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Study of cultural properties of canine enteric coronavirus isolate in different cell cultures

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SUMMARY

The etiology of the diseases affecting gastrointestinal tract of dogs is variable. The second most important enteric viral pathogen in the world after parvovirus is coronavirus (CCoV). Vast studies by scientists from different countries evidence the ubiquitous spread of coronavirus infection in dog populations. In this regard, the prevention of this disease is still an acute problem; firstly this means the development of effective vaccines, which can provide a reliable protection from the infection. The aim of this work was to study the CCoV isolate cultural properties, the selection of cell cultures most suitable for its reproduction and optimization of virus cultivation parameters in sensitive cell lines. The CCoV isolate recovered from the pathological material of a dead puppy with enteritis symptoms was used in the study. Seven continuous and two primary trypsinized cell cultures were tested during the study and it was established that the most sensitive cell culture for the reproduction of this isolate was continuous Crandell-Rees Feline Kidney Cells (CRFK) in which a typical cytopathic effect was noted on Day 2 of the cultivation. Virus infectivity titer in this culture was 3.58 ± 0.14 lg $TCID_{50}/cm^3$. It was established that such cell lines as feline spleen cells (FS) and primary trypsinized kitten spleen cells (KS) are also sensitive to the CCoV isolate but less than CRFK cells. Effect of such parameters as multiplicity of infection, cultivation time, inoculation technique, adsorption period and cell line age on coronavirus growth rate was studied for some selected cell cultures. It was concluded that CRFK and FS cells can be used for the propagation of viral material to develop diagnostic tools and vaccines against canine enteric coronavirus.

Keywords: canine coronavirus (CCoV), canine enteric coronavirus, canine intestinal infections, virus culture, continuous cell lines, primary trypsinized cells

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Изучение культуральных свойств изолята возбудителя коронавирусного энтерита собак в различных культурах клеток

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РЕЗЮМЕ

Этиология заболеваний, сопровождающихся поражением желудочно-кишечного тракта у собак, многообразна. Вторым наиболее распространенным у собак энтеропатогеном вирусной природы в мире после парвовируса является коронавирус CCoV. Обширные исследования, проведенные учеными из разных стран, доказывают повсеместное распространение коронавирусной инфекции в популяции собак. В связи с этим не теряют актуальности вопросы профилактики данного заболевания, в первую очередь изготовление эффективных иммунобиологических препаратов, обеспечивающих надежную защиту от данной инфекции. Целью настоящей работы являлось изучение культуральных свойств изолята CCoV, выбор наиболее подходящей для его репродукции клеточной системы и оптимизация параметров культивирования вируса в чувствительных клеточных линиях. В опытах использовали изолят CCoV, выделенный из патологического материала, полученного от погибшего щенка с симптомами энтерита. В ходе исследований было испытано семь перевиваемых и две первично трипсинизированные культуры клеток и установлено, что наиболее чувствительной клеточной системой для репродукции данного изолята является перевиваемая культура клеток почки кошки CRFK, в которой на 2-е сут культивирования отмечалось характерное цитопатическое действие. Титр инфекционной активности вируса в данной культуре был на уровне 3,58 ± 0,14 lg $ТЦД_{50}/см^3$. Определено, что такие клеточные линии, как перевиваемая культура клеток селезенки кошки (FS) и первично трипсинизированная культура клеток селезенки котенка (СК), также чувствительны к изоляту CCoV, но в меньшей степени, чем CRFK. Также в ходе опытов изучалось влияние на накопление коронавируса таких показателей, как множественность заражения, время культивирования, способ заражения, срок предварительного контакта (адсорбции) вируса с культурой клеток и возраст клеточной системы для некоторых выбранных культур клеток. Сделан вывод, что линии клеток CRFK и FS могут быть использованы для получения вирусного материала с целью разработки средств диагностики и специфической профилактики коронавирусного энтерита собак.

Ключевые слова: коронавирус собак (CCoV), коронавирусный энтерит собак, кишечные инфекции собак, культивирование вирусов, перевиваемые культуры клеток, первично трипсинизированные культуры клеток

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INTRODUCTION

Symptoms of gastrointestinal disorders in puppies are a frequent reason for visits to veterinary clinics. The etiological factors responsible for their occurrence are diverse. Canine coronavirus (CCoV) is the second most common enteric pathogen in the world after canine parvovirus (CPV) responsible for the viral diseases [1–3].

