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Determination of reproductive properties of virulent and vaccine classical swine fever virus strains in primary and continuous cell cultures

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SUMMARY

Classical swine fever (CSF) is a highly dangerous porcine disease. CSF outbreaks are annually notified in several countries. Despite the availability of specific prevention tools, the disease spread risk still persists both at country level and at world level. Hence, the disease surveillance and eradication require highly sensitive methods for early diagnosis of the infection and for tests for the virus circulation in the environment. Development of up-to-date diagnostic methods is based on well-established virus cultivation system; therefore, CSF virus reproduction enhancement, tests of new cell lines without endogenous contamination for their possible use are still of current importance. The said study was aimed at testing of primary and continuous cell cultures for their susceptibility to classical swine fever virus (vaccine virus strains and some field virus isolates recovered in the Russian Federation) and detection of the virus reproduction dynamics with real-time polymerase chain reaction with fluorescent hybridization probes used for detection. Virus replication intensity in primary and continuous cell cultures was also analyzed. The CSF virus was found incapable of replicating in some cell cultures without its preliminary adaptation. Primary porcine and lamb testicle cell cultures grown in minimal essential medium supplemented with 10% normal CSFV-negative porcine serum instead of fetal bovine serum were shown to be useful for the virus accumulation, both for vaccine strains and field isolates. Cultivation parameters and optimal minimal essential medium composition contributing to the 4–10-fold increase in the virus accumulation both in primary and continuous cell cultures were determined.

Keywords: classical swine fever (CSF), CSF virus isolates, vaccine virus strains, cell cultures, real-time reverse transcription-polymerase chain reaction (RT-PCR) with fluorescent hybridization probes used for detection, direct immunofluorescence test

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Определение репродуктивных свойств вируса классической чумы свиней вирулентных и вакцинных штаммов в первичных и перевиваемых культурах клеток

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РЕЗЮМЕ

Классическая чума свиней относится к особо опасным болезням этого вида животных, вспышки которой ежегодно нотифицируют в ряде стран. Несмотря на наличие средств специфической профилактики, опасность распространения заболевания в отдельной стране или во всем мире в целом сохраняется до сих пор. В связи с этим применение высокочувствительных методов ранней диагностики инфекции и определение наличия циркуляции вируса в природе являются необходимыми при надзоре и эрадикации данной болезни. Разработка новейших методов диагностики опирается на хорошо разработанную систему культивирования вируса, поэтому повышение уровня репродукции вируса классической чумы свиней, изучение возможности использования новых, лишенных эндогенной контаминации линий клеток до сих пор остается весьма актуальной задачей. Данная работа посвящена изучению чувствительности первичных и перевиваемых культур клеток к вирусу классической чумы свиней (вакцинных штаммов и ряда полевых изолятов, выделенных на территории России) с детекцией динамики его репродукции при помощи полимеразной цепной реакции с гибридизационно-флуоресцентной детекцией в режиме реального времени. Также проведен анализ интенсивности репродукции вируса в первичных и перевиваемых культурах клеток, при этом установлено, что вирус классической чумы свиней не обладает способностью к размножению в некоторых из них без предварительной адаптации. Показано, что для накопления вируса как полевых изолятов, так и вакцинных штаммов целесообразно применять первичные культуры клеток тестикул свиньи и тестикул ягнят при добавлении в питательную среду 10%-й нормальной серонегативной к вирусу классической чумы свиней сыворотки свиньи, а не фетальной бычьей сыворотки. Определены параметры культивирования и оптимальный состав поддерживающих питательных сред, использование которых способствует увеличению накопления вируса в 4–10 раз как в первичных, так и в перевиваемых культурах клеток.

Ключевые слова: классическая чума свиней, изоляты вируса классической чумы свиней, вакцинные штаммы, культуры клеток, обратно-транскриптазная полимеразная цепная реакция с гибридизационно-флуоресцентной детекцией в режиме реального времени, реакция прямой иммунофлуоресценции

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INTRODUCTION

Classical swine fever (CSF) is a highly contagious infectious disease characterized by fever, blood-vascular system, respiratory and gastrointestinal tract disorders, high morbidity and lethality in domestic pigs and wild boars [1].

