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Statistical analysis of determining the reproducibility of the study of the developed test system for detecting African swine fever virus DNA

Análisis estadístico de la determinación de la reproducibilidad del estudio del sistema de prueba desarrollado para la detección del ADN del virus de la peste porcina africana

Larisa Gnezdilova[1,*], Sergey Pozyabin[1], Marina Selina[1], Seidfatima Borunova[1,2]

[1] Moscow State Academy of Veterinary Medicine and Biotechnology named after K.I. Skryabin. Russia.
[2] All-Russian State Center for Quality and Standardization of Veterinary Drugs and Feed. Russia.
*Corresponding author: larisa.a.gnezdilova@mail.ru

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ABSTRACT

The study aimed to develop a loop-mediated isothermal amplification-based test system with hybridization-fluorescence detection to detect the DNA of the African swine fever virus in the presence of an internal control sample. As part of the study, optimal amplification conditions were determined, a method of nucleic acid extraction was selected, and the amplification of positive and negative samples was analyzed. Statistical analysis was carried out to determine the reproducibility of the study of the developed test system. The genetic features of the African swine fever virus were studied, and the most promising fragment of the genome for the specific identification of this pathogen, the major capsid protein p72 gene, was identified. Oligonucleotides were selected for isothermal amplification of the target. The results of the study are valuable from the point of view of further developments in virology, epidemiology, and diagnostics and can also be used by organizations and institutions involved in the control and prevention of animal diseases, in particular African swine fever.

Keywords: infectious diseases, animals, genetics, molecular genetic studies, polymerase chain reaction, statistical data analysis.

RESUMEN

El objetivo del estudio era desarrollar un sistema de prueba basado en la amplificación isotérmica mediada por bucle con detección por hibridación-fluorescencia para detectar el ADN del virus de la peste porcina africana en presencia de una muestra de control interno. Como parte del estudio, se determinaron las condiciones óptimas de amplificación, se seleccionó un método de extracción de ácidos nucleicos y se analizó la amplificación de muestras positivas y negativas. Se llevó a cabo un análisis estadístico para determinar la reproducibilidad del estudio del sistema de prueba desarrollado. Se estudiaron las características genéticas del virus de la peste porcina africana y se identificó el fragmento del genoma más prometedor para la identificación específica de este patógeno, el gen de la proteína p72 de la cápside mayor. Se seleccionaron oligonucleótidos para la amplificación isotérmica de la diana. Los resultados del estudio son valiosos desde el punto de vista de futuros avances en virología, epidemiología y diagnóstico, y también

pueden ser utilizados por organizaciones e instituciones implicadas en el control y la prevención de enfermedades animales, en particular la peste porcina africana.

Palabras claves: enfermedades infecciosas, animales, genética, estudios de genética molecular, reacción en cadena de la polimerasa, análisis estadístico de datos.

1. INTRODUCTION

African swine fever (ASF) is a highly contagious viral disease found in pigs and characterized by fever, skin cyanosis, and extensive hemorrhages in internal organs. It refers to particularly dangerous infectious animal diseases, according to the World Organization for Animal Health (WOAH) list of the International Classification of Infectious Animal Diseases, which are defined as "infectious (transmissive) diseases that can spread dangerously and rapidly regardless of state borders, are accompanied by serious consequences in the field of public economy and health and are important in international trade in animals and products animal husbandry" (WOAH, n.d.).

Studies devoted to African swine fever (ASF) are of utmost importance due to several reasons. ASF is a highly contagious viral disease that affects domestic and wild pigs, leading to significant economic losses in the pig farming industry (Sánchez-Cordón, et al., 2018). Outbreaks of ASF can result in the mass culling of infected animals to prevent further spread, leading to severe disruptions in the pork supply chain and trade restrictions on affected regions (Khanna, 2022).

ASF poses a threat to food security and livelihoods, especially in regions where pork is a staple food and pig farming is a major source of income for communities (Ruiz-Saenz et al., 2022). The disease can devastate pig populations, leading to a decrease in the availability of affordable protein sources and affecting the livelihoods of small-scale farmers who rely on pig farming for their sustenance.

