Part 2. Biotechnology

UDC 619:602.3:578.825.15.083.224'21:636.22/.28 **DOI 10.36016/JVMBBS-2021-7-4-4**

STUDY OF ISOLATE OF INFECTIOUS RYNOTRACHEITIS VIRUS IDENTIFIED IN THE ACUTE COURSE OF THE DISEASE

Kornieikov O. M. [1], Stegniy M. Yu. [1], Arefiev V. L. [1], Oleshko A. Yu. [1], Borodai N. I. [1], Dunaiev Yu. K. [1], Gerilovych A. P. [1], Al Jabari M. [2]

[1] National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine, e-mail: korneykov@ukr.net
[2] State Biotechnological University, Kharkiv, Ukraine

Summary. Isolation of the virus from biological material from a two-month-old calf with pathology of the respiratory system from a herd with a morbidity rate of 48% was performed. After detection the presence of IRT antigens in the lungs of the dead animal, the pathogen was isolated on a continuous culture of calf kidney cells, where a characteristic cytopathic effect was observed. The genetic material of the bovine herpesvirus type 1 (Bovine herpesvirus-1, BHV-1) was identified by polymerase chain reaction in the test sample. The virus isolate was adapted to continuous cell cultures of calf kidneys, sheep kidney, cow embryo lung and calf trachea, and the most suitable biological system was determined, where adsorption and reproductive properties of the virus were more pronounced. It was found that the highest titer of infectious activity of BHV-1 isolate (6.1 lg TCD_{50}/cm^3) was obtained on continuous culture of lung embryonic cells of a cow embryo after its reproduction during three consecutive passages (observation period)

Keywords: virus isolation, herpesvirus type 1, infectious activity, infectious rhinotracheitis, cell culture, cytopathic effect

Introduction. Pathogens of bovine respiratory diseases, including infectious rhinotracheitis (IRT) virus, are known to have significant adverse effects on animal health and productivity (Headley et al., 2018), and to limit international trade in animals worldwide (Fernandes et al., 2018).

Due to the fact that according to domestic and foreign researchers (Headley et al., 2018; Kornieikov et al., 2019; Barrett et al., 2018) a complex of viral diseases of the respiratory group (infectious rhinotracheitis (IRT), viral diarrhea (VD), parainfluenza-3 (PI-3) and respiratory syncytial infection (RSI)) occurs both in the form of monoinfections and in their associations, their timely diagnosis is of paramount importance.

Thus, several methods are commonly used to detect IRT-infected animals, namely enzyme-linked immunosorbent assay (ELISA), neutralization reaction (NR), polymerase chain reaction (PCR) (OIE, 2018), and immunofluorescence assay (IFA).

It should be noted that modern diagnostic enzyme-linked immunosorbent assay systems are used in two ways — to assess the level of total antibodies and to differentiate animals immunized with marker vaccines from those infected with an epizootic strain of the virus (Van Oirschot et al., 1997).

Regarding PCR and IFA, these methods allow to effectively identify the etiological cause in sick animals and to differentiate from other diseases (Maidana et al., 2020; Hou et al., 2017). However, effective non-costly means for diagnostics and specific prevention of the disease are needed to implement large-scale IRT control measures in cattle herds. For this purpose it is necessary to use strains of the pathogen circulating on the territory of Ukraine. Knowledge of the biological features of this pathogen will allow to give epizootological and biotechnological answers.

Analysis of local strains allows to study the peculiarities of the course, to establish local associations of microorganisms and to conduct effective immunoprophylaxis (Headley et al., 2018; Barrett et al., 2018). Thus, Brock et al. (2020) studied the dependence of the prevalence of infectious rhinotracheitis of the first type in different age groups of vaccinated and unvaccinated livestock population.

The study by Barrett et al. (2018) in 161 meat farms established seroprevalence of the herd and the relationship in the associated course with viral diarrhea and neosporosis. Depending on the characteristics of the association of pathogens, an individual program of disease control in the herd, immunoprophylaxis schemes, risk assessment of the introduction of pneumoenteritis and measures to prevent the spread of disease are developed (Benavides et al., 2020). Knowledge of the etiological component of pneumoenteritis in cattle herds allows to develop individual effective eradication programs, including programs at the state level with the involvement of state and private manufacturers (Hostnik et al., 2021).

