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# Investigation of bovine coronavirus strain CV-315 cultural properties

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The purpose of this study was to investigate the features of cultivation of bovine coronavirus strain CV-315 isolated in Ukraine from a calf with coronavirus infection and to select optimal methods of virus cultivation to obtain viral material with the highest possible titers of infectious activity in order to develop manufacturing technology of means of immunoprophylaxis and specific diagnostics. During the study, the influence of a number of factors on the accumulation of strain CV-315 was studied: the presence and concentration of trypsin in the nutrient medium, the effect of fetal bovine serum, the degree of cell culture monolayer during virus infection, also the virus dose, temperature and the term of cultivation. According to the results, it was established that bovine coronavirus strain CV-315 has the highest infectious activity when cultured for 72 hours before the manifestation of CPE of 70–80%, without the addition of trypsin and fetal bovine serum content of 2% at 37±0.5°C. It was also found that the optimal infective dose is 0.1–0.01 viral particles per cell for infection of the fully formed monolayer of MDBK cell culture. The obtained results will be used in the development of veterinary vaccines against bovine coronavirus.

**Key words:** bovine coronavirus, virus cultivation, cell culture, infectious activity of viruses, culture regimens

Coronaviruses are widespread in the environment and cause acute diseases not only in livestock and poultry, but also in humans. That is why studying the biological properties of coronaviruses and ascertainment of the conditions and modes of its cultivation should help solve global problems that arise in the world and are associated with the spread of dangerous socio-economic trends and also for a deeper understanding of epizootic processes.

Bovine coronavirus (BCoV) is a virus that causes disease in domestic and wild cows. BCoV belongs to the family *Coronaviridae*, an order of *Nidovirales* and a subfamily of *Orthocoronavirinae*, which is divided into 3 groups, which are formed depending on natural hosts, serological reactions (epitopes present in the glycoprotein shell) and nucleotide sequence of positive single-stranded RNA. BCoV belongs to 2nd group [3].

BCoV was first isolated in a primary trypsinized culture of bovine kidney cells [9], and then propagated in various cell lines, in particular: BEK-1 (bovine embryonic kidney) [7], VERO (African green monkey kidney) [6], MDBK (Madin-Darby bovibe kidney), PK-15 (pig kidney), HRT-18 (human rectal tumor) [12] with trypsin in a nutrient medium useful for enhancing replication.

Kapil et al. noted that the age of HRT-18 cells after reseeding affected the accumulation of the BCoV in the monolayer and the manifestation of the cytopathic effect (CPE). It was found that the best time to infect cell culture HRT-18 by BCoV is 24 hours after the monolayer of cells formation, because in such circumstances, cytopathic changes caused by bovine coronavirus, according to the authors, were most severe [8].

Conditions necessary for optimal accumulation of cell culture-adapted strains of BCoV have been repeatedly described by Dea et al. [4]. In their work, they showed that a weakly acidic inoculum (pH 6.5 to 7.0), cultivation in basic medium (pH 8.0 to 8.5) with trypsin (5 μg/cm$^3$) and treatment of cells with DEAE-dextran (25 μg/cm$^3$) are important factors influencing the increase in the accumulation of bovine coronavirus and the manifestation of CPE in Vero cells.

Storz et al. [13] also emphasized the importance of using trypsin for virus replication and manifestation of the cytopathic action of cell culture-adapted BCoV strain L9. Addition of trypsin (10 μg/cm$^3$) accelerated the appearance of CPE and plaques, promoted cell fusion, increased the amount of hemagglutinin released by cells and in-

creased the accumulation of virus in cultures of thyroid cells and brain of embryonic calves using the Minimum Essential Medium Eagle (MEM) antibiotics and 10% of fetal serum of calf or lamb, inactivated by heating. In this case, when the virus was pre-treated with trypsin or trypsin was present only during the adsorption of the virus, the increase in plaque formation was not observed.

Treatment of cells with trypsin may facilitate the attachment of virions to otherwise inaccessible receptor sites. Treatment of the virus with trypsin can change the configuration of protein molecules in the envelope of the virus, making them more compatible with the receptor sites of cells. It has been suggested that trypsin may neutralize the active inhibitor of the virus, which is produced by cell culture, and cause multiple replications of the virus [5].

Okulova et al. [10] highlighted the possibility of obtaining a double 'harvest' of bovine coronavirus in cultures of MDBK and Vero cells at 34°C.