Furthermore, ASF has the potential to spill over into wild boar populations, creating a reservoir of the virus and perpetuating its spread (Denstedt et al., 2021). This poses a significant challenge for disease control and eradication efforts since wild boars can act as a source of infection for domestic pigs (Gnezdilova et al., 2022b; Suárez et al., 2018).

Studies dedicated to African swine fever are crucial for understanding the disease, developing effective control measures, safeguarding food security, and protecting the livelihoods of communities dependent on pig farming. By advancing our knowledge of ASF, researchers aim to mitigate the impact of the disease and prevent its spread, thereby ensuring the stability and sustainability of the pig farming industry.

The main purpose of the study was to develop a set of protective measures against economically and socially significant animal diseases based on the production strains of microorganisms selected by genomic sequencing methods.

2. MATERIALS AND METHODS

In this work, the following research methods were applied: multiple comparisons of nucleotide sequences, selection of oligonucleotide primers for DNA amplification, search for nucleotide sequences using the basic local alignment search tool (BLAST) algorithm, loop isothermal DNA amplification, isolation of DNA from biological material and viral strains, as well as the use of the MedCalc diagnostic evaluation calculator to analyze the results (MedCalc, n.d.).

70 samples of blood plasma and 42 samples of tissue suspension (tonsil, spleen, lung tissue) obtained from pigs were used to test the loop-mediated isothermal amplification (LAMP)-based test system under development.

The reproducibility of the study was determined in two independent experiments, using rotary (Rotor-Gene Q) and flatbed (CFX96) devices.

LAMP mixtures for these two experiments were prepared independently from oligonucleotide primers of different production batches. The positive samples were ASF virus DNA at a concentration of 5x10^3 copies/ml mixed with DNA isolated from biological material obtained from animals (blood plasma, tissue suspension extract). These DNA samples were tested in 12 repetitions in each experiment (on rotary and flatbed amplifiers).

The evaluation of reproducibility occurred:

- by results agreement (ASF virus DNA detected/not detected),

- by the difference between the average values of threshold cycles ((ΔCtav) along the ASF DNA detection channel,

- by the coefficient of variation (CoV), %.

The difference between the average values of the threshold cycles (ΔCtav) of the ASF DNA detection channel should not exceed two cycles.

For polymerase chain reaction (PCR) techniques, the CoV usually does not exceed 5%. However, given the fact that the isothermal amplification method is characterized by a large spread of threshold cycle values, in our opinion, the value of the coefficient of variation can be assumed to be no more than 10-15% as a criterion.

The calculation of the CoV in the reproducibility conditions, %, was carried out according to the formula:

$$CoV, \% = SD / Ctav \times 100 \%, \quad (1)$$

where SD is the mean standard deviation of threshold cycles (Ct) between tests performed under reproducibility conditions.

3. RESULTS AND DISCUSSION

As a result of the experiment, sufficient repeatability and reproducibility of independent research results using two different LAMP mixtures prepared at different times and tested on different amplifiers (rotary and flatbed type) were shown (Table 1).

In the first experiment using a LAMP mixture of ASF 21.06.22 and amplification on Rotor-Gene Q (QIAGEN, Germany), all 12 samples containing 5x10^3 copies/ml of ASF DNA in the DNA of biological material obtained from animals were identified.

In the second experiment using a LAMP mixture of ASF 19.08.22 and amplification at CFX96 (Bio-Rad Laboratories, USA), all 12 samples containing 5x10^3 copies/ml of ASF DNA in the DNA of biological material obtained from animals were also identified.

Thus, the following result was obtained for all positive samples: ASF virus DNA was detected in all samples in all formulations.

Table 1. Evaluation of reproducibility of the results of the study of samples containing 5x10^3 copies/ml of ASF virus DNA.

ASF DNA 5x10^3 copies/ml, ASF Ct		
Repetition No.	Experiment 1, LAMP mixture 21.06.22, Rotor-Gene Q	Experiment 2, LAMP mixture 19.08.22, CFX96
1	24.13	22.67
2	24.45	23.03
3	24.64	24.05
4	24.98	22.66
5	25.24	22.26
6	25.35	25.14
7	25.41	24.34
8	25.55	25.51
9	25.57	25.16
10	25.97	24.83
11	26.75	25.21
12	27.16	23.67
Average Ct value over the ASF channel	25.43	24.04
Difference of average ASF Ct values for two experiments	1.39	
Ct SD	0.88	1.16
CoV (Ct), %	3.47	4.83

The average values of the threshold cycles, the standard deviation, and the coefficients of variation for each of the experiments were calculated (Table 1).