According to the World Organization for Animal Health (OIE) data for 2021, only 38 countries located in different continents are CSF free, there are CSF free zones in three countries. CSF situation in Asian and South American countries are complicated.

In the disease-affected countries, CSF causes significant economic losses in pig industry due to decreased animal performance or animal deaths as well as mandatory stamping out carried out for the disease outbreak eradication. Successful pig industry development requires permanent improvement of the available diagnostic test methods, scientifically justified approaches to this highly dangerous disease control and prevention [2–3].

Modern diagnostic methods are essential tools for the agent detection and study of the infectious process progression features in the infected territory. Based on the analysis of currently used methods, they can be classified by either detection target (detection of virus, antigens, antibodies and genome analysis) or test purpose (detection, identification, monitoring, retrospective diagnosis, etc.) [3].

The following main methods are used for CSF virus and CSFV antigen detection: virus isolation in cell cultures followed by detection with direct immunofluorescence test (DIFT), immunohistochemistry (IHC), polymerase chain reaction (PCR) and, of course, bioassay in susceptible animals. Indirect immunofluorescence test (IIFT) is used for retrospective diagnosis and enzyme-linked immunosorbent assay (ELISA) is used for virus-specific antibodies detection [4–5].

In case of CSF suspicion, the disease is finally diagnosed with laboratory tests demonstrating presence of

the agent/genome or specific antibodies when the animals were not vaccinated against CSF [6].

Generally, two or three serial passages in various primary or continuous CSF-susceptible cell cultures are required to increase the pathogen amount in order to overcome sensitivity limit of the diagnostic method used for testing of the sample for the virus [7].

Since the CSF virus has no destructive effect on cells, an additional detection method is used for rapid detection of CSF reproduction in the culture, for example, such as real-time reverse transcription polymerase chain reaction (rtRT-PCR) [8].

Bioassay in susceptible animals is often used for CSF virus isolate diagnosis and tests of CSFV isolates for their biological properties at research laboratories [9].

Virus isolation by inoculation of the potentially virus-containing material in susceptible cell culture is generally used for tests for viable virus. As CSF virus is capable of replicating in continuous porcine kidney cell culture (PK-15), swine peripheral blood leukocyte (PBLs) culture, porcine splenocyte (PS) culture, porcine bone marrow (PBM) cell culture, the CSF virus isolation is carried in one of the said cultures [10].

Since CSF virus does not induce any apparent cytopathic effect (CPE) during its reproduction in different cell lines, additional method is required for detection of the virus reproduction, for example, direct immunofluorescence test (DIFT) or RT-PCR.

Cell cultures are also used for CSF virus accumulation for diagnostic purposes (for antigen scaling-up and analysis of the genome structure, etc.). However, there are some disadvantages when CSF virus is scaled up in primary cell cultures: tissue cell culture preparation is laborious, cell source for the culture preparation is not standardized that hampers reproducibility of individual experiment results, etc.

New effective CSF virus cultivation systems are selected for standardization of diagnostic, virological and molecular-biological tests that allow stable virus antigen and genome accumulation up to the large amounts [11–12].

Owing to live attenuated vaccine development and improved methods for CSF virus cultivation in different cell lines, CSF virus can be successfully accumulated *in vitro* in continuous porcine kidney cell line (IBRS), lamb testicle (LT) cell line, porcine kidney (PK) cell line, etc. [13–14].

Nevertheless, high-quality reproduction system development requires solving of the problems associated with the primary cell cultures used for virus-containing material scaling up for research purposes, or CSFV adaption to reproduction in continuous cell lines [11].

Use of the CSF virus preliminary adapted to growth in continuous cell culture lines could be helpful for partial overcoming of the said challenges and significantly simplifies cultivation process and standardizes the results of many diagnostic and experimental studies, but the number of adapted virus strains is limited [13, 15].