Given the conflicting results of other researchers regarding different strains of coronavirus, we decided to determine the features of culturing of bovine coronavirus strain CV-315 and select the optimal methods of culturing the virus in order to obtain viral material with the highest possible titers of infectious activity to develop technology for monoprophylaxis and specific diagnostic means production.

## Materials and Methods

*Cell culture*

MDBK cell culture (CC) obtained from the collection of cell cultures of *Biotestlab* LLC was used for these studies. Cell culture was adapted to Dulbecco's Modified Eagle Medium (DMEM — manufactured by *Sigma-Aldrich*®) with the addition of 10% fetal bovine serum (FBS — manufactured by *Sigma-Aldrich*®). Cell culture was incubated in culture flasks with a ventilated lid (manufactured by *SARSTEDT AG & Co. KG*®, Germany) at a temperature of 37±0.5°C and a $CO_2$ content of 5±0.1%.

*Virus*

Bovine coronavirus strain CV-315, obtained in Ukraine from a calf with diarrhea caused by coronavirus [1, 2], which was confirmed by Real-Time PCR (*VetMAX*® *Ruminant Respiratory Screening Kit*, *Thermo Fisher Scientific*®, USA) was used in our work. While determining antigenic affinity and dominance, strain CV-315 dominated the standard KL-2 and other field isolates, so it was selected for study and further development of vaccines.

Material with an infectious activity titer of 6.2 lg $TCID_{50}/cm^3$ and a hemagglutinating activity titer of 10,3 $log_2/cm^3$ was used for the studies.

*Infection of cell culture*

In accordance with this goal, we evaluated the impact on the cultivation of bovine coronavirus of the following parameters:

— the presence and concentration of trypsin in the nutrient medium;

— the presence and concentration of FBS in the nutrient medium;

— temperature of virus cultivation;

— the degree of cell culture monolayer formation during virus infection;

— term of culturing the virus;

— the dose of virus to infect cell culture.

In order to adequately assess the effect of these parameters, 3 consecutive passages of virus were performed on MDBK cell culture in T25 culture flasks. Prior to the infection, cell culture with 90–100% formed monolayer (except for experiments where another degree of monolayer formation is indicated) was washed with Hanks' solution (HBSS, pH 7.2 — *Sigma-Aldrich*®) from growth media with fetal serum. The virus was introduced in a pre-calculated volume (infection dose (ID) was 0.1 VP/cell (1 virus particle per 10 cells), except for experiments where another ID is indicated) in the maintenance medium — Minimum Essential Medium Eagle (MEM — *Sigma-Aldrich*®). Cultivation was performed at a temperature of 37±0.5°C (except for experiments where another temperature is indicated). Infected cell cultures were examined daily visually under a light microscope to detect CPE (cytopathic effect) of the virus and to record the time of its manifestation by 80–90% (except for experiments where the fixation of another % of CPE manifestations is indicated).

The CPE of bovine coronavirus manifested itself as vacuolation and accumulation of cells, which subsequently led to the formation of syncytia and exfoliation of most cells from the surface of the culture vessel.

After the manifestation of CPE culture flasks with infected cells were frozen at a temperature minus 20±2°C, then thawed to collect virus-containing suspension and sampling for research. Until the results of the studies, all samples were stored at a temperature minus 20±2°C.

*Determination of infectious activity of the virus*

Infectious activity of the virus was determined by titration of the virus in the CC MDBK with the formed monolayer in a 96-well culture plate for 7 days (taking into account the results from the 3rd day after titration). The virus titer was calculated by the Reed and Mench method [11].

## Results and Discussion

In this work, a study of the cultural properties of bovine coronavirus, strain CV-315 and evaluation of the influence of a number of factors on the level of accumulation of this virus in MDBK cell culture was performed.

*The effect of trypsin on the titer of infectious activity of bovine coronavirus strain CV-315*

Trypsin was added in a pre-calculated amount to the maintenance medium. During the study, the characteristic CPE was observed in all experimental samples.

The data presented in fig. 1 indicate that the addition of trypsin at concentrations of 1, 5 and 10 μg/cm³ in the maintenance medium does not increase the titer of infectious activity of bovine coronavirus strain CV-315. The increase in trypsin concentration, in contrast, directly proportionally contributed to the reduction of the level of accumulation of the obtained viral material during 3 consecutive passages. Therefore, further cultivation of bovine coronavirus CV-315 was performed without the addition of trypsin solution.