However, all these works are impossible without isolation of the pathogen from animals, preceded by indication and differentiation from other viruses and types (in the case of herpesvirus), isolation from biological material from animals, identification by molecular-genetic methods and subsequent adaptation and study of biological properties on continuous cell cultures (Dagalp et al., 2020; d'Offay, Mock and Fulton, 1993). In the future, this will allow the development of effective domestic vaccines and diagnostic systems, such as ELISA based on monoclonal antibodies (Teixeira et al., 2001), using the experience of previous developments of researchers of NSC 'IECVM'.

The aim of the study was to isolate the pathogen of bovine respiratory disease of viral etiology with further study of its biological properties in the laboratory.

Materials and methods. The analysis of the epizootic situation in the farm was carried out with the help of clinical and epizootological examination of the cattle herd, the results of which revealed animals with signs of respiratory disease. Samples of biological material were taken from laboratory animals for laboratory confirmation of the diagnosis, namely nasal washes and serum from calves up to 6 months of age, as well as pathological material (lungs, spleen, mesenteric and mediastinal lymph nodes) from dead animals with signs of respiratory disease (2–6 months of age calves).

Indication and identification of viruses in biological material. In the laboratory, biological material from animals was studied using serological (bovine serum), virological and molecular-genetic methods (pathological and clinical material from cattle).

Detection of specific antibodies to viruses of infectious rhinotracheitis (IRT), viral diarrhea (VD), parainfluenza-3 (PI-3), and respiratory syncytial infection (RSI) was performed using enzyme-linked immunosorbent assay (IRT, VD and RSI) using test-systems manufactured by IDEXX (France) and hemagglutination inhibition (HI) assay (PI-3) using a test system manufactured by SRE 'Veterinary Medicine' LLC (Ukraine). Indication and identification of antigens of IRT, VD, PI-3, and RSI viruses was performed by immunofluorescence assay (IFA) following the generally accepted method, which included preparing of touch smears from the organs of a dead animal (spleen, lungs and lymph nodes), as well as nasal washes of sick animals. After fixation they were stained with specific fluorescent immunoglobulins and washed in phosphate-buffered saline with the addition of 1% solution of Evans blue.

Subsequently, the smears were examined under a fluorescent microscope at a magnification of ×40 at the eyepiece ×10, in the blue-green spectrum of rays at a wavelength 490–510 nm. The reaction was recorded by the intensity of the fluorescent glow of the cells (Syurin, Belousova and Fomina, 1984). Under the conditions of obtaining a positive result of the study of biological material in IFA, virological studies on cell culture were conducted.

Cell cultures. Continuous cultures of cow embryonic lung cells (CELC), calf kidney (CK), sheep kidney (ShK-2) and calf trachea (CT) stored in a cryobank of the NSC 'IECVM' were used as sensitive biological test objects for virus isolation and study of biological properties of pathogens. DMEM and 199 mediums were used as growth medium in equal proportions with the addition of 10% native inactivated bovine serum and antibiotics (penicillin 100 IU/cm^3 and streptomycin 100 μg/cm^3) (Krasochko et al., 2016). DMEM and 199 nutrient medium in a 1:1 ratio without the addition of bovine serum was used as a supportive medium.

Isolation of viruses on cell culture. The essence of this method is to detect the cytopathogenic effect (CPE) of the virus isolate on cell culture with its subsequent identification. For this purpose, pieces of organs from a dead animal were homogenized to form a 20% suspension in phosphate-buffered saline and centrifuged at 3000 rpm for 20 min.

The supernatant was transferred to vials and 200 IU/cm^3 of benzylpenicillin sodium and 200 μg/cm^3 of streptomycin sulfate were added. After incubation for 2–4 h at 4.0 ± 0.1°C, the resulting suspension was used to infect sensitive cell culture.

The study was performed in test tubes. 0.2 cm^3 (1:10) of each test material was added to five tubes with a monolayer of continuous CK cell culture after removal of the nutrient medium. Tubes with material were incubated at 37.0 ± 0.5°C for one hour, after that the test fluid was removed from each tube and 2.0 cm^3 of supportive nutrient medium for cell culture was added.

As a control, five tubes with intact cell culture were used, in which the nutrient medium was similarly replaced with a supportive one. Experimental and control tubes were incubated at a temperature of 37.0 ± 0.5°C for 5–6 days. The cytopathic effect of field viral isolates was recorded daily using a light inverted microscope (Syurin, Belousova and Fomina, 1984).