New CSF virus isolates are analyzed for their reproduction levels by cultivation in cell cultures that allows their biological properties to be determined and the virus-containing material to be accumulated for the virus genome extraction widely used for the determination of the agent relationship and possible disease spread routes [16].

The study was aimed at selection of the virus strains having high replication rates *in vitro* and testing of CSF virus strains for their reproduction properties in different cell cultures.

MATERIALS AND METHODS

The following CSF virus strains were used:

– "Sinlak" vaccine strain prepared from lapinized "K" strain through intermittent passages in PK-13, A_4C_2 cell cultures and adult rabbits (the strain was provided by the Laboratory for Porcine and Horned Livestock Diseases of the FGBI "ARRIAH");

– "CSF Amur 19-10/WB-12555" strain isolated from wild boar in the Amur Oblast in 2019;

– reference "Shi-Mynn" strain at 48th passage in pigs.

The following cell cultures obtained from the Cell Culture Unit of the FGBI "ARRIAH" Innovation Department were used for tests of different cell cultures for their susceptibility to CSF virus: primary cell cultures (PK – porcine kidney, PT – porcine testicle, LT – lamb testicle cell cultures) and continuous cell cultures (PEK – porcine embryo kidney, PSGK – Siberian ibex kidney, SSs – porcine spleen (a subculture, underwent more than 70 passages), IBRS – porcine kidney cell cultures).

The following medium was used as a minimal essential medium (MEM) for CSF virus cultivation in different cell cultures: Eagle-MEM prepared according to the FGBI "ARRIAH" procedure, supplemented with 10% fetal bovine serum and containing 50 μg/cm^3 of gentamycin, 2.5 mg/1,000 cm^3 of amphotericin B and 0.3 mg/cm^3 of glutamine.

Confluent and subconfluent cell monolayers were infected with CSF virus at multiplicity of infection of at least 0.1–1.0 $CCID_{50}$/cell.

The cell cultures were infected as follows:

1. Infection without adsorption – the virus was inoculated in culture flasks containing completely formed monolayer of 2–3-day-grown primary and continuous cell cultures.

2. Infection without adsorption – the cell culture was inoculated with the virus and left at 37 °C for 60 minutes, then, a required amount of the minimal essential medium was added to the culture flask. The flask with uninfected cell culture where the minimal essential medium was changed only served as control. The cell culture was daily observed for changes and detachment of the cells from the flask walls under CKX41 PhP FL US50 inverted laboratory microscope with binocular tube (Olympus, Japan).

3. Infection with minimal essential medium changing – Eagle-MEM supplemented with 10% normal porcine serum (freshly prepared and frozen serum was used for comparative testing) was used as a minimal essential medium. The medium was changed before cell culture infection by adding of 10 mL of Eagle-MEM containing 10% normal porcine serum.

The cell culture-containing plastic flasks with 25 cm^2 growth surface (T25) were incubated in thermostat or in CO_2 incubator at (37 ± 2) °C for 72–96 hours. Then, the following samples were taken from the culture flasks for testing with rtRT-PCR:

– from culture medium, at least 100 μL;

– from cell suspension, at least 100 μL.

Table 1
Results of rtRT-PCR tests of culture fluid and cell suspension samples collected on day 3–4 of CSF virus cultivation in primary and continuous cell cultures ($n = 3$)

CSF virus strain	Cell culture	Average Ct*	
		for cell culture	for cell suspension
"CSF Amur 19-10/WB-12555"	PK	16.4	18.27
	PSGK	18.25	20.01
	PEK	19.66	21.48
	SSs	15.52	17.02
"Shi-Mynn"	PK	17.83	19.11
	PSGK	17.63	19.64
	PEK	17.94	19.86
	SSs	15.85	17.19

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

Table 2
Results of rtRT-PCR tests of culture fluid samples collected during CSF virus cultivation in primary and continuous cell cultures when the cell monolayer was infected with or without adsorption ($n = 3$)

