UDC 619:616.98:578.831.31:616-078:578.74
DOI: 10.29326/2304-196X-2020-2-33-109-114

Development and use of indirect liquid-phase ELISA test system for detection of PRRS virus antigen during in-process control of raw materials intended for vaccine production

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SUMMARY
Porcine reproductive and respiratory syndrome (PRRS) being endemic and reported in the most countries in the world remains one of the most challenging diseases in pig industry. The main disease control measures include preventive vaccination and animal movement control within and outside the country as well as diagnostic testing of pigs in the population. Live and inactivated vaccines are used for specific prevention of porcine reproductive and respiratory syndrome. Complete and irreversible infectious agent inactivation with maximum epitope preservation and protective immunity in immunized animals are the main requirements for inactivated vaccines. Therefore, continuous improvement of methods for vaccine quality control at various vaccine production stages is of current importance. Results of development of the test system based on indirect liquid-phase enzyme-linked immunosorbent assay (ELISA) for PRRS virus antigen detection and activity testing in infectious and inactivated virus-containing cell cultures at intermediate stages of the vaccine production process are described in the paper. The test-system development process included purified and concentrated virus antigen as well as hyperimmune rabbit sera preparation. Specificity of purified and concentrated virus antigen was confirmed with real-time polymerase chain reaction. The developed test-system was shown to detect the virus antigen at initial infectivity titre of 4.87–7.21 lg $TCID_{50}/cm^3$ corresponding to ELISA titre (dilution) of 1:4 up to 1:64. Methodical Guidelines for detection of porcine reproductive and respiratory syndrome virus antigen with indirect liquid-phase enzyme-linked immunosorbent assay (ELISA) (2019) were developed based on the work results, commissioned and approved by the FGBI "ARRIAH" Scientific Board.

Key words: porcine reproductive and respiratory syndrome, enzyme-linked immunosorbent assay, antigen.

Acknowledgements: The work was funded by the FGBI "ARRIAH" as part of the research activities "Animal health and welfare".

For citation: Baborenko Ye. P., Byadovskaya O. P., Biryuchenkov D. A., Konstantinov A. V., Yashin R. V. Development and use of indirect liquid-phase ELISA test system for detection of PRRS virus antigen during in-process control of raw materials intended for vaccine production. *Veterinary Science Today*, 2020; 2 (33): 109–114. DOI: 10.29326/2304-196X-2020-2-33-109-114.

Conflict of interest. The authors declare no conflict of interest.

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УДК 619:616.98:578.831.31:616-078:578.74

Разработка и применение тест-системы на основе непрямого жидкофазного блокирующего варианта ИФА для определения антигена вируса РРСС при технологическом контроле вакцинного сырья

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РЕЗЮМЕ

Одной из наиболее актуальных проблем свиноводства остается репродуктивно-респираторный синдром свиней, который является эндемичным заболеванием и регистрируется в большинстве стран мира. К основным мерам борьбы с заболеванием относятся профилактическая вакцинация, контроль за передвижением животных как внутри страны, так и за ее пределами, а также постоянное проведение диагностических исследований в популяции свиней. В качестве средств специфической профилактики репродуктивно-респираторного синдрома свиней применяются живые и инактивированные вакцины. Основным требованием, предъявляемым к инактивированным препаратам, является полная и необратимая инактивация инфекционного агента при максимальной сохранности антигенной детерминанты и иммунная защита привитых животных. Поэтому актуальной задачей является постоянное совершенствование методов контроля качества вакцин на различных технологических стадиях их производства. В статье представлены результаты разработки тест-системы на основе непрямого жидкофазного блокирующего иммуноферментного анализа для выявления и определения активности антигена вируса репродуктивно-респираторного синдрома свиней в инфекционных и инактивированных вируссодержащих препаратах клеточных культур на промежуточных этапах производства вакцинных биопрепаратов. Разработка тест-системы включала получение очищенного и концентрированного препарата антигена вируса, а также специфических гипериммунных сывороток крови кроликов. Специфичность очищенного и концентрированного антигена вируса подтверждали с помощью полимеразной цепной реакции в режиме реального времени. Показано, что разработанная тест-система позволяет выявлять антиген вируса с исходным титром инфекционной активности в диапазоне от 4,87 до 7,21 lg $ТЦД_{50}/см^3$, соответствующий значению титра (разведения) при постановке иммуноферментного анализа от 1:4 до 1:64. В результате проведенной работы были разработаны, прошли комиссионные испытания и утверждены ученым советом ФГБУ «ВНИИЗЖ» «Методические рекомендации по выявлению антигена вируса репродуктивно-респираторного синдрома свиней в непрямом жидкофазном блокирующем варианте иммуноферментного анализа» (2019 г.).