*The effect of FBS on the titer of infectious activity of bovine coronavirus strain CV-315*

FBS was added to the maintenance medium in a pre-calculated amount of the total volume. During the study, the characteristic CPE was observed in all experimental samples. The results shown in fig. 2 indicate that the cultivation of bovine coronavirus strain CV-315 in cell culture MDBK with a maintenance medium and 2% of fetal bovine serum content receive the highest titers of infectious activity of the virus — 6,64–6,7 lg $TCID_{50}$/cm³ for 3 consecutive passages. The difference in titers for virus cultivation without fetal bovine serum and with a content of 2 and 5% was ±0.3 lg $TCID_{50}$/cm³ at the level of the 3rd passage. That is why the feasibility of using fetal bovine serum in the cultivation of bovine coronavirus strain CV-315 in the culture of MDBK cells requires further discussion.

*The effect of cultivation temperature on the titer of infectious activity of bovine coronavirus strain CV-315*

After inoculation of the virus, cultivation of the virus in the CC was performed at a temperature of 34±0.5°C and at a temperature of 37±0.5°C. The data in table 1 indicates that the temperature of coronavirus cultivation 34±0.5°C does not lead to the manifestation of cytopathic effect of the virus for 168 hours (observation period) while at a temperature 37±0.5°C CPE of the virus was manifested after 71–74 hours of culturing the virus in cell culture.

The infectious activity of the virus did not differ significantly during three consecutive passages at a temperature of 37±0.5°C. Infectious activity of the virus cultured at a temperature of 34±0.5°C was not detected, indicating the absence of accumulation of the virus at this temperature. Therefore, further studies were performed only by culturing the virus at a temperature of 37±0.5°C.

**Table 1**. The effect of cultivation temperature on the bovine coronavirus infectious activity titer

| Temperature, °C | Passage no. | The virus CPE presence | Cultivation period, h | Infectious activity, lg $TCID_{50}$/cm³ |
|---|---|---|---|---|
| 34±0,5 | 1 | – | 168 | 0 |
| | 2 | – | 168 | 0 |
| | 3 | – | 168 | 0 |
| 37±0,5 | 1 | + | 71 | 6.2±0 |
| | 2 | + | 74 | 6.45±0.25 |
| | 3 | + | 72 | 6.45±0.25 |