CSF virus strain	Cell culture	Average Ct*	
		inoculation without adsorption	inoculation with adsorption
"CSF Amur 19-10/WB-12555"	SSs	16.27	20.91
	PK	16.22	16.26
	PEK	13.76	17.74
	PSGK	15.18	15.50
"Shi-Mynn"	SSs	10.78	10.82
	PK	12.97	14.37
	PEK	16.03	16.51
	PSGK	12.14	12.17

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

The subsequent passaging was carried out by direct transfer of the virus-containing suspension to fresh cell culture or after thrice freezing-thawing of the culture fluid of the previous passage.

In case of absence of apparent CSFV-induced cytopathic effect the samples were tested for the virus and virus antigen with direct immunofluorescence test in accordance with the "Methodical Guidelines for classical swine fever virus isolation in different cell cultures followed by the virus identification with immunofluorescence test" [17], or for the virus RNA with rtRT-PCR in accordance with "Methodical Guidelines for classical swine fever virus isolation in primary cell cultures (PS, PBM, PK, PT, LT) followed by the virus identification with real-time polymerase chain reaction including detection using fluorescent hybridization probes" [18].

RESULTS AND DISCUSSION

Comparative analysis of virulent "CSF Amur 19-10/WB-12555" strain and reference "Shi-Mynn" strain as well as vaccine "Sinlak" strain accumulation levels was carried out for determination of CSF virus replication peculiarities in primary and continuous cell cultures.

The replication rate was estimated based on the time of maximum virus release in the minimal essential medium. For this purpose, culture fluid and cell monolayer samples collected on day 3–4 of cultivation were tested with rtRT-PCR (according to the test-kit manufacturer's instruction) providing that the highest cycle threshold (Ct) values correlated to the minimum CSF virus accumulation [19].

The test results given in Table 1 show that more than 90% of the virus-containing material were found in the culture fluid rather than in the cells on day 3–4 of the CSF virus cultivation. Maximum virus accumulation with the virus release in the minimal essential medium was observed in PK and SSs cell cultures.

At the next stage, two methods for cell infection: virus inoculation directly to the minimal essential medium and virus inoculation with adsorption on the cell monolayer for one hour were analyzed for their effectiveness. Accumulation levels were assessed with rtRT-PCR based on Ct values as in previous tests.

Based on the data given in Table 2 there were no significant differences in the virus accumulation in the cell culture when the virus-containing material was inoculated with adsorption on monolayer and directly to the minimal essential medium. The only one significant difference was observed for "CSF Amur 19-10/WB-12555" strain during its replication in SSs cell culture when Ct value was 20.91 in the cells infected with adsorption and 16.27 in the cells infected without adsorption.

Thus, analysis of the results of cultivation in four different cultures showed that effectiveness of the cell culture infection by CSF virus inoculated with adsorption on the cell monolayer was higher by 8.8% as compared to the infection by CSF virus inoculated directly to the minimal essential medium.

Ten percent inactivated normal porcine serum was added to the minimal essential medium to enhance the cell culture regenerative capacity and to increase the number of the cells attached to the culture flask walls. CSFV "Sinlak" vaccine strain was additionally used for comparative tests of the virulent CSFV strains.

Before the experiment and data analysis, "Sinlak" vaccine strain of CSF virus was subjected to three serial passages in primary PT and LT cell cultures to achieve desired Ct values (10–12) when it was tested with rtRT-PCR. Then, the virus was accumulated in the minimal essential medium supplemented with 10% inactivated normal porcine serum and parallelly in minimal essential medium supplemented with 10% fetal bovine serum for comparative analysis.

Analysis of the data given in Table 3 shows that CSF virus replication rate was the highest when the nutrient medium was changed and supplemented with 10% normal porcine serum. Significant decrease in Ct values indicative of increase in the virus titres was observed under the said conditions both for virulent and vaccine CSF virus strains.