Ключевые слова: репродуктивно-респираторный синдром свиней, иммуноферментный анализ, антиген.

Благодарность: Работа выполнена за счет средств ФГБУ «ВНИИЗЖ» в рамках научно-исследовательских работ по теме «Ветеринарное благополучие».

Для цитирования: Баборенко Е. П., Бьядовская О. П., Бирюченков Д. А., Константинов А. В., Яшин Р. В. Разработка и применение тест-системы на основе непрямого жидкофазного блокирующего варианта ИФА для определения антигена вируса РРСС при промежуточном контроле вакцинного сырья. *Ветеринария сегодня*. 2020; 2 (33): 109–114. DOI: 10.29326/2304-196X-2020-2-33-109-114.

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) has been and remains a significant challenge for the majority of the countries where pig faming is well-developed including Russia. PRRS is currently endemic and almost all countries in the world including Russia, USA, China as well as West European countries suffer great economic losses [1, 2].

Analysis of anti-PRRS measures taken worldwide shows that the main measures include preventive vaccination, animal movement control within and outside the country as well as permanent diagnostic testing of pigs in the population [3]. Preventive vaccination covers more and more pigs every year. Therefore, continuous improvement of the vaccines and methods for control at various vaccine production stages is of current importance.

It is known that inactivated antiviral vaccine production is a complex technological process and complete and irreversible infectious agent inactivation with maximum epitope preservation and protective immunity in vaccinated animals are the main requirements for inactivated vaccines.

Qualitative and quantitative characterization of the virus antigen is a fundamental prerequisite for produced anti-PRRS vaccine effectiveness. If the virus infectivity can be controlled at the stage of the vaccine raw material scaling up with titration in susceptible cell culture, then, after inactivation, quantification of PRRS antigen, a parameter characterizing the ability to induce immune response, is difficult.

The study was aimed at development of the method for determination of PRRS virus antigen activity both in cultural virus-containing materials intended for the vaccine production and in the semi-finished vaccine.

MATERIALS AND METHODS

Antigen. Aminoethylethyleneimine-inactivated KPR-96 strain of European genotype PRRS virus (FGBI "ARRIAH" Collection of Microorganism Strains) grown in Marc-145 continuous cell culture (rhesus macaque kidney), a trophovariant of MA-104 cell culture, (infectivity titre – 4.87–7.21 lg $TCID_{50}/cm^3$) was used.

Animals. The following laboratory animals were used for tests: seronegative Chinchilla rabbits (live weight – 2–2.5 kg) and clinically healthy piglets at the age of 2 months (live weight – 20–25 kg) obtained from the PRRS-free holdings.

All tests in the animals were carried out in strict compliance with the interstate standards on laboratory animal keeping and handling: GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council on Standardization, Metrology and Certification as well as in accordance with the requirements laid down in Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on protection of the animals used for scientific purposes.

Specific hyperimmune sera. Specific hyperimmune rabbit sera prepared by thrice immunization of rabbits with preliminary purified and concentrated emulsified antigen supplemented with oil adjuvant were used.

Anti-species conjugate. Horseradish peroxidase-conjugated anti-rabbit IgG (H+L) immunoglobulins (Sigma, USA) were used as anti-species conjugate.

Tested preparations: cultural virus and antigen specimens prepared during the vaccine raw material scaling-up, exhibiting different infectivity levels and collected at different production stages. Suspension of normal uninfected cell culture equivalent to the tested specimen as well as antigens of Aujezsky's disease virus (ADV) and porcine parvovirus (PPV) were used as negative controls. Cultural virus-containing preparation with known infectivity titre was used as positive control.

Methods. Common methods for PRRSV cultivation, infectivity titre determination, antigen preparation through the virus inactivation and low speed-centrifugation concentration; microneutralization assay (MNA), Bradford protein assay; real-time polymerase chain reaction (qPCR) were used.

Enzyme-linked immunosorbent assay (ELISA) was carried out in accordance with the Methodical Guidelines for detection of porcine reproductive and respiratory syndrome virus antigen with indirect liquid-phase enzyme-linked immunosorbent assay developed in the FGBI "ARRIAH" [4].

Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit (IDEXX, USA) was used for testing porcine sera for specific antibodies against PRRS virus, the test results were read in accordance with the test-kit manufacturer instruction.