Serological and virological tests have shown that CCoV is widespread in the dog population, especially in kennels and animal shelters [4]. It is also reported in wild canines, including foxes, raccoon dogs and wolves [5–7]. Since the late 1990s and in the 2000s, scientists from different countries have repeatedly identified highly virulent strains of the coronavirus in dogs [8–10]. Pantropic CCoV (pCCoV) isolated in Italy in 2005 causes systemic disease in puppies followed by death [11]. In 2008, pantropic CCoV-associated disease was reported in France and Belgium [12], in 2010 in Greece [13]. In 2020, a large number of gastroenteritis cases caused by canine coronavirus infection were reported in the UK [14, 15].

The results of molecular tests conducted in Italy and Australia suggest that the virus is changing and its virulence is gradually increasing [16]. In this regard, the development of vaccines that provide reliable protection against new, including pantropic, strains of canine coronavirus remains critical, since existing vaccines have shown limited effectiveness [17].

The purpose of this work was to study the cultural properties of the canine coronavirus isolate in primary and continuous cell cultures for further use in immunobiological production.

MATERIALS AND METHODS

The CCoV isolate recovered from pathological samples from a dead puppy with enteritis symptoms was used in the tests.

To study the cultural properties of the virus, the following cell cultures were selected:

– trypsinized primary cells and subcultures: kitten kidney (KK) cells and kitten spleen (KS) cells;

– continuous cell cultures: feline kidneys (CRFK), feline spleen (FS), canine kidneys (MDCK), as well as African green monkey kidney (Vero-76), mouse neuroblastoma (Neuro-2a), porcine kidney (IB-RS-2) and domestic goat gonad (YADK-04).

Cell cultures were obtained from the Cell Culture Sector of the FGBI "ARRIAH".

Two methods of cell culture inoculation were used:

– inoculation of a cell culture monolayer: the virus was inoculated in a completely formed monolayer of primary trypsinized and continuous cell cultures;

– inoculation of cell suspension: the virus was inoculated in cell suspension during seeding.

The virus was cultured in 25 cm^2 polystyrene cell culture flasks (T25).

Before inoculation of the cell monolayer with the virus, the nutrient medium was removed from the flasks. Then the virus was inoculated and cell culture flasks were incubated for 60 minutes in CO_2 incubator at (37.0 ± 0.5) °C for contact (adsorption) of the virus to the cell monolayer. After contact (adsorption), a maintenance medium was added to the flasks. For maintenance a semi-synthetic nutrient medium was used, prepared according to the formula by the FGBI "ARRIAH", with the addition of 2% fetal bovine serum, 2% L-glutamine, streptomycin (100 μg/cm^3) and penicillin (100 U/cm^3).

At the end of the cultivation time, each flask was frozen at a temperature of minus (45 ± 5) °C and thawed at (20 ± 2) °C. Monolayer was disaggregated by periodic shaking of the flask, then samples were taken from the flasks to determine the titer of the CCoV isolate infectivity.

The CCoV isolate infectivity was determined in CRFK cell line grown in 96-well plates Costar® (Corning, USA) by microtitration using a generally accepted procedure. Microplates were examined daily using Olympus SKX53 inverted microscope (Japan), the number of wells with a typical cytopathic effect (CPE) was recorded. The infectivity titer was calculated using the Kerber method and expressed in lg $TCID_{50}$/cm^3. Samples were additionally tested for canine enteric coronavirus using commercial chromatographic immunoassay kits Asan Easy Test® CCV Ag (Asan Pharmaceutical Co., Ltd., Republic of Korea).