In the absence or weak manifestation of cytopathic action in cell culture in the first passage, the second and third passages were carried out with an interval of 5–6 days. The material of each passage was stored at a temperature of minus 20.0 ± 2.0°C. The result of the study of pathological material was considered negative if after the third passage the CPE of virus was not detected.

In the presence of a stable CPE of the same type in cell culture in the last passage the accumulation of viral culture biomass was conducted for further identification of the viral field isolate by polymerase chain reaction.

Virological studies of the selected virus isolate were performed on cell cultures by determining the dose dependence of infection and susceptibility of continuous cell lines (CEL, CK, ShK-2, and CT), as well as by determination the level of infectious activity of pathogens and their rate of accumulation.

The susceptibility of cell cultures to the virus was determined by the degree of cytotopathic effect, which was assessed in crosses (from '++++' to '–'), where '–' — complete absence of CPE, '+' — violation of cell

morphology and integrity of the monolayer, not more than 25%, '++' — affected no more than 50% of monolayer cells, '+++' — no more than 75% of monolayer cells affected, and '++++' — complete cell degeneration and destruction of the monolayer.

In each experiment, four samples of continuous cell culture were used. The integrity of the cell membrane was determined using 0.2% trypan blue solution (in the absence of cell coloring) (Krasochko et al., 2016). In addition, the rate of virus accumulation and its infectious activity in the 3rd passage were taken into account. Infectious activity of viral isolates was determined by the level of cytopathic effect (CPE) by titration in one-day cell culture with ten-fold dilution of the virus. The titer of infectious activity was calculated by the Reed and Mench method (Syurin, Belousova and Fomina, 1984).

Identification of the genetic material of the IRT virus by PCR. Detection of genetic material of the virus was performed by polymerase chain reaction (PCR).

Extraction of nucleic acid from the material was performed using the sorbent method in our modification, which is based on the lysis of guanidine cells by thiocyanate in the presence of Triton X-100, sorption of DNA into silicon dioxide, final washing with a solution containing chloroform, and elution of DNA into a solution with low ionic strength (Boom et al., 1990; Stegniy and Gerilovych, 2014).

The amplification reaction to detect the genetic material of the IRT virus was performed using the polymerase chain reaction method using primer systems flanking a section of glycoprotein E-gene length of 325 bp herpesvirus type 1 (Weiss et al., 2015):

BoHV-1 gE_F GCCAGCATCGACTGGTACTT
BoHV-1 gE_R GCACAAAGACGTAAAGCCCG

PCR conditions provided for 40 cycles of initial denaturation at 95°C for 0.5 min; followed by annealing at 57°C for 0.5 min, elongation at 72°C for 0.5 min, and final elongation for 10 min at 72°C. Ten microliters of each reaction were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide.

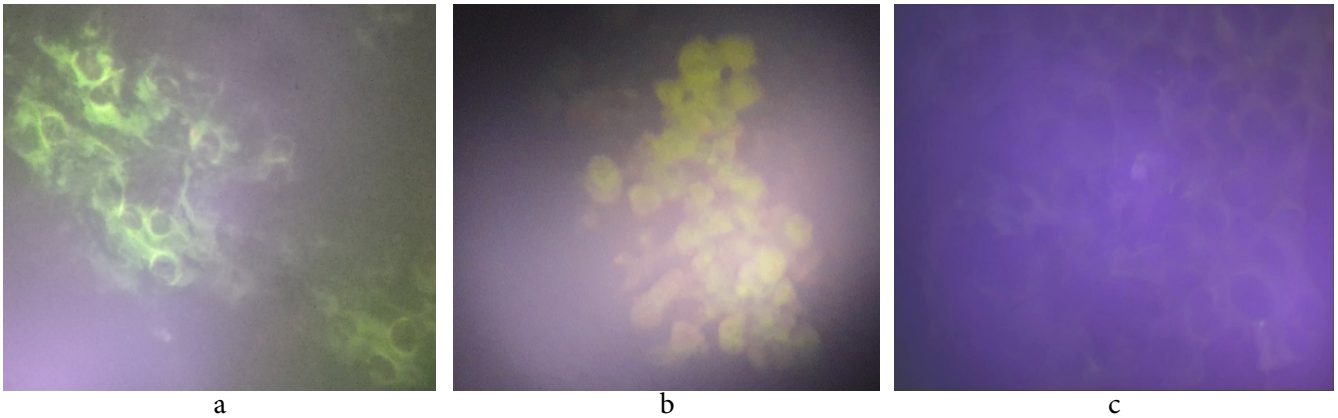
Amplification of nucleic acids was performed using a programmable amplifier T 3000 (Biometra, Germany). Agarose produced by Biozym (Germany) was used for electrophoretic analysis at a voltage of 10 V/cm for 45–50 min.

