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COMPARATIVE STUDIES OF FELINE VIRAL RHINOTRACHEITIS VIRUS FOR ITS REPLICATION PROPERTIES IN DIFFERENT CELL CULTURES

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SUMMARY

The results of comparative studies of feline viral rhinotracheitis (FVR) virus for its culture properties in primary and continuous cell cultures of feline origin (FK, FK (subculture), CrFK, FS, CC-81, FC/Tg) are presented. It was found that viral rhinotracheitis virus replication, irrespective of the route of infection and the culture technique, was consistent and practically equal in susceptible cell cultures. The most pronounced cytopathic effect (more than 75% monolayer degeneration) was observed in all types of cell cultures in 48–72 hours of cultivation. However, the accumulation of feline viral rhinotracheitis virus Grand strain was the highest when preliminary adsorption occurred within the specified period of time, monolayer cell cultures were infected with the virus at a dose of 5.5 lg $TCID_{50}$/ml and roller bottle cultivated, and the pH of the medium was maintained at 7.0–7.4. Single freezing of the virus at a temperature of minus 60 degrees Celsius upon the completion of the cultivation cycle (during 60–72 hours) and its thawing were found to significantly increase the virus titre by 0.5 lg $TCID_{50}$/ml.

Key words: feline viral rhinotracheitis (FVR), virus replication, primary cell culture, continuous cell culture.

INTRODUCTION

Feline viral rhinotracheitis (feline viral rhinitis, epizootic coryza, feline herpes, "sniffing tracheitis") is a widely spread contagious disease caused by FeHV-1 herpesvirus belonging to *Herpesviridae* family. Herpesvirus infection is one of the most important feline infectious diseases.

Upper respiratory infection was classified as a separate disease in kittens and described for the first time by R. A. Grandell et al. in the USA in 1958 [3, 4]. In Russian Federation the virus was isolated and it etiological role was described by the FGBI "VGNKI" staff-members in 1995 [2].

Development of modern highly efficient biologicals plays an important role in the specific disease prevention. Testing feline viral rhinotracheitis virus for its replication in homologous cell cultures and optimization of culture parameters are currently important issues.

The studies were aimed at testing feline viral rhinotracheitis virus grown in homologous primary and continuous cell lines for its cultural and infective properties.

MATERIALS AND METHODS

Cultured Grand strain of feline viral rhinotracheitis virus (FVR virus) was used for testing [2]. Primary feline kidney cells and their subcultures (FK FKS) as well as continuous feline spleen (FS), feline kidney (CrFK), feline tongue epithelium (FC/Tg) and murine Moloney sarcoma virus-transformed feline embryo cells (CC-81) were used for tests of various cell cultures for their susceptibility to FVR virus [1].

The following nutrient media were used as growth media: Hank's solution-based lactalbumin hydrolyzate, DMEM, MEM, EMEM with single or double amino acids supplemented with 5–10% fetal bovine serum, L-glutamine, 50 μm/ml gentamycin. Cells were cultivated at (37.0 ± 0.5) °C. The same media without serum were used as maintenance media.

Cell culture was grown in flasks and bottles of different volumes. Two-litre bottles were used for roller cultivation.

Primary-trypsinized and continuous cell culture plating concentrations were 300 ths cells/ml and 70 ths cells/ml when they subjected to stationary cultivation and 700 ths cells/ml and 150 ths cells/ml when they subjected to roller cultivation, respectively.

Media at the following pH levels were used for FVR virus cultivation in cell cultures: 6.8; 7.0; 7.2; 7.4; 7.6 and 7.8.

The virus was inoculated in cell cultures at ratio of 3.5–5.0 lg $TCID_{50}$ per 1 ml of suspension.

Two methods were used for cell culture inoculation.

1. Inoculation of monolayer cell culture – the virus was inoculated on completely formed monolayer of 3–5-day primary and 3–4-day continuous cell cultures. The virus was inoculated without its preliminary adsorption by the cells or after its adsorption (contact) by cells for 30, 60

Table 1
FVR virus Grand strain infectivity in homologous primary and continuous cell cultures
n = 3

Cell culture	Inoculation dose	Number of passages	Virus titre, lg $TCID_{50}$/ml
FK	5.5 lg $TCID_{50}$/ml	5	7.44 ± 0.06
FK subculture		5	7.50 ± 0.10
CrFK		5	7.39 ± 0.06
FS		5	7.50 ± 0.10
CC-81		5	7.44 ± 0.06
FC/Tg		5	7.30 ± 0.17

and 120 min. The cells were left to contact with the virus at 37 °C. Then, maintenance medium at the required volume was added. Non-inoculated culture where the medium was changed only served as control.

The monolayer was observed daily for cytopathic effect: changed cell shape, cell detachment from glass and its extent.

2. Inoculation of cell suspension – the virus was inoculated in cell suspension during its plating. The same control was used as described above and the cells were assessed for changes daily.

Samples of culture fluid were collected immediately after virus inoculation and then every 24 hours during the whole cultivation period (120 hours) for examination of FVR virus accumulation dynamics in inoculated cell cultures.