RESULTS AND DISCUSSION

To study the susceptibility of various cell cultures to the CCoV isolate, five serial passages were made in continuous (CRFK, MDCK, FS, Vero-76, YADK-04, Neuro-2a, IB-RS-2) and trypsinized primary cells and sub-cultures (KK, KS). The presence of the CCoV antigen in the culture fluid after each passage was determined

Table 1
Sensitivity of different continuous cell lines to the CCoV isolate ($n = 3$)

Passage	Infectivity titer, lg $TCID_{50}/cm^3$						
	CRFK	MDCK	FS	Vero-76	YADK-04	Neuro-2a	IB-RS-2
1	3.08 ± 0.14	1.25 ± 0.25	2.50 ± 0.25	< 1.0	< 1.0	< 1.0	< 1.0
2	3.33 ± 0.14	< 1.0	2.92 ± 0.14	< 1.0	< 1.0	< 1.0	< 1.0
3	3.58 ± 0.14	< 1.0	3.08 ± 0.14	–	–	–	–
4	3.58 ± 0.14	–*	3.08 ± 0.14	–	–	–	–
5	3.58 ± 0.14	–*	3.08 ± 0.14	–	–	–	–

* not tested.

by immunochromatographic test kits (ICT). The results of the tests performed are given in Table 1.

It was found that CCoV isolate actively replicated and accumulated only in CRFK and FS lines. In other cell cultures, CCoV did not replicate. The maximum viral growth rate was observed from Passage 3 in CRFK cell culture (3.58 ± 0.14 lg $TCID_{50}/cm^3$), and microscopic examination revealed typical CPE in the cell culture starting from Passage 1 (Fig. 1–4). It was manifested by cell rounding, cytoplasm degeneration, symplast formation and gradual destruction of the monolayer.

In FS cell lines, the virus accumulated in smaller quantities, starting from Passage 3 the titer was 3.08 ± 0.14 lg $TCID_{50}/cm^3$, no typical CPE was observed during microscopic examination. When culturing the virus in MDCK cell line, minimal viral growth rate in Passage 1 was noted equal to 1.25 ± 0.25 lg $TCID_{50}/cm^3$, however, further microtitration in CRFK cell line did not reveal a typical CPE, and at Passage 4 ICT gave a negative result. In other cell lines, the passaging of the CCoV isolate turned out to be impractical after Passage 2 since microtitration in CRFK-containing wells showed no typical CPE, and the ICT demonstrated the lack of the viral antigen.

Results of the CCoV isolate cultural properties analysis in KS and KK trypsinized primary and subculture cells are presented in Table 2.

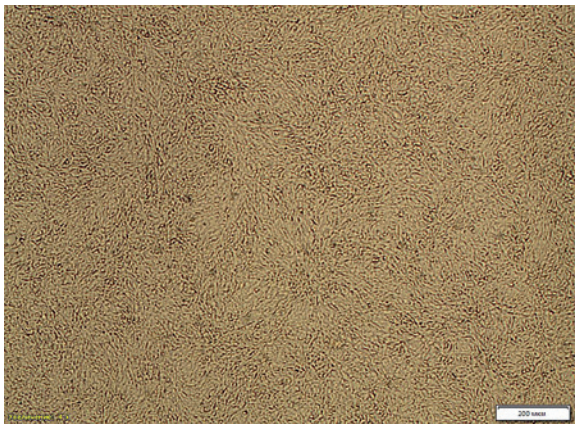

Fig. 1. CCoV not-inoculated CRFK monolayer after 3 days (40× magnification)

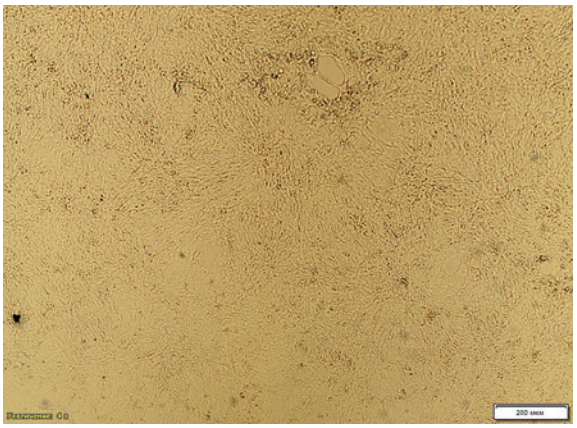

Fig. 2. CRFK monolayer 2 days post inoculation with CCoV isolate (40× magnification)