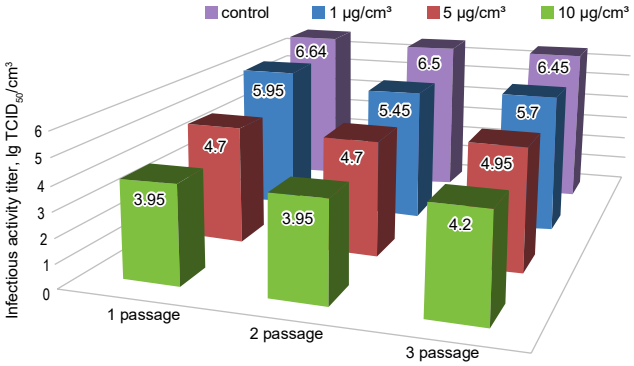


**Fig. 1.** The effect of trypsin concentration on the accumulation of bovine coronavirus strain CV-315

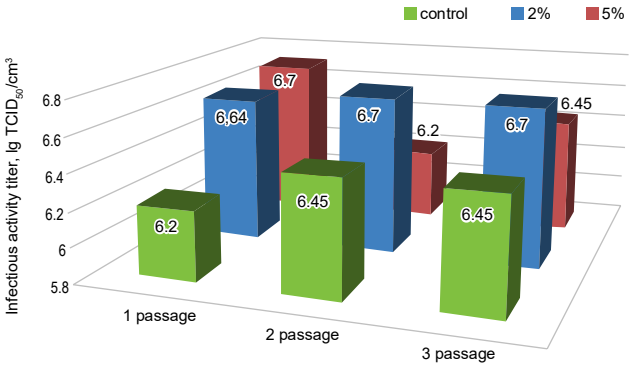


**Fig. 2**. The effect of FBS concentration on the accumulation of bovine coronavirus strain CV-315

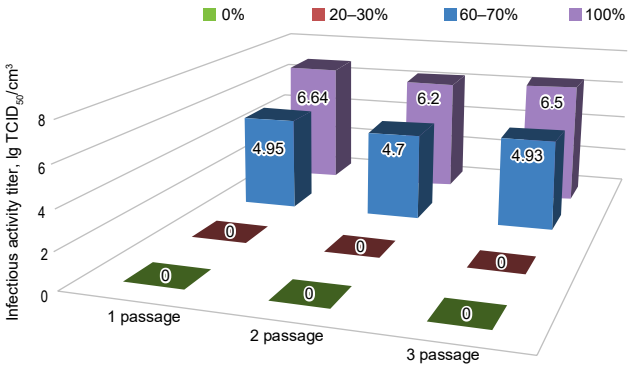


**Fig. 3.** The influence of the monolayer formation degree on the accumulation of bovine coronavirus strain CV-315

*Influence of the degree of cell culture monolayer formation during virus infection on the titer of infectious activity of bovine coronavirus strain CV-315.*

The results of the study are presented in fig. 3. The diagram shows that infection of MDBK cell culture in the early stages of cell monolayer formation (up to 30%) does not allow the accumulation of virus for three consecutive passages — these experiments did not reveal the cytopathic effect of the virus. Infection of cells of the monolayer formed by 60–70% revealed cytopathic activity of the virus in titers of 4.7–4.95 lg $TCID_{50}$/cm³. The highest activity of the virus in CC were obtained

when the virus was introduced into a 100% formed monolayer of cells — the titer of infectious activity of the virus was 6.2–6.64 lg $TCI_{50}/cm^3$, which confirms the data of other researchers.

Subsequent studies used a method of infecting cell culture into a completely formed monolayer of cells.

*Influence of the cultivation term in cell culture on the titer of infectious activity of bovine coronavirus strain CV-315*

During the study, the characteristic CPE of the virus was observed in all experimental samples. Presented in fig. 4 results show that the cultivation of bovine coronavirus strain CV-315 in the culture of MDBK cells at a dose of 0.1 VP/cell for 72 hours had the highest titers of infectious activity — 6.36±0.41 lg $TCID_{50}/cm^3$ for 3 passages. During 48 hours of cultivation, the titers of infectious activity were only 5.11±0.1 lg $TCID_{50}/cm^3$, and for 96 hours — 5.5±0.2 lg $TCID_{50}/cm^3$.

The obtained results prove that the optimal time for culturing bovine coronavirus strain CV-315 in the culture of MDBK cells at a dose of 0.1 VP/cell is 72 hours of cultivation.

*The effect of the infectious dose on the titer of infectious activity of bovine coronavirus strain CV-315*

During the study, the characteristic CPE was observed in all experimental samples (fig. 5). The diagram shows that the cultivation of bovine coronavirus strain CV-315 with a dose of 0.01 VP/cell had titers of infectious activity of 6.1±0.1 lg $TCID_{50}/cm^3$, with a dose of infection of 0.1 VP/cell — 6,38±0.2 lg $TCID_{50}/cm^3$, and with a dose of 1.0 VP/cell — 5.42±0.3 lg $TCID_{50}/cm^3$. The infectious activity of the virus increased during 3 consecutive passages. Bovine coronavirus had the highest activity when cultured with an infection dose of 0.1 VP/cell.

Therefore, further studies and production of bovine coronavirus strain CV-315 were performed with a dose of 0.1 VP/cell.

As a result of the research, the optimal temperature for culturing bovine coronavirus strain CV-315, the required degree of formation of a monolayer of cell culture during inoculation of the virus and also the time of CPE manifestation in compliance with certain parameters were determined.

Research results were summarized and optimal conditions for bovine coronavirus strain CV-315 culturing were determined. These data are presented in table 2.

Most researchers have studied the effects of proteolytic enzymes, including trypsin, on the accumulation of bovine coronavirus. According to studies by [4], trypsin concentration of 5 μg/cm³ in the nutrient medium increased the accumulation of bovine coronavirus in Vero cell culture, and in the studies of [13], trypsin concentration of 10 μg/cm³ in the nutrient medium increased plaque formation of bovine coronavirus strain L9. However, our results indicate that the addition of trypsin to the nutrient medium, on the contrary, does not increase the titer of infectious activity of bovine coronavirus strain CV-315 in MDBK cell culture.

