologous sera.

Anti-species antibody conjugate. Peroxidase conjugate against porcine immunoglobulin G (IgG) was used (Sigma, USA).

Indirect ELISA. Development of recombinant antigen-based indirect ELISA was the research objective, thus the PED-ELISA protocol is described in Results and Discussion section.

Commercial ELISA test-kits. Imported commercial test-kit ID Screen PEDV Indirect (IDvet, France) was used. The ELISA was performed according to the test-kit manufacturer's instructions.

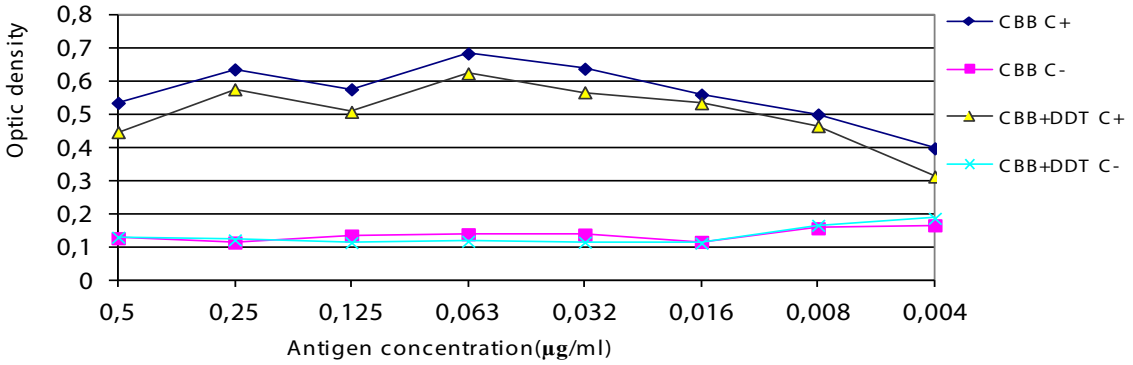

Fig. 1. Recombinant protein antigenicity tested using PED-ELISA with control sera and antibody removal

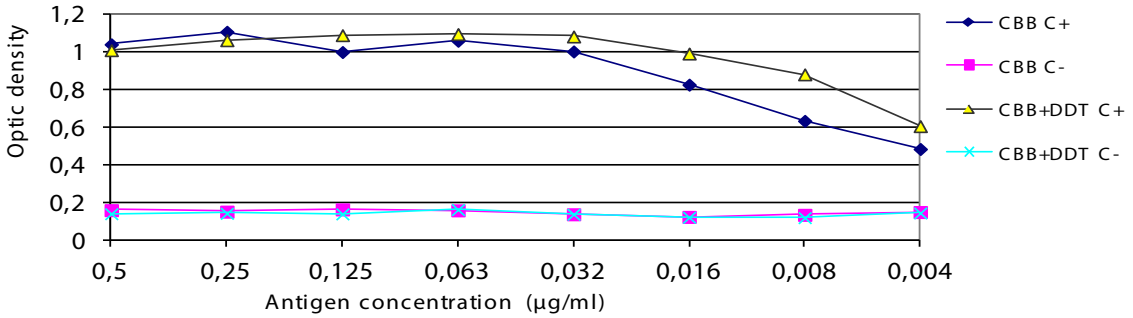

Fig. 2. Recombinant protein antigenicity tested using PED-ELISA with control sera but without antigen removal

RESULTS AND DISCUSSION

Development of indirect ELISA for PEDV antibody detection included several stages.

Determination of antigenic activity of PEDV recombinant protein and indirect ELISA protocol optimization. Purified PEDV recombinant protein preparation was used for examination of its antigenic activity and specificity. For this purpose, serial dilutions of the antigen-containing coating buffer were applied. The following solutions were used for coating: carbonate-bicarbonate buffer, pH 9.6 (CBB) and carbonate-bicarbonate buffer supplemented with 50 mM 1,4-Dithio-DL-threitol, pH 9.6 (CBB+DDT). The coated plates were incubated at 4–8 °C for 16–18 hours. Hereafter, unbound antigen was removed from one of the plates by 3× addition of 300 µl of phosphate-buffered solution (PBS) in each well. The other plate was only dried and transferred to the blocking solution. Samples pre-tested using commercial test-kit ID Screen PEDV were used as positive and negative control sera.

Antigenicity test results of recombinant PEDV protein are graphically demonstrated in Figures 1 and 2.

Figures 1 and 2 demonstrate that the coating buffer composition made no effect on the recombinant protein antigenicity – the antigen working dilution used as coating buffer amounted to 1:800 standing for 0.032 µg/ml protein concentration. This was the plate washing for unbound antigen removal that made effect. Therefore, carbonate-bicarbonate buffer was used as coating buffer and the plate was washed for unbound antibody removal.