The flasks and roller bottlers were transferred to thermal room or in thermostat for further cultivation ((37.0 ± 0.5) °C).

Two cultivation methods were used.

1. Roller method – mixing was performed in two-litre bottles (Rollerbottle Cellmaster Cat. No. 680060 produced by Greiner Bio-One Company (Germany)) rotated at the rate of 8–12 rph in Wheaton roller apparatus (USA).

2. Stationary method – cell suspension was added to culture flasks and grown without mixing.

The flasks were put horizontally. The medium pH was maintained at 7.2–7.4 with glycolic buffer. Cell cultures, inoculated and non-inoculated, were observed under low-magnification microscope for cytopathogenic effect.

Inoculated cell cultures were tested for cytopathic effect (CPE) intensity. Changes in cell monolayers were recorded as follows:

+ – separate cell degeneration foci (25%);
++ – degeneration up to 50% of monolayer;
+++ – degeneration up to 75% of monolayer;
++++ – complete degeneration of monolayer surface.

When more than 75% monolayer degeneration was observed the virus-containing suspension was frozen at –60 °C until the next passage.

The virus infectivity was determined by micromethod in 96-well culture plates (Costar) using at least four parallel rows.

Ten-fold virus dilutions (from 10^{-1} to 10^{-8}) were made with maintenance medium for the virus titration. Each dilution starting with 10^{-8} was inoculated in four wells.

Growth medium was removed from each culture-containing well and 0.2 ml of the corresponding virus dilution was added. Each virus dilution starting with 10^{-8} was inoculated in four wells. Four non-inoculated wells with completely formed cell monolayer served as controls. Titration results were recorded for 7 days of incubation in CO_2-incubator at 37 °C and 5% CO_2.

Virus infectivity titre was expressed as $TCID_{50}$ (50% tissue culture infective dose) and calculated using Reed-Muench method.

RESULTS AND DISCUSSION

Results of tests of various cell cultures for their susceptibility to FVR virus are given in Table 1.

It was found that FVR virus replication in FK, FK subculture, CrFK, FS, CC-81, FC/Tg was consistent. No significant difference in susceptibility of the above-mentioned cell cultures to tested FVR virus was detected. The said differ-

Table 2
Tests of FVR virus Grand strain inoculated by different routes and cultivated by different methods in CrFK and FS cell cultures after cultivation for 48–72 hours
n = 3

Cultivation method	Inoculation method	Cell culture	Inoculation dose	Virus titre, lg $TCID_{50}$/ml
Roller	Monolayer	CrFK	5.5 lg $TCID_{50}$/ml	7.71 ± 0.11
	Suspension			5.78 ± 0.11
Stationary	Monolayer			6.61 ± 0.06
	Suspension			5.11 ± 0.11
Roller	Monolayer	FS		7.78 ± 0.11
	Suspension			5.89 ± 0.22
Stationary	Monolayer			6.72 ± 0.15
	Suspension			5.61 ± 0.06

Table 3
Dynamics of FVR virus Grand strain accumulation in continuous CrFK cell line depending on inoculation dose and cultivation time

Inoculation dose lg $TCID_{50}$/ml	Values	Virus titre, lg $TCID_{50}$/ml			
		Cultivation time, hours			
		48	72	96	120
5.5	$M \pm m$	7.00 ± 0.14	7.10 ± 0.08	6.92 ± 0.16	6.54 ± 0.08
	p	> 0.5	control	> 0.4	> 0.005
4.5	$M \pm m$	6.67 ± 0.10	6.75 ± 0.08	6.63 ± 0.04	6.42 ± 0.05
	p	> 0.5	control	> 0.2	> 0.01
3.5	$M \pm m$	5.37 ± 0.10	5.84 ± 0.10	6.38 ± 0.13	6.08 ± 0.02
	p	< 0,001	< 0.001	< 0.001	> 0.05

ence was not higher than 0.2 $TCID_{50}$/ml under the same cultivation and inoculation conditions.

The virus replication caused CPE. CPE was observed starting from the 1st passage onwards and was similar in the susceptible cell cultures.

First signs of herpesvirus-characteristic CPE were recorded 24 hours after the monolayer inoculation. They were as follows: cell swelling and rounding without monolayer disruption. Thirty-six hours after cell culture inoculation the CPE was manifested by formation of large round cell foci. At its onset the CPE was focal and then became diffuse. Thereafter, monolayer destruction progressed rapidly with gradual cell detachment and void formation. The most apparent CPE manifestation (degeneration of more than 75% of the monolayer) and maximum virus accumulation in all types of the cell cultures were observed 48–72 hours after inoculation (7.3–7.5 lg $TCID_{50}$/ml).

Obtained results showed that homologous cell cultures (FK, FK subculture, CrFK, FS, CC-81, FC/Tg) could be used for FVR virus cultivation.

Additionally, tests of FVR virus for its infectivity and period of its maximum accumulation in cell cultures, as well as tests of the effect of inoculation route and cultivation method, inoculation dose, preliminary virus adsorption time, medium pH on the virus infectivity and accumulation rate as well as effect of the virus-containing fluid freezing and thawing methods on the virus isolation from the inoculated cells were carried out.