Kapil et al. also investigated the effect of the degree of monolayer formation on the level of accumulation of bovine coronavirus. According to their results, 24 hours after the formation of a monolayer of HRT-18 cell culture is the optimal time for infection of bovine coronavirus, which coincides with the results obtained in this study [8].

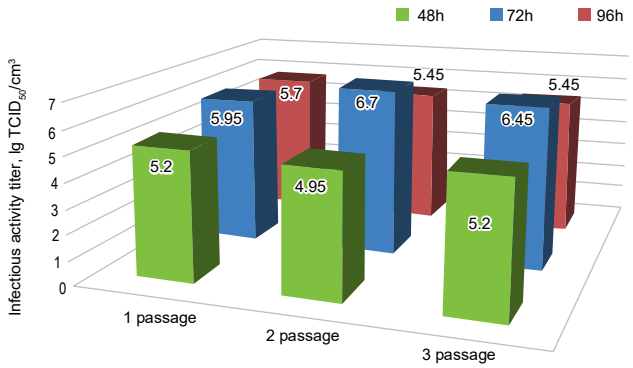


**Fig. 4.** The effect of the cultivation term on the accumulation of bovine coronavirus strain CV-315

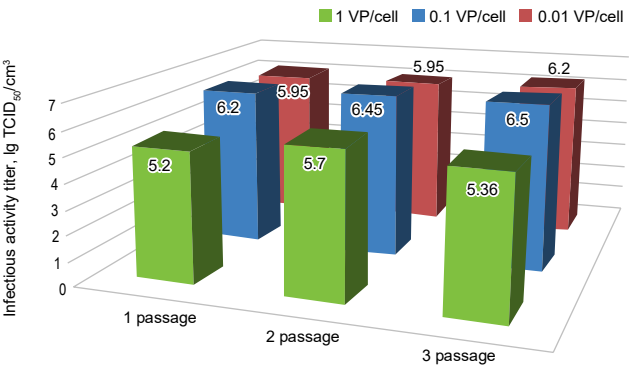


**Fig. 5.** The effect of the infective dose on the accumulation of bovine coronavirus strain CV-315
*Note*. *VP/cell — viral particles per cell

**Table 2**. The results of evaluation of the culture conditions influence on the degree of bovine coronavirus accumulation in MDBK cell culture

| Parameter | | The result obtained after 3 passages on the cell culture, lg $TCD_{50}/1.0$ $cm^3$ |
|---|---|---|
| Trypsin influence | Without trypsin | 6.45±0.25 |
| Fetal bovine serum influence | 2% | 6.7±0,46 |
| Infective dose | 0.1 VP/cell* | 6.5±0.32 |
| Cultivation temperature influence | 37±0,5 °C | 6.45±0.25 |
| Degree of monolayer formation | 100 % | 6.5±0.3 |
| The cultivation term and the degree of the virus CPE manifestation | 72 h | 6.45±0.38 |

*Note*. *VP/cell — viral particles per cell.

## Conclusions

As a result of the research it was found that the highest infectious activity — 6.45±0.3 lg $TCID_{50}/cm^3$ bovine coronavirus strain CV-315 has with cultivation within 72 hours before the manifestation of CPE by 70–80% without addition trypsin and fetal bovine serum content of 2% at a temperature of 37±0,5°C. Also founded that the optimal dose of infection is 0.1–0.01 VP/cell for infection of the fully formed monolayer of MDBK cell culture allows to obtain material with the highest titers of infectious activity in this cell culture.

Selected cultivation regimes allow to optimize the bovine coronavirus cultivation conditions for the prophylactic and diagnostic drugs production.

## Prospects for further research

The obtained results are further planned to be used in the development of veterinary immunobiological means against bovine coronavirus.

Strain CV-315 is deposited in the collection of the State Research and Control Institute of Biotechnology and Strains of Microorganisms of Ukraine in Kyiv.