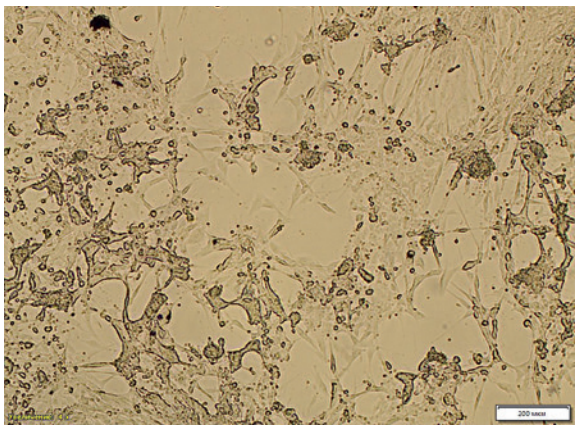

Fig. 3. CRFK monolayer 3 days post inoculation with CCoV isolate (40× magnification)

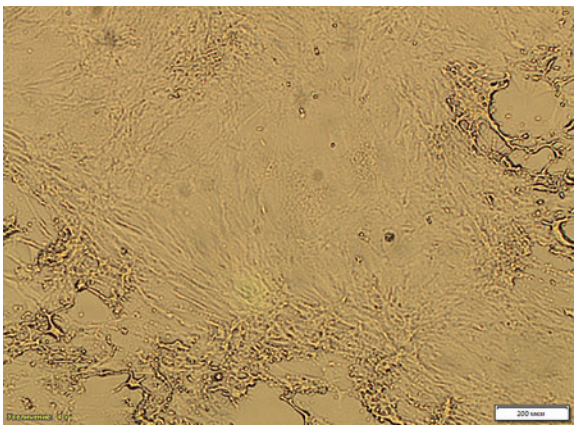

Fig. 4. CRFK monolayer 3 days post inoculation with CCoV isolate (100× magnification)

Virus cultivation in KK trypsinized primary and subculture cells led to a gradual decrease in its infectivity. At Passage 2, ICT showed a positive result for the canine enteric coronavirus antigen. However, the titration in CRFK cell line did not demonstrate typical CPE in the plate. At Passage 3, the ICT result was negative, therefore, further cultivation of the virus in the KK trypsinized primary and subculture cells became impractical. Cultivation of CCoV isolate in KS trypsinized primary and subculture cells resulted in a gradual viral growth, with the infectivity titer of 3.42 ± 0.14 lg $TCID_{50}/cm^3$ at Passage 5. At the same time, no typical CPE was observed in this cell culture.

It should be noted that continuous CRFK and FS cell lines can be considered most suitable of all the tested cell cultures for this CCoV isolate reproduction.

Table 2
CCoV isolate reproduction in primary trypsinized and subculture cells (KS and KK), *n* = 3

Cell culture	Passage	Cultivation time, h	Infectivity titer, lg $TCID_{50}/cm^3$	ICT result
KS	1	72	1.50 ± 0.25	+
	2	72	2.58 ± 0.14	+
	3	72	2.92 ± 0.14	+
	4	72	2.92 ± 0.14	+
	5	72	3.42 ± 0.14	+
KK	1	72	2.33 ± 0.14	+
	2	72	< 1.0	+
	3	72	–*	–

* not tested.

Table 3
CCoV isolate growth dynamics depending on culture time in continuous, primary and subculture cells (*n* = 3)

Cell culture	Infectivity titer, lg $TCID_{50}/cm^3$			
	48 hours	72 hours	96 hours	120 hours
FS	2.75 ± 0.25	3.17 ± 0.14	2.83 ± 0.29	2.67 ± 0.14
KS	3.00 ± 0.25	3.42 ± 0.14	3.17 ± 0.14	3.08 ± 0.29

Table 4
Effect of multiplicity of infection on CCoV isolate infectivity (*n* = 3)

Cell culture	MOI, $TCID_{50}$/cell	Cultivation time, hours	Infectivity titer, lg $TCID_{50}/cm^3$
CRFK	0.1	48	3.25 ± 0.25
	0.01	72	3.58 ± 0.14
	0.001	96	1.50 ± 0.25
	0.0001	96	1.08 ± 0.14
FS	0.1	72	3.17 ± 0.14
	0.01	72	3.08 ± 0.14
	0.001	72	2.92 ± 0.38
	0.0001	72	2.08 ± 0.14

MOI – multiplicity of infection.