Ct values detected with rtRT-PCR were found to be the lowest in primary PT and LT cell cultures (for the vaccine strain), that correlated with the higher virus accumulation in the said cell cultures. The said correlation was observed for both vaccine and virulent virus strains. Analysis of Ct values for the samples taken from PK and IBRS cell cultures allows us to conclude that these cultures are ineffective for the virus accumulation, therefore, CSF virus requires preliminary adaptation to these cell cultures.

Thus, PT cell culture was found to be 58% more effective for CSF virus accumulation (virulent strains and vaccine strain) as compared to LT, PK and IBRS cell cultures.

In subsequent experiments, the effectiveness of the freshly prepared and frozen normal pig sera use in the minimal essential medium changed during the cultivation was compared.

Test results showed that the highest Ct values were detected with rtRT-PCR when the frozen porcine serum was used. However, it cannot be excluded that freshly prepared porcine serum used in this experiment could contain a large number of anti-CSFV antibodies and, consequently, reduced the virus replication rate. Hence, porcine serum should be tested for anti-CSFV antibodies with ELISA prior to its adding to the minimal essential medium.

According to the results of the set of experiments, it was found that the CSF virus demonstrated the best reproductive properties in the primary PT cell culture, the average cycle threshold (Ct) value was 12.89 when the said cell culture was used.

Table 3
Results of rtRT-PCR tests of culture fluid samples collected during CSF virus cultivation in primary and continuous cell cultures with or without changing of minimal essential medium supplemented with 10% normal porcine serum (*n* = 3)

CSF virus strain	Cell culture	Average Ct*	
		without MEM changing	with MEM changing
"Sinlak"	PT	9.41	8.22
	LT	12.18	14.29
	PK	23.84	21.93
"CSF Amur 19-10/WB-12555"	PT	11.09	10.69
	PK	14.98	14.52
	IBRS	16.56	16.10
"Shi-Mynn"	PT	8.08	7.17
	PK	12.37	11.71
	IBRS	13.83	13.74

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

Table 4
Results of rtRT-PCR tests of culture fluid samples collected during CSF virus cultivation in primary porcine testicle and lamb testicle cell cultures in the minimal essential medium supplemented with freshly prepared or frozen 10% porcine serum (*n* = 3)

CSF virus strain	Cell culture	Average Ct*	
		when freshly prepared porcine serum was added	when frozen porcine serum was added
"Sinlak"	LT	17.57	13.26
	PT	16.48	11.16
"CSF Amur 19-10/WB-12555"	PT	15.63	14.71
"Shi-Mynn"	PT	15.09	12.08

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

CONCLUSION

Since diagnosticum development requires accumulation of CSFV antigen and genome in large amounts, the main goal of the study was to achieve high CSF virus reproduction in cell culture. Maximum CSFV accumulation with the virus release in the minimal essential medium was registered on day 3–4 of cultivation. The virus inoculation of the cell culture with adsorption was found to be ineffective. It is reasonable to add 10% porcine CSFV antibody-negative sera instead of fetal bovine serum.

Based on the tests of vaccine and virulent CSFV strains for their reproductive properties in primary and continuous cell cultures, the following strains are selected for further development of diagnostica: the "Sinlak" vaccine strain that demonstrated effective accumulation in PT and LT cell cultures when the minimal essential medium was supplemented with 10% normal porcine serum and virulent "CSF Amur 19-10/WB-12555" strain originating from the isolate recovered from a wild boar in the Amur Oblast of the Russian Federation in 2019 that better accumulated in PT cell cultures.

Genetic analysis of these CSF virus variants is required for determination of unique genetic markers allowing differentiation between CSFV strains and isolates for further tests of the selected strains for their use for diagnostic purposes.

In the above context, it should be noted that the development of DIVA strategy as an improved tool that can be successfully used in CSF-enzootic countries for CSFV circulation monitoring as well as in CSF-affected countries for minimization of the virus spread and economic losses in case of the disease outbreak is critical for CSF eradication and spread control.

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