Statistical processing of the results. Standard methods for processing of the selected variables as well as elements of correlation-regression analysis were used. All calculations and graphical plottings were performed using Microsoft Office Excel applications.

RESULTS AND DISCUSSION

Preparation of active and specific antigen is one of the important stages of the ELISA test-kit development. The virus-containing cultural fluid was purified from ballast proteins and cell fragments with low-speed centrifugation using Beckman Coulter J-26 XP centrifuge (USA) equipped with JA-14 rotor at 4,500 rpm for 40 min.

Resulting clarified supernatant was centrifuged with Beckman Coulter J-26 XP centrifuge (USA) with JA-18 rotor at 17,000 rpm for 2.5 hours and then the supernatant was removed and pellet was resuspended with 10 ml of TNE-buffer.

For the further PRRS virus antigen purification and concentration the resuspended pellet was centrifuged in 30% sucrose-layer gradient with high-speed Beckman Coulter Optima L-80 XP (USA) centrifuge with SW-28 rotor at 27,000 rpm for 2.5 hours. The resulting pellet was resuspended with TNE-buffer at the ratio of 100:1 of the initial volume.

The specificity of the purified and concentrated PRRS virus antigen was confirmed with qPCR. C_t-values of all antigen samples tested with qPCR were less 35 (C_t) and within the range of 17–23 (C_t), that was indicative of high virus levels in the preparations.

Protein concentration in the PRRS virus antigen preparations measured with Bradford protein assay using bovine serum albumin standard solutions at wavelength of 595 nm was 0.5–1.0 mg/ml. The purified and concentrated antigen was used for preparation of hyperimmune sera required for ELISA test- system development.

The test-system development comprised selection of optimal antigen dilution, specific hyperimmune serum and anti-species conjugate. The test results were read with Sunrise spectrophotometer (Tecan, Austria) at wavelength of 405 nm. The test-system development included selection of suitable buffer systems and blocking solutions, determination of the component adsorption time and temperature.

Optimal antigen dilution was determined for each batch with serial-dilution ELISA. The last antigen dilution that gave the following optical density (OD) values was considered to be working dilution: control positive serum OD_S – within the range of 1.0–1.5 AU (when tested sera were diluted at 1:100) and negative serum OD_S – not higher than 0.25 AU. The data are given in Figure 1.

Based on the obtained data, PRRS virus antigen dilutions giving control positive serum OD_S of 1.0–1.2 (at 1:100 dilution of the tested sera) that were equal to the antigen dilutions of 1:50 – 1:100 (P/N 8.8–8.75) were used as working PRRS virus antigen dilutions.

As a result, the Methodical Guidelines for detection of porcine reproductive and respiratory syndrome virus antigen with indirect liquid-phase enzyme-linked immunosorbent assay (2019) were developed, commissioned and approved by the FGBI "ARRIAH" Scientific Board [4].

Testing of the developed test-system for its sensitivity and specificity was the next stage of the study. Tests of the virus-containing specimens before and after their inactivation as well as virus antigen at different stages of its purification and concentration with the proposed method were carried out. Test results are given in Table 1.

The data given in Table 1 show direct correlation between infectivity titres and ELISA dilutions. For example, infectivity titre of the 10×-concentrate of the virus was 7.21 ± 0.11 lg $TCID_{50}/cm^3$, and its ELISA titre was 1:32 – 1:64 both before and after inactivation; infectivity titre of the 3×-concentrate of the virus was 6.15 ± 0.14 lg $TCID_{50}/cm^3$, its ELISA titre was 1:16 – 1:32 both before and after inactivation. While supernatants with infectivity titres of 1.15 ± 0.09 and 1.35 ± 0.12 lg $TCID_{50}/cm^3$ demonstrated negative ELISA values – < 1:2.

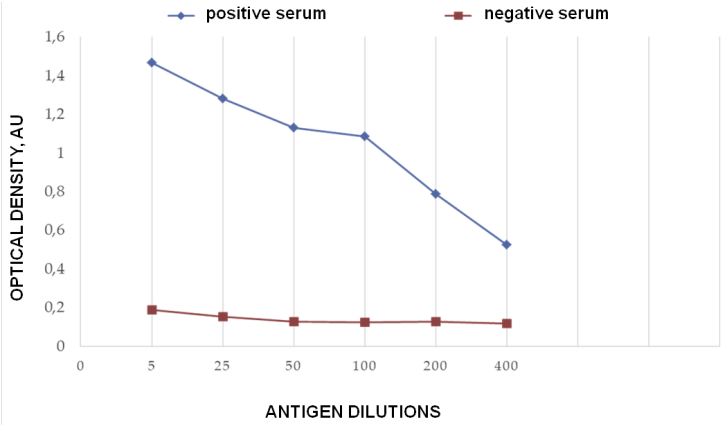

Fig. 1. Specific and normal rabbit serum activity, 1:100 dilution

Рис. 1. Активность специфической и нормальной сывороток крови кролика в разведении 1:100