Further studies included the analysis of the effect of the following factors on viral growth rate: the time of virus cultivation in cell cultures in which the CCoV isolate did not produce CPE, the multiplicity of infection, inoculation method, the period of viral contact (adsorption) to cells and the age of cell lines.

To study the dynamics of the CCoV isolate growth rate depending on the cultivation time, cell cultures in which the CCoV isolate did not produce typical CPE and the monolayer remained compact throughout the entire cultivation were used. The above mentioned cultures included continuous FS cell line and KS trypsinized primary and subculture cells. CCoV isolate was cultured for 48, 72, 96 and 120 hours. The results of the tests performed are given in Table 3.

Analysis of the results showed that the cultivation of the CCoV isolate for 48 hours is insufficient for its maximum growth rate, the infectivity titer after Day 2 was the lowest. It was found that the most optimal time for coronavirus cultivation is 72 hours (3 days). The infectivity titer of the CCoV isolate was the highest and was equal to 3.17 ± 0.14 and 3.42 ± 0.14 lg $TCID_{50}/cm^3$ in FS and KS cell lines, respectively. With further cultivation after 96 and 120 hours in both cell cultures, the infectivity titer of the virus decreased.

To study the effect of the multiplicity of infection on the infectivity of the CCoV isolate, the following infection doses were used: 0.0001; 0.001; 0.01 and 0.1 $TCID_{50}$/cell. The tests were performed using two continuous CRFK and FS cell lines. Incubation of the virus in CRFK cell culture was stopped when 80% of the monolayer was destructed and cells detached from the glass. In FS cell lines, the virus was incubated for 72 hours, since earlier experiments showed that this time is optimal for maximum growth rate of the CCoV isolate in the specified cell culture. The results of the tests performed are given in Table 4.

It found that the CCoV isolate infectivity in CRFK cell culture with a multiplicity of infection of 0.01 $TCID_{50}$/cell was 3.58 ± 0.14 $TCID_{50}$/cell. At lower inoculation doses (0.0001 and 0.001 $TCID_{50}$/cell), the infectivity decreased significantly, and the time of CPE manifestation increased to 96 hours. At a multiplicity of infection of 0.1 $TCID_{50}$/cell, a faster CPE manifestation and 80% destruction of the monolayer was observed. However, the titer of the virus was lower and was at the level of 3.25 ± 0.25 lg $TCID_{50}/cm^3$, which is probably due to too rapid destruction of the monolayer, as a result of which the virus did not have enough time to accumulate in maximum concentration. Thus, the optimal multiplicity of infection of CRFK cell line with the CCoV isolate is 0.01 $TCID_{50}$/cell.

The infectivity of CCoV isolate in FS cell culture at multiplicity of infection of 0.1 and 0.01 $TCID_{50}$/cell was 3.17 ± 0.14 and 3.08 ± 0.14 lg $TCID_{50}/cm^3$, respectively. At lower inoculation doses (0.0001 and 0.001 $TCID_{50}$/cell), the infectivity of the virus decreased, therefore it is advised to inoculate FS cell line with a dose of 0.01 $TCID_{50}$/cell.

At the next stage of the work, two methods of inoculation of CRFK and FS cell lines were compared: direct inoculation of cell suspension with the virus and adsorption on the formed cell monolayer for an hour.

The results presented in Table 5 demonstrate that the titer of the CCoV isolate infectivity was maximal when it was

inoculated on the cell monolayer. In CRFK and FS cell lines, it was 3.58 ± 0.14 and 3.17 ± 0.14 lg $TCID_{50}/cm^3$, respectively. Inoculation of CRFK and FS cell suspension gave a lower infectivity of the virus.

Also in this study, the effect of such a factor as the time of preliminary contact (adsorption) of the virus with the CRFK monolayer on the dynamics of CCoV isolate accumulation was analysed. The results of the tests performed are given in Table 6.