The difference between the values of threshold cycles (ΔCtav) along the ASF DNA detection channel under reproducibility conditions does not exceed 1.4 cycles.

CoV of the values of threshold cycles along the detection channel of the results of ASF virus DNA amplification (CoV(Ct)) under repeatability conditions did not exceed 4.9%, which meets even the requirements for PCR diagnostics (no more than 5%).

The obtained results indicate the repeatability and reproducibility of independent test results of the developed ASF virus diagnostic technique.
The reproducibility of the studies was also evaluated based on the results of the study of samples of biological material that do not contain ASF virus DNA (negative samples).

The developed test system for detecting ASF virus DNA is based on the study of biological samples by extracting DNA together with the DNA of an exogenous control sample and conducting isothermal loop amplification of the resulting DNA. In the conditions of competition with ASF virus DNA amplification in the sample, the internal control sample (ICS) DNA amplification reaction is most often suppressed and does not happen. However, in the absence of ASF virus DNA amplification, the ICS DNA amplification reaction indicates the absence of inhibition of the reaction and loss of DNA during extraction, which serves to exclude false negative results.

Due to the importance of controlling ICS amplification in the absence of ASF virus DNA amplification, the presence of ICS DNA amplification in samples not containing ASF virus DNA was evaluated. The study was conducted under reproducibility conditions. Three experiments were carried out on CFX96 and one experiment on RotorGene. According to the results of studies in these independent formulations for all 61 samples prepared based on different biological materials (blood plasma, tissue suspension), ICS DNA was detected (Table 2).

Table 2. Evaluation of the reproducibility of the results of the study of images that did not contain the ASF virus DNA by amplification of the ICS added at the extraction stage to biological samples.

DNA sample, ASF DNA not detected		Ct, ICS DNA	DNA sample, ASF DNA not detected		Ct, ICS DNA
3	blood plasma	33.61			
4	blood plasma	33.49	46	blood plasma	36.9
5	blood plasma	33.67	47	blood plasma	35.9
10	blood plasma	34.37	48	blood plasma	34.43
11	blood plasma	27.83	49	blood plasma	32.91
15	blood plasma	43	50	blood plasma	33.41
16	blood plasma	32.32	51	blood plasma	34.47
17	blood plasma	35.71	52	blood plasma	38.91
18	blood plasma	35.4	53	blood plasma	34.52
19	blood plasma	32.84	57	tissue suspension	35.27
20	blood plasma	37.58	58	tissue suspension	36.31
21	blood plasma	34.54	59	tissue suspension	35.22
22	blood plasma	36.35	62	tissue suspension	38.49
23	blood plasma	34.68	81	tissue suspension	42.1
25	blood plasma	34.61	82	tissue suspension	39.94
26	blood plasma	36.21	83	tissue suspension	40.63
27	blood plasma	34.97	84	tissue suspension	41.37
28	blood plasma	33.6	85	blood plasma	41.92
29	blood plasma	32.51	86	blood plasma	44.61
30	blood plasma	33.39	90	tissue suspension	42.6
33	blood plasma	34.24	97	tissue suspension	42.22
35	blood plasma	38.84	98	tissue suspension	45.54
36	blood plasma	41.11	99	tissue suspension	43.86
37	blood plasma	34.96	100	tissue suspension	47.58
38	blood plasma	34.54	101	tissue suspension	42.52
39	blood plasma	36.35	102	blood plasma	38.45
40	blood plasma	34.68	103	blood plasma	41.45
41	blood plasma	33.03	104	blood plasma	34.25
42	blood plasma	34.77	105	blood plasma	32.50
43	blood plasma	32.9	106	blood plasma	36.89
44	blood plasma	39.08	108	tissue suspension	38.59
45	blood plasma	33.87	109	tissue suspension	31.86

We calculated the average value of threshold cycles along the ICS DNA detection channel under reproducibility conditions (ICS Ctav = 36.74), its SD (4.0), and CoV common for all experiments (Table 1), which equaled 10.8%.