The obtained data were analyzed statistically using the Student's *t*-test and the Mann-Whitney-U-test (Lakin, 1990).

Results. According to the results of clinical and epizootiological examination regarding bovine viral pneumoenteritis in cattle of the Kirovohrad Region, it was found that animals 2–6 months of age showed respiratory disease, characterized by increased salivation, cough, redness of mucous membranes, including redness of the nasal mirror. The incidence among animals was 48% and the mortality rate was 12%. The analysis of plans of anti-epizootic measures established that the means of specific prevention have not been used in the farm for the last three years. Examination of serum from animals of different age groups (calves up to 2 months of age, calves 6 months of age, and cows) for the presence of specific antibodies to IRT, VD, and PI-3 viruses found that seropositivity of animals (n = 21) to IRT virus was 76.2%, to the VD virus — 38.1%, and to the pathogen PI-3 — 57.1%. Considering that serological diagnosis is an indirect method of identifying infected animals and requires the study of 'paired samples' of serum, which in the context of exacerbation of the epizootic situation was impractical, a preliminary diagnosis was made taking into account clinical signs of the disease. According to the previous diagnosis, the cause of respiratory disease in animals was the bovine infectious rhinotracheitis (IRT) virus.

For the purpose of laboratory confirmation of the previous diagnosis, samples of pathological material (lungs, spleen, mesenteric and mesenteric lymph nodes) were taken from the dead 2-month-old calf with signs of respiratory syndrome.

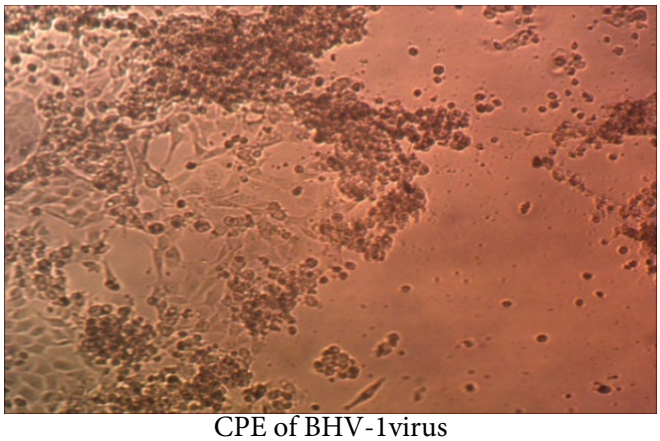
In addition, nasal washes (n = 7) were selected from calves with clinical signs of respiratory syndrome. This biological material was investigated by immunofluorescence assay (IFA) for the presence of antigens of IRT, VD, PI-3 and RSI viruses. In samples of pathological (lungs) and clinical material (nasal washes) from animals, antigens of the IRT virus were identified (Fig. 1).



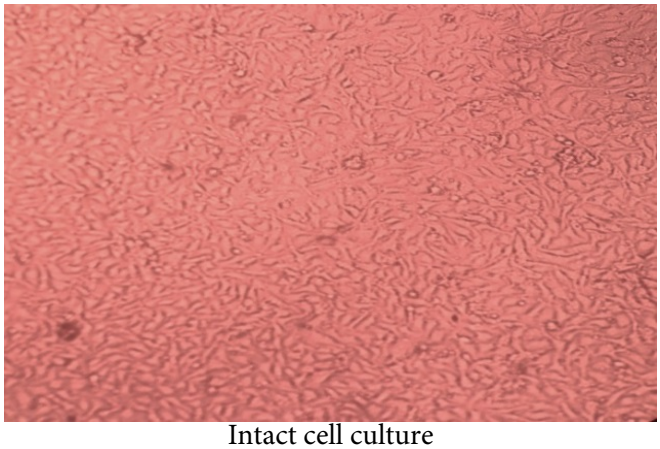
a b c

Figure 1. Results of indication and identification of the IRT pathogen in biological material (a — lungs; b — nasal washes; c — negative control) using IFA