It was found that the cultivation of the CCoV isolate without prior contact (adsorption) with the CRFK monolayer was accompanied by a low viral growth, the infectivity titer was at the level of 2.42 ± 0.14 lg $TCID_{50}/cm^3$. With an increase in the time of preliminary contact (adsorption) of the virus with the monolayer, the infectivity titer of the CCoV isolate gradually increased from 3.08 ± 0.14 lg $TCID_{50}/cm^3$ if adsorption lasted for 30 minutes and to 3.33 ± 0.14 lg $TCID_{50}/cm^3$ with 60 minute adsorption. Subsequently an increase in the adsorption time to 90 minutes led to a slight decrease in the infectivity. Thus, 60 minutes can be considered the optimal time of the viral contact (adsorption) with the monolayer.

The final stage of the work was to study the effect of the cell culture age on the CCoV isolate infectivity. For this purpose, CRFK cell line was chosen, since this is the line in which the virus accumulates in maximum concentration and manifests visible CPE. For testing, a cell culture with a fully formed monolayer was used.

As it can be seen from Table 7, inoculation of the CCoV isolate on a one- and two-day CRFK monolayer, the infectivity titers of the virus did not differ and was equal to 3.08 ± 0.14 lg $TCID_{50}/cm^3$. When the virus was inoculated into three-day CRFK cells, the infectivity of the CCoV isolate increased insignificantly and amounted to 3.17 ± 0.14 lg $TCID_{50}/cm^3$. When a four-day cell culture was inoculated with the CCoV isolate, the growing of the virus decreased to 2.92 ± 0.14 lg $TCID_{50}/cm^3$, and the cultivation time increased to 96 hours. A decrease in the viral infectivity may be associated with aging of the CRFK cells and with a slowdown of metabolic processes in cells. Thus, to accumulate the CCoV isolate in the maximum amount, it is advisable to use CRFK cells with a fully formed 1–3-day monolayer.

CONCLUSION

In this work, the possibility of the CCoV isolate reproduction in various continuous and trypsinized primary cell lines of homologous and heterologous origin was studied. The study of the susceptibility of continuous cells to CCoV isolate revealed that CRFK and FS cell lines are effective for the preparation of a highly active viral suspension. These cell lines can be used to prepare viral material for developing diagnostic tools and vaccines against canine enteric coronavirus. Studies have shown that the optimal dose of CRFK cell inoculation is 0.01 $TCID_{50}$/cell, the viral infectivity titer was 3.58 ± 0.14 lg $TCID_{50}/cm^3$. For FS cells, it is advisable to use an infection dose of 0.01 $TCID_{50}$/cell. It was established that the virus accumulates in maximum amounts when CRFK cells are inoculated into a fully formed 1–3-day cell monolayer and cultured for 48–72 hours. For FS cell culture, the optimal conditions included inoculation of the virus in the formed cell monolayer and cultivation for 72 hours.

Table 5
Effect of inoculation technique on CCoV isolate infectivity in continuous cells ($n = 3$)

Inoculation method	MOI, $TCID_{50}$/cell	Cultivation time, hours	Cell culture	Infectivity titer, lg $TCID_{50}/cm^3$
Monolayer	0.01	72	CRFK	3.58 ± 0.14
Suspension	0.01	48		3.17 ± 0.14
Monolayer	0.01	72	FS	3.17 ± 0.14
Suspension	0.01	72		2.75 ± 0.25

MOI – multiplicity of infection.

Table 6
Effect of contact (adsorption) period on CCoV isolate growth rate in CRFK cells ($n = 3$)

Parameters	Adsorption time, minutes			
	no contact (adsorption)	30	60	90
MOI, $TCID_{50}$/cell	0.01			
Infectivity titer, lg $TCID_{50}/cm^3$	2.42 ± 0.14	3.08 ± 0.14	3.33 ± 0.14	3.25 ± 0.25

MOI – multiplicity of infection.

Table 7
Effect of CRFK cell age on infectivity of CCoV isolate ($n = 3$)

Age of culture, days	Time of cultivation, hours	Infectivity titer, lg $TCID_{50}/cm^3$
1	48	3.08 ± 0.14
2	72	3.08 ± 0.14
3	72	3.17 ± 0.14
4	96	2.92 ± 0.14

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