Eight different compositions were used for the selection of the blocking buffer: 10%, 5% and 3% bovine serum albumin (BSA) solutions; 10% and 5% normal horse serum solutions and 10%, 3% and 1% dry skim milk solutions. The components were dissolved in PBS and pH was adjusted to 7.3–7.6. After a set of experiments, the number of suitable blocking buffers was reduced to three: 10%, 1% milk solutions and 10% BSA solution. These three buffers were used for determination of the optimal period of serum and conjugate incubation. Optical density (OD) was measured in three negative and four positive samples tested in quadruplicate in 30, 45 and 60 min. Then serum OD arithmetic mean ($\bar{x}$), mean square deviation (δ) and coefficient of variation (c) were calculated. The best results (coefficient of variation below 10%) were demonstrated at 30 min incubation of sera and conjugate with 1% milk solution.

Based on the experimental results the following PED-ELISA protocol was chosen: recombinant PEDV antigen was diluted in the coating buffer (CBB, pH 9.6) and 50 µl was added into each well. The plate with the antigen was incubated at 4–8 °C for 16–18 hours and washed 3× 250–300 µl/well. After drying, the blocking buffer (1% milk and PBS solution, pH 7.3–7.4) was added and incubated in incubator shaker at 37 °C for 60 min. The plate was washed again, and the buffer-diluted serum samples and conjugate (1% milk and PBS solution, pH 7.3–7.4) were added. The plate was incubated in the incubator shaker at 37 °C for 30 min at 750 rpm. After the plate washing, the conjugated working dilution was added and the plate was incubated as described above. 2,2′-azino-di-[3-ethyl]-bensothiazoline-6 sulfonic acid solution was added for color development and the reaction was stopped with 1% sodium dodecylsulfate solution. The reaction was recorded with spectrophotometer at 405 nm.

Choice of pig serum working dilution for single dilution testing. Serum sample containing different amount of PEDV antibodies were ELISA tested in different dilutions ranging from 1:20 to 1:1280.

Table 1
PED-ELISA repeatability while testing one sample on three plates

Test run	Min OD C^+ (x_{min})	Max OD C^+ (x_{max})	Mean OD C^+ ($\bar{x}$)	Mean square deviation (σ)	Coefficient of variation (c), %
Plate 1	0.672	0.920	0.817	0.049	6.0
Plate 2	0.557	0.891	0.759	0.067	8.83
Plate 3	0.572	0.911	0.802	0.068	8.48

PED-ELISA was performed according to the procedure described above.

The percent positivity (PP) and end-point titre were calculated for all test serum dilutions (85 samples).

The final serum concentration was considered to be the end-point titre and its OD was equal or above the 2× mean OD of the negative control serum.

Percent positivity (PP) was calculated as follows

$$PP = (OD - NC_x)/(PC_x - NC_x) \times 100\%,$$

where OD – test serum optical density;
NC_x – negative control optical density;
PC_x – positive control optical density.

The obtained results were processed using Statistica software, and thus correlation coefficient and standard error were calculated. The serum working solution was chosen basing on these parameters. The highest correlation coefficient (R = 0.9199) and the lowest standard error 0.07718 were demonstrated at 1:40 dilution with 0.95 confidence interval.

Determination of cutoff value. In order to justify the cutoff between non-specific (negative) and specific (positive) PED-ELISA reactions the OD values were determined for 92 samples of PEDV antibody-free sera.

The cutoff value was determined through calculation of negative serum mean OD at selected 1:40 dilution with due account of two or three values of standard deviation (σ). It was determined that sera should be considered negative at PP < 20% and positive at PP ≥ 25%. PP values within the range of 20–25% shall be indicative of inconclusive result.

Determination of acceptable control serum OD. Twenty replicates of 1:40 diluted positive and negative pig sera were tested. The resulted OD values allowed for calculation of relative mean OD, standard deviations and 95% confidence interval.

The positive control mean OD was demonstrated to correspond to 0.999 (standard deviation – 0.223), negative control mean OD – 0.137 (standard deviation – 0.036). Acceptable values of control serum OD at 95% confidence interval were within the following range: for positive control – from 0.776 to 1.222 AU, for negative control – from 0.062 to 0.212 AU.

Thus, the investigations resulted in determination of all necessary test conditions.

The next stage included investigations aimed at determination of the key validation properties of PED-ELISA: analytical and diagnostic sensitivity and specificity, stability upon change of reagents, precision under repeatability and reproducibility.