Table 1
Comparative testing of PRRS virus for its infectivity and antigenicity with titration and ELISA

Таблица 1
Сравнительная оценка инфекционной и антигенной активности вируса РРСС, определенной методом титрования и с помощью ИФА

Type of sample	Number of samples	Mean titre, lg $TCID_{50}/cm^3$	Mean ELISA titre* (dilutions)
Virus-containing suspension (before inactivation)	25	4.87 ± 0.12	1:4
Virus-containing suspension (before inactivation)	75	5.67 ± 0.19	1:8 – 1:16
Antigen-containing suspension (inactivated)	75	not tested	1:8
Virus-containing suspension (×10 concentrate of PRRSV antigen) (before inactivation)	10	7.21 ± 0.11	1:32 – 1:64
Antigen-containing suspension (×10 concentrate of PRRSV antigen) (inactivated)	10	not tested	1:32 – 1:64
Supernatant (before inactivation)	10	1.15 ± 0.09	< 1:2
Virus-containing suspension (×3 concentrate of PRRSV antigen) (before inactivation)	15	6.15 ± 0.14	1:16 – 1:32
Supernatant (before inactivation)	15	1.35 ± 0.12	< 1:2
Antigen-containing suspension (×3 concentrate of PRRSV antigen) (inactivated)	15	not tested	1:16 – 1:32
Supernatant (inactivated)	15	not tested	< 1:2
Negative control (cultural fluid of normal non-PRRSV-infected cell culture)	5	0	< 1:2
Negative control (PPV antigen)	5	0	< 1:2
Negative control (AD virus antigen)	5	0	< 1:2

* ELISA test result in titres: > 1:2 – positive result; < 1:2 – negative result.

* Результат ИФА в титрах: > 1:2 – положительно; < 1:2 – отрицательно.

Correlation between developed indirect liquid-phase ELISA-derived results and virus infectivity titre (T, $TCID_{50}$) calculated for the tested virus-containing cultural fluid specimens was determined based on results of numerous tests. For ELISA the following was taken as a value indicating the relative amount of reacting antigen: $D = K^-/S$, where K^- and S – negative control and tested sample optical density, respectively; D – estimated independent parameter and lgT – estimated dependent parameter.

Obtained results are presented in the form of graph in Figure 2.

Figure 2 shows the following regression model for tested parameters: lgT″ = 1.808(D) + 0.769, where lgT″ – expected titre for the specified D value; the coefficient of model validity is as follows: $R^2 = 0.891$.

Obtained data are indicative of apparent correlation between relative amount of ELISA-reactive antigen (D) and logarithmic titres of the virus (lgT) expressed as correlation coefficient (R = 0.944). Plotted model of correlation between specified parameters was as follows: lgT″ = 1.808(D) + 0.769 and covered at least 89.1% of empirical points. Within the $2 \le D \le 3.15$ range the model allows infectivity titre of the sample to be predicted with statistical uncertainty (regression error) of $m \le 0.18$ lg.

Tests shows that developed sensitive and specific competitive liquid-phase ELISA can be used for in-process laboratory control carried out at the stages of PRRS virus antigen preparation for determination of semi-finished vaccine activity.

During the laboratory experiment specific antibody levels were determined in piglets immunized intramuscularly at a dose of 2 cm^3 with tested vaccine specimens prepared based on the virus antigen with different infectivity levels determined with titration and developed ELISA test-system. Piglet sera were tested in accordance with the instruction for use of Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit (IDEXX, USA). The results are given in Table 2.