4. CONCLUSION

The developed method allows reproducible detection of ASF virus DNA from various biological materials (blood plasma, tissue suspension).

A statistical analysis of the data was carried out to determine the reproducibility of the study of the developed test system for detecting ASF virus DNA.

The obtained results indicate the repeatability and reproducibility of independent test results of the developed ASF virus diagnostic technique.

The rapidly developing industry of portable devices for isothermal amplification with fluorescence detection (Gnezdilova et al., 2022a; Zidovec Lepej, Poljak, 2020), which often provide for a full cycle of research, including the stage of extraction of nucleic material on microfluidic cartridges, shows that in the future this test can be successfully used not only in laboratory conditions but also in livestock farms for the rapid detection of a dangerous pathogen.

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REFERENCES

Denstedt, E., Porco, A., Hwang, J., Nga, N.T.T., Ngoc, P.T.B., Chea, S., Khammavong, K., Milavong, P., Sours, S., Osbjer, K., Tum, S., Douangngeun, B., Theppanya, W., Van Long, N., Thanh Phuong, N., Tin Vinh Quang, L., Van Hung, V., Hoa, N.T., Le Anh, D., … Pruvot, M. (2021). Detection of African swine fever virus in free-ranging wild boar in Southeast Asia. Transboundary and Emerging Diseases, 68(5), 2669-2675. https://doi.org/10.1111/tbed.13964

Gnezdilova, L., Pozyabin, S., Borunova, S., Stoyan, T., Selina, M., Davydova, E. (2022a). An application of loop isothermal amplification to detect African swine fever virus. International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies, 13(9), 13A9I. https://doi.org/10.14456/ITJEMAST.2022.177

Gnezdilova, L.A., Panin, A.N., Pozyabin, S.V., Selina, M.V., Borunova, S.M. (2022b). Diagnosis and prevention of infectious animal diseases based on monitoring, molecular diagnostics, and genomics. International Journal of Ecosystems and Ecology Science, 12(3), 459-470. https://doi.org/10.31407/ijees12.3

Khanna, K. (2022, April 1). African swine fever virus: A global concern. The American Society for Microbiology. Retrieved from https://asm.org/Articles/2022/March/African-Swine-Fever-Virus-Is-A-Global-Concern

MedCalc. (n.d.). Diagnostic test evaluation calculator. Retrieved from https://www.medcalc.org/calc/diagnostic_test.php

Ruiz-Saenz, J., Diaz, A., Bonilla-Aldana, D.K., Rodríguez-Morales, A.J., Martinez-Gutierrez, M., Aguilar, P.V. (2022). African swine fever virus: A re-emerging threat to the swine industry and food security in the Americas. Frontiers in Microbiology, 13, 1011891. https://doi.org/10.3389/fmicb.2022.1011891

Sánchez-Cordón, P.J., Montoya, M., Reis, A.L., Dixon, L.K. (2018). African swine fever: A re-emerging viral disease threatening the global pig industry. The Veterinary Journal, 233, 41-48. https://doi.org/10.1016/j.tvjl.2017.12.025

Suárez, J., Carreño, L., Paniz-Mondolfi, A., Marco-Canosa, F.J., Freilij, H., Riera, J.A., Risquez, A., Rodriguez-Morales, A.G., Hernández-Rincón, E., Alvarado-Socarras, J., Contreras, G., Cabrera, M., Navas, R.M., Díaz-Quijano, F., Rosales, T., Barbella-Aponte, R.A., Villamil-Gómez, W.E., Diaz, M., Carrero, Y., … Rodriguez-Morales, A.J. (2018). Infectious diseases, social, economic and political crises, anthropogenic disasters and beyond: Venezuela 2019 - Implications for public health and travel medicine. Revista Panamericana de Enfermedades Infecciosas, 1(2), 73-93. https://doi.org/10.13140/RG.2.2.13082.90562/1

WOAH. (n.d.). Animal diseases. Retrieved from https://www.woah.org/en/what-we-do/animal-health-and-welfare/animal-diseases/

Zidovec Lepej, S., Poljak, M. (2020). Portable molecular diagnostic instruments in microbiology: Current status. Clinical Microbiology and Infection, 26(4): 411-420. https://doi.org/10.1016/j.cmi.2019.09.017