Determination of PED-ELISA analytical specificity. Analytical specificity of the recombinant protein was tested in pig sera containing antibodies against TGEV, PRRSV, CSFV, porcine circovirus type 2 and Aujeszky's disease virus. The protein reactivity with heterogenic sera did not exceed the background level demonstrated in the reaction with non-immune serum.

Determination of analytical sensitivity. Blood serum from the convalescent pig was used. Presence of antibodies was demonstrated using IDvet commercial test-kit.

Using serial two-fold dilutions the serum was PED-ELISA tested in triplicates. The serum titre amounted to 9.32 $\log_2$, and this value was the test limit of detection [6]. As for IDvet commercial test-kit, its limit of detection amounted to 8.32 $\log_2$.

Determination of precision. Under repeatability conditions one serum sample was tested three times in 96 rep-

Table 2
Repeatability while testing six samples within the same test run

Sample	Repeatability	Optic density	Mean square deviation (σ)	Coefficient of variation (c), %
1	1	0.786	0.005	0.6
	2	0.795		
	3	0.787		
2	1	0.446	0.011	2.41
	2	0.465		
	3	0.457		
3	1	0.598	0.052	9.5
	2	0.510		
	3	0.527		
4	1	0.125	0.006	4.65
	2	0.135		
	3	0.128		
5	1	0.185	0.014	7.6
	2	0.172		
	3	0.195		
6	1	0.105	0.002	1.9
	2	0.103		
	3	0.107		

Table 3
PED-ELISA stability upon change of recombinant antigen batch

Recombinant antigen batch	Sample 1	Sample 2	Sample 3
Batch 1	1.095	0.998	0.116
Batch 2	1.062	0.926	0.112
Batch 3	1.005	0.855	0.119
Mean OD ($\bar{x}$)	1.054	0.926	0.116
Mean square deviation (σ)	0.053	0.085	0.004
Coefficient of variation (c), %	5.03	9.18	3.45

licates (on three plates). The results are demonstrated in Table 1. PED-ELISA repeatability was also determined while testing six samples with different antibody levels in triplicates within the same test run (Table 2).

Coefficients of variation in both cases were below 10% thus being indicative of high repeatability of PED-ELISA results.

PED-ELISA precision under inter-laboratory repeatability conditions was determined by testing control serum sample on different days. For this purpose, the positive serum sample was subsequently tested for 20 days and arithmetic mean positivity percentage, mean square deviation and coefficient of variation were calculated. These values amounted to 100.0; 7.43 and 7.43. Low coefficient of variation (below 10%) is indicative of high reproducibility of the developed method [10].

Therefore, the estimated PED-ELISA precision amounted to over 90% under both the repeatability and reproducibility conditions.

Determination of stability. Such PED-ELISA components as dry milk, buffer salts, etc. are calibrated by the manufacturers.

One of the key components of the reaction is a recombinant antigen. Three pig sera were tested in order to determine the test stability upon the change of recombinant antigen batches (Table 3).

Low coefficients of variation of PED-ELISA results with three different recombinant antigen batches are indicative of high stability of the test upon the change of the key reagent.

Determination of PED-ELISA diagnostic specificity. Field serum samples collected from pigs on PED free farms were used. Out of 1,510 PED-ELISA tested samples eight samples demonstrated positive results, while the same samples were negative when tested using IDvet commercial test-kit. Therefore, the diagnostic specificity of the developed test amounted to 99.47%.

Determination of PED-ELISA sensitivity. As PEDV field strains cannot be isolated from the cell culture, there was no opportunity to perform experimental infection of pigs for production of sera known to be positive and essential for diagnostic sensitivity determination. However, available commercial test-kits for PEDV antibody detection allowed for determination of sensitivity of the developed method against the imported diagnostic test-kits.

One hundred ID Screen PEDV Indirect test-kit-positive sera were PED-ELISA tested to this effect. The sera were collected on four farms, where PED clinical signs were reported and laboratory RT-PCR results demonstrated PEDV in the pathological samples. The developed PED-ELISA identified PEDV antibodies in 92 samples, four samples demonstrated inconclusive results and four samples demonstrated negative results. Hence, under very good compatibility of the two tests (k-criterion – 0.88) PED-ELISA sensitivity for this serum panel amounted to 92% against IDvet test-kit.

CONCLUSION

Recombinant antigen-based indirect ELISA was developed for the detection of antibodies against PED virus. The test validation demonstrated that the developed test-kit is characterized by very good result reproducibility, stability upon the change of key component of the reaction, high specificity and sensitivity, i.e. it can be used as a tool for PED serological diagnosis.

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