According to the results of the identification of the IRT virus in the biological material, the specified pathogen was isolated from a suspension made from the lungs of a dead animal. After two days of incubation of the calf kidney cell culture, on the monolayer of which a virus-containing suspension of biological material was previously applied, the manifestation of the cytopathic effect of the virus was observed. CPE was characterized by the appearance of round cells in the monolayer, which accumulated in the form of conglomerates and resembled grape clusters, with subsequent detachment of individual cells and their conglomerates from glass, the formation of windows, the number of which increased rapidly and ended with complete destruction of the monolayer of cells of the continuous calf kidney line (Fig. 2).



CPE of BHV-1virus



Intact cell culture

Figure 2. Cytopathic effect of infectious rhinotracheitis virus isolate on continuous calf kidney cell culture (52 hours of incubation)

After adaptation of the virus isolate to the specified cell culture for three consecutive passages, it was determined that the infectious activity of the herpesvirus pathogen type 1 in the sensitive cell line was 4.7 lg TCD_{50}/cm^3. In order to further identify the viral isolate adapted to the biological object at the molecular-genetic level, the genetic material of the pathogen was detected by PCR. For this purpose, a sample of virus-containing fluid obtained from a continuous culture of CK cells after co-culturing it with a virus isolate was studied during three consecutive passages.

According to the results of molecular-genetic studies, it was found that the genetic material of herpesvirus type 1 (BHV-1) was identified in the studied sample (Fig. 3).

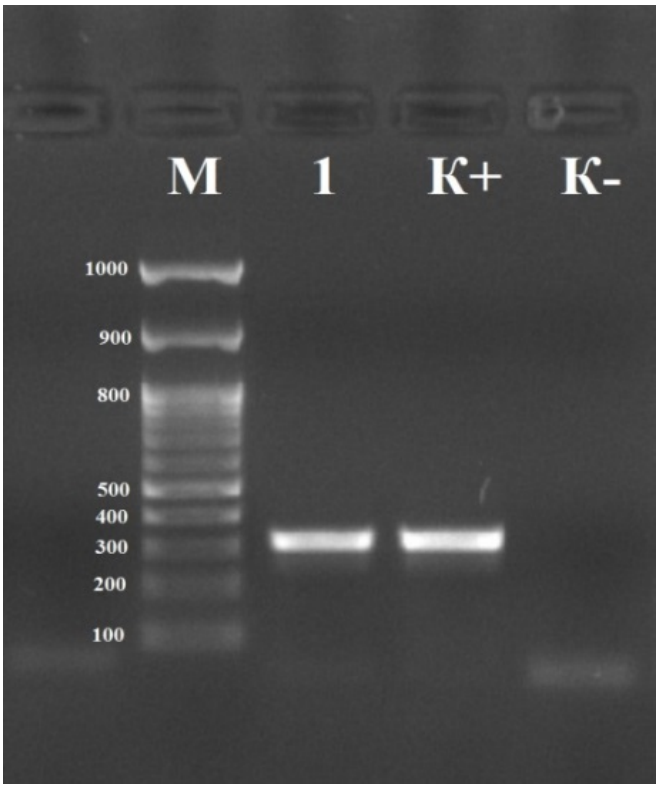


Figure 3. Results of molecular genetic studies of IRT virus isolate using polymerase chain reaction (1 — amplicon formation (325 bp) in the study of IRT virus isolate; K– — negative control; K+ — positive control; M — molecular mass marker with a step of 100 bp)

Thus, the results of molecular genetic studies confirmed the data of virological studies on the belonging of the field isolate of infectious rhinotracheitis virus to the family Herpesviridae and allowed to identify it as herpesvirus type 1 (BHV-1).

In order to determine the sensitivity of continuous cell cultures to the BHV-1 isolate, its cultivation was performed on the corresponding cultures of CK, ShK-2, CEL, and CT. The results of the research are presented in Table 1.