1. Berezenko A, Nedosekov V, Godovskiy O. Isolation of bovine coronavirus (BCoV) in cell cultures. *Sci. Rep. NULES Ukraine*. 2021; 4 (92). DOI: 10.31548/dopovidi2021.04.001.
2. Berezenko AS, Nedosekov VV, Vabishchevych FS, Godovskiy OV. Selection of candidate strain for vaccine against bovine coronavirus production. *Sci. Tech. Bull. State Sci. Res. Cont. Inst. Vet. Med. Prod. Fodder Add. Inst. Anim. Biol.* 2021; 22 (2): 41–47. DOI: 10.36359/scivp.2021-22-2.04.
3. Brandão PE, Gregori F, Heinemann MB, Lima CHA, Rosales CAR, Ruiz VLA, Jerez JA. Animal coronaviruses. *Virus Rev. Res.* 2001; 6 (1): 7–13. DOI: 10.17525/vrrjournal.v6i1.186.
4. Dea S, Roy RS, Begin ME. Bovine coronavirus isolation and cultivation in continuous cell lines. *Amer. J. Vet. Res.* 1980; 41 (1): 30–38. PMID: 6767425.
5. Debiaggi M, Perduca M, Romero E, Cereda PM. Phosphatidylserine inhibition of $OC_{43}$ and NCDCV coronavirus infectivity. *Microbiologica*. 1985; 8 (4): 313–317. PMID: 6767425.
6. Hansa A, Rai RB, Dhama K, Wani MY, Saminathan M, Ranganath GJ. Isolation of bovine coronavirus (BCoV) in Vero cell line and its confirmation by direct FAT and RT-PCR. *Pakistan J. Biol. Sci.* 2013; 16 (21): 1342–1347. DOI: 10.3923/pjbs.2013.1342.1347.
7. Inaba Y, Sato K, Kurogi H, Takahashi E, Ito Y, Omori T, Goto Y, and Matumoto M. Replication of Bovine Coronavirns in Cell Line BEK-I Culture. *Archives of Virology*. 1976; 50: 339–42.
8. Kapil S, Richardson KL, Radi C, Chard-Bergstrom C. Factors affecting isolation and propagation of bovine coronavirus in Human Rectal Tumor-18 cell line. *J. Vet. Diagnost. Invest.* 1996; 8 (1): 96–99. DOI: 10.1177/104063879600800115.
9. Mebus CA, Stair EL, Rhodes MB, Twiehaus MJ. Neonatal calf diarrhea: propagation, attenuation, and characteristics of a coronavirus-like agent. *Amer. J. Vet. Res.* 1973; 34 (2): 145–150. PMID: 4568246.
10. Okulova ON, Dumova VV, Mishchenko VA, Ponomarev AP. Bovine coronavirus reproduction in the continuous cell cultures at a temperature of 34 degrees C. *Virol. Issues.* 2007; 52 (4): 37–40. PMID: PMID: 17722610.
11. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Amer. J. Epidemiol.* 1938; 27 (3): 493–497. DOI: 10.1093/oxfordjournals.aje.a118408.
12. Saif LJ, Heckert RA, Miller KL, Tarek MM. Cell culture propagation of bovine coronavirus. *J. Tissue Cult. Method*. 1988; 11 (3): 139–145. DOI: 10.1007/BF01404267.
13. Storz J, Rott R, Kaluza G. Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. *Infect. Immun*. 1981; 31 (3): 1214–1222. DOI: 10.1128/iai.31.3.1214-1222.1981.


## Дослідження культуральних властивостей коронавірусу ВРХ штаму CV-315


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Метою дослідження було вивчити особливості культивування коронавірусу ВРХ штаму CV-315, виділеного на території України від хворого на коронавірусну інфекцію теляти, та підібрати оптимальні способи культивування вірусу з метою отримання вірусного матеріалу з високими титрами інфекційної активності і для розроблення технології виробництва засобів імунопрофілактики та специфічної діагностики. Під час проведення досліджень було вивчено вплив на накопичення вірусу низки факторів: наявність та концентрація трипсину в поживному середовищі, фетальної бичачої сироватки, ступеня формування моношару культури клітин за проведення зараження вірусом, а також вплив дози вірусу, температури та терміну культивування. Відповідно до отриманих результатів, було встановлено, що найбільш високу інфекційну активність штам CV-315 має за культивування впродовж 72 год. до прояву цитопатичного ефекту на 70–80% без додавання трипсину та вмістом фетальної бичачої сироватки 2% за температури 37±0,5°С. Також встановлено, що оптимальною дозою зараження є 0,1–0,01 вірусних частинок на клітину для інфікування повністю сформованого моношару культури клітин MDBK. Отримані результати буде використано для розроблення ветеринарних вакцин проти коронавірусу ВРХ.



**Ключові слова:** коронавірус ВРХ, культивування вірусів, культура клітин, інфекційна активність вірусів, режими культивування



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