Results of comparative tests of FVR virus grown under roller and stationary conditions for its infectivity depending on cultivation method and inoculation route are given in Table 2.

Results given in Table 2 show that the virus accumulation depended on used cultivation method and inoculation route.

The virus titre was maximum when it was inoculated in monolayer cell cultures. In particular, it was 7.71 ± 0.11 and 7.78 ± 0.11 lg $TCID_{50}$/ml in roller-cultivated CrFK and FS cell cultures, respectively, and 6.61 ± 0.06 and 6.72 ± 0.15 lg $TCID_{50}$/ml in stationary-cultivated CrFK and FS cell cultures, respectively. The virus inoculated in the suspension of the same cell lines did not accumulate to the titres higher than the above-mentioned: the virus titre was 5.78 ± 0.11 and 5.89 ± 0.22 lg $TCID_{50}$/ml in roller-cultivated cell suspensions and 5.11 ± 0.11 and 5.61 ± 0.06 lg $TCID_{50}$/ml in stationary-cultivated cell suspensions, respectively. No completely formed monolayer was observed.

Dynamics of FVR virus accumulation in CrFK cells depending on inoculation dose and cultivation time were examined for 4 days at a 24-hour interval (Table 3).

Analysis of the obtained results showed that the higher was inoculation dose of the virus the higher was the virus accumulation titre. It should be noted that time of maximum virus accumulation at minimum inoculation dose (3.5 lg $TCID_{50}$/ml) increased up to 4 days. Thus, it was shown that inoculation dose mainly had an effect on time of maximum virus accumulation. Virus inoculated at a dose of 5.5 lg $TCID_{50}$/ml caused FVR virus accumulation at titres of (7.0 ± 0.14)–(7.1 ± 0.08) lg $TCID_{50}$/ml after cultivation for 48–72 hours.

Preliminary contact (adsorption) of the virus to monolayer of tested cell lines is of great importance for optimal animal virus replication. During the said study the effect of duration of concentrated FVR virus Grand strain contact to CrFK cell culture monolayer was tested.

Test results were given in Table 4.

The Table 4 shows that cultivation of the virus without preliminary adsorption by cells and after adsorption for

Table 4
Effect of preliminary adsorption on FVRV Grand strain accumulation in CrFK cell culture after cultivation for 60–72 hours
n = 4

Criterion	Time of adsorption, min			
	Without adsorption (control)	30	60	120
Inoculation dose	5.5 lg $TCID_{50}$/ml			
Virus titre, lg $TCID_{50}$/ml $M \pm m$	6.54 ± 0.04	6.59 ± 0.05	7.00 ± 0.14	7.08 ± 0.16
p	–	> 0.4	> 0.01	> 0.01

Table 5
Replication of FVRV Grand strain in CrFK cell culture at different medium pH levels
n = 3

pH level of medium	Inoculation dose	Virus accumulation, lg $TCID_{50}$/ml	Difference significance (*p*)
6.8	5.5 lg $TCID_{50}$/ml	5.61 ± 0.06	< 0.001
7.0		7.00 ± 0.19	> 0.5
7.2 (control)		7.00 ± 0.19	–
7.4		7.11 ± 0.11	> 0.5
7.6		5.89 ± 0.11	< 0.005

30 minutes resulted in low virus accumulation at titres not higher than (6.54 ± 0.04)–(6.59 ± 0.05) lg $TCID_{50}$/ml. Whereas, the virus accumulation was maximum (virus titres: (7.00 ± 0.14)–(7.08 ± 0.16) lg $TCID_{50}$/ml) when the virus had been adsorbed by cells for 60–120 min.

It is known that optimal medium pH is important for creation of favorable conditions for the virus replication in cells. Medium pH levels for various viruses vary significantly depending on cell type (from 6.8 to 7.6).

It was found that FVR virus replicated in CrFK cell cultures at the highest titres when pH level of maintenance medium was 7.0–7.4 (Table 5).

Tests showed that single freezing of the cultured virus at –60 °C upon the cultivation procedure completion (for 60–72 hours) significantly increased the virus titre by 0.5 lg $TCID_{50}$/ml. The virus titre remained almost unchanged when the virus had been frozen-thawed two-three times.

CONCLUSION

Primary feline kidney cell cultures and feline kidney cell subcultures, continuous CrFK, FS, CC-81, FC/Tg cell cultures were equally susceptible to feline viral rhinotracheitis virus and suitable for its cultivation and the virus replicates in the said cultures causing apparent CPE.

The virus accumulated at the highest titres (7.71–7.78 ± 0.11 lg $TCID_{50}$/ml) under the following conditions:

- inoculation of monolayer cultures;
- roller cultivation;
- medium pH level maintained at 7.0–7.4;
- inoculation dose was 5.5 lg $TCID_{50}$/ml;
- preliminary adsorption of the virus by cells for 60–120 min;
- cultivation time was 48–72 hours;
- single freezing at –60 °C and thawing of the harvested virus upon the cultivation completion.

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