According to the results of studies on the adaptation of the obtained virus isolate to cultures of CK, ShK-2, CT and CEL cells, as well as determining their susceptibility to the pathogen, it was found that reproduction and adsorption properties of BHV-1 were more pronounced on continuous CK and CEL cultures — complete destruction of the monolayer was observed on the 2nd–3rd days of incubation, even under conditions of application of the pathogen at a dilution of 1:1000. The lowest activity of the virus was observed in the continuous culture of calf tracheal cells and was

characterized by the manifestation of the cytopathic effect of the pathogen from 25 to 100% after 4 days of incubation. The total number of affected cells in the corresponding repeats (n = 4) of each of the test modes did not differ statistically (U = 4.5, p < 0.05).

Table 1 — Determination of the sensitivity of continuous cell cultures to the isolate of BHV-1 virus

Cell culture	Dose of infection	Cytopathic effect of the virus, hours after infection, n = 4			
		24	48	72	96
Calf kidney	1:10	++++	++++	++++	++++
	1:100	++	++++	++++	++++
	1:1000	–	++	++++	++++
Sheep kidney	1:10	++	++++	++++	++++
	1:100	+	+++	++++	++++
	1:1000	–	+	+++	++++
Calf trachea	1:10	+	+++	++++	++++
	1:100	–	+	++	+++
	1:1000	–	–	+	++
Lungs of cow embryo	1:10	+++	++++	++++	++++
	1:100	+++	++++	++++	++++
	1:1000	–	+++	++++	++++

Notes: '–' — CPE of the virus is absent, '+' — CPE not more than 25%, '++' — CPE not more than 50%, '+++' — CPE not more than 75%, '++++' — CPE at the level of 100%.

Confirmation of the presence of genetic material BHV-1 after reproduction of the pathogen on these cell cultures was performed by PCR, examining the virus-containing fluid obtained after the third passage of the virus on them (Fig. 4).

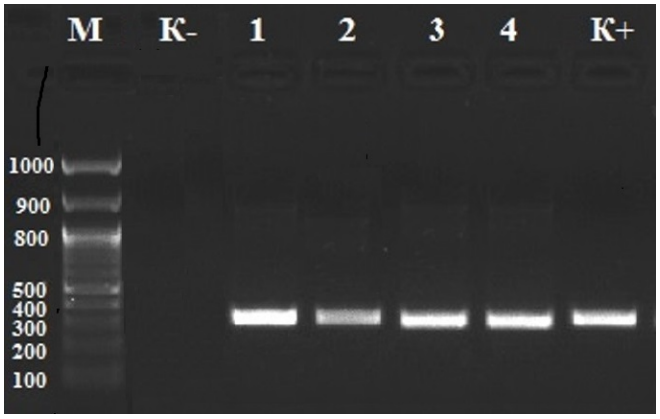


Figure 4. Formation of amplicon (325 bp) in the study of isolate BHV-1 after passage on continuous cell cultures: 1 — CK; 2 — CT; 3 — ShK-2; 4 — CEL; K– — negative control; K+ — positive control; M — molecular mass marker with a step of 100 bp.

According to the results of the PCR study, it was found that the genetic material of herpesvirus type 1 (BHV-1) was identified in the studied samples obtained from infected cell cultures. That is, the results of molecular genetic studies confirmed the reproduction of BHV-1 isolate on continuous cell cultures of CK, ShK-2, CEL, and CT during three consecutive passages. Further, in order to determine the infectivity of the obtained virus isolate, the titer of its infectious activity was determined (Fig. 5).

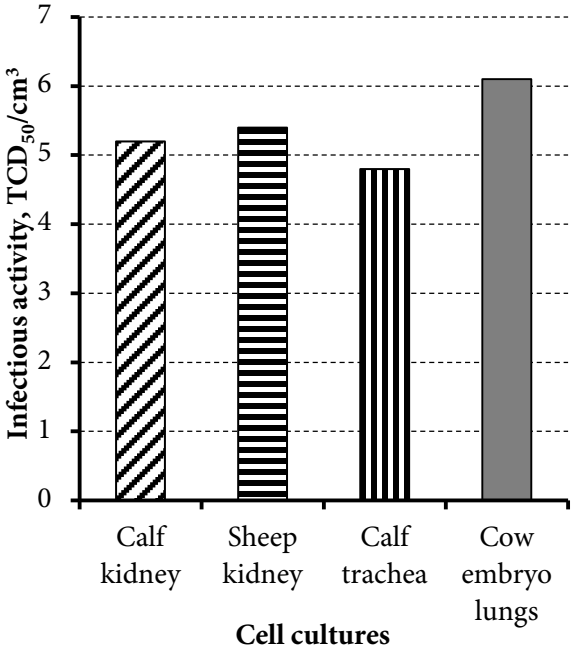


Figure 5. Infectious activity of BHV-1 isolate on continuous cell cultures at the level of the 3rd passage.