The animals were PRRS virus-seronegative before vaccination. Twenty-eight days after vaccination specific antibodies were detected only in 30% of the piglets immunized with vaccine specimen 1 (ELISA virus antigen activity – 1:4)

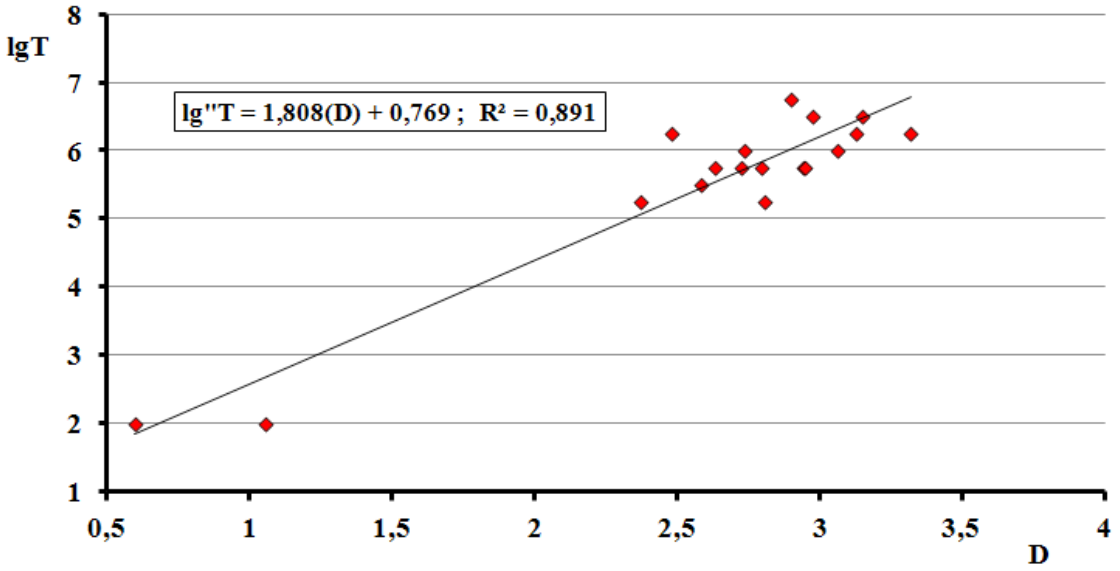

Fig. 2. Logarithmic titres of the virus infectivity (lgT, ◆) determined for tested cultural fluid samples corresponding to relative amounts of ELISA-reacting antigen (D)

Рис. 2. Логарифмические оценки титра инфекционной активности вируса (lgT, ◆), установленные в тестируемых образцах культуральной жидкости соответственно показателям относительного количества реагирующего антигена (D) в ИФА

whereas vaccine specimens 2 and 3 (ELISA virus antigen activity – 1:8 and higher) induced pronounced immune response in the piglets detectable both with ELISA and MNA.

CONCLUSION

ELISA test-system to quantitate porcine reproductive and respiratory syndrome virus antigen in tested specimens of semi-finished anti-PRRS vaccine during in-process laboratory control was developed based on test results. Specific ELISA components were prepared, cut-off values were determined, correlation between relative amount of ELISA-reactive antigen and logarithmic titre of the virus expressed as the following correlation coefficient: R = 0.944 was established. Inactivated PRRSV antigen with ELISA activity of at least 1:8 – 1:16 induces specific immunity that is evidenced by presence of diagnostic levels of specific anti-PRRSV antibodies in animals.

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Table 2
Tests of inactivated emulsion anti-PRRS vaccine samples demonstrating different antigen activity for their antigenic properties

Таблица 2
Изучение антигенных свойств вакцины против РРСС инактивированной эмульгированной с различной активностью антигена

Inactivated emulsion vaccine against PRRS	PRRS virus/antigen activity		Vaccine antigenicity (mean antibody level in the group of piglets 28 days after vaccination	
	before inactivation (lg $TCID_{50}/cm^3$)	after inactivation (ELISA)	ELISA (s/p)	MNA (log_2)
Specimen No. 1	5.0	1:4	10/3* 0.31 ± 0.06	< 2.0
Specimen No. 2	5.75	1:8	10/8* 0.58 ± 0.1	2.74 ± 0.12
Specimen No. 3	6.25	1:16	10/10* 1.1 ± 0.1	3.57 ± 0.11

* Number of tested/positive samples.
ELISA result: $s/p < 0.4$ – no specific antibodies; $s/p \geq 0.4$ – presence of specific antibodies.
MNA results: < 2.0 – no microneutralization antibodies; ≥ 2.0 – presence of specific antibodies.

* Количество исследованных/положительных проб.
Значение ИФА: $s/p < 0,4$ – специфические антитела отсутствуют; $s/p \geq 0,4$ – наличие специфических антител.
Значение РМН: < 2,0 – специфические антитела отсутствуют; ≥ 2,0 – наличие специфических антител.

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Received on 28.03.2020
Approved for publication on 19.05.2020

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