As can be seen in Fig. 5, the highest titer of infectious activity (6.1 lg TCD_{50}/cm^3) of BHV-1 isolate was obtained after its adaptation and reproduction during three consecutive passages on continuous cell culture of cow embryonic lungs. Whereas the lowest infectious activity was observed under the conditions of reproduction of the BHV-1 pathogen on the continuous culture of calf tracheal cells — 4.8 lg TCD_{50}/cm^3. With regard to the infectious activity of the IRT virus isolate obtained after reproduction on continuous cultures of calf kidney and sheep kidney cells, this indicator did not differ significantly and was at the level of 5.2–5.4 lg TCD_{50}/cm^3. It should be noted that the obtained level of infectious activity of the virus is mostly correlated with the intensity of its cytopathic effect on continuous cell cultures. In addition, the obtained titer of infectious activity is slightly lower than that of the IRT virus, which may be due to the small number of passages performed on the respective cell lines. According to the results of this work, the obtained isolate of infectious rhinotracheitis virus after certification, was deposited in the Depository of the NSC 'IECVM' under the name 'K-17' ('Kirovohrad-2017').

Conclusions. 1. As a result of virological studies, a field isolate of the virus was identified and adapted to continuous cultures of CEL, CK, SHk, and CT cells. The isolate was identified as a herpesvirus type 1 (BHV-1) using a molecular-genetic research method.

2. Reproduction and adsorption properties of BHV-1 isolate were more pronounced on continuous cultures of CK and CEL cells — complete destruction of the monolayer was observed on the 2nd–3rd days of incubation, even under conditions of application of the pathogen at a dilution of 1:1000.

3. The highest titer of infectious activity (6.1 lg TCD50/cm3) of BHV-1 isolate was observed after its adaptation and reproduction on continuous culture of cow embryonic lung cells.

References

Barrett, D., Parr, M., Fagan, J., Johnson, A., Tratalos, J., Lively, F., Diskin, M. and Kenny, D. (2018) 'Prevalence of Bovine Viral Diarrhoea Virus (BVDV), Bovine Herpes Virus 1 (BHV 1), Leptospirosis and Neosporosis, and associated risk factors in 161 Irish beef herds', *BMC Veterinary Research*, 14(1), p. 8. doi: 10.1186/s12917-017-1324-9.

Benavides, B., Casal, J., Diéguez, J. F., Yus, E., Moya, S. J., Armengol, R. and Allepuz, A. (2020) 'Development of a quantitative risk assessment of bovine viral diarrhea virus and bovine herpesvirus-1 introduction in dairy cattle herds to improve biosecurity', *Journal of Dairy Science*, 103(7), pp. 6454–6472. doi: 10.3168/jds.2019-17827.

Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M. and van der Noordaa, J. (1990) 'Rapid and simple method for purification of nucleic acids', *Journal of Clinical Microbiology*, 28(3), pp. 495–503. doi: 10.1128/jcm.28.3.495-503.1990.

Brock, J., Lange, M., Guelbenzu-Gonzalo, M., Meunier, N., Vaz, A. M., Tratalos, J. A., Dittrich, P., Gunn, M., More, S. J., Graham, D. and Thulke, H.-H. (2020) 'Epidemiology of age-dependent prevalence of Bovine Herpes Virus Type 1 (BoHV-1) in dairy herds with and without vaccination', *Veterinary Research*, 51(1), p. 124. doi: 10.1186/s13567-020-00842-5.

Dagalp, S. B., Farzani, T. A., Dogan, F., Alkan, F. and Ozkul, A. (2020) 'Molecular and antigenic characterization of bovine herpesvirus type 1 (BoHV-1) strains from cattle with diverse clinical cases in Turkey', *Tropical Animal Health and Production*, 52(2), pp. 555–564. doi: 10.1007/s11250-019-02042-6.

Fernandes, L. G., Pituco, E. M., de Campos Nogueira Romaldini, A. H., De Stefano, E., Clementino, I. J., Maia, A. R. A., de Sousa Américo Batista Santos, C., Alves, C. J. and de Azevedo, S. S. (2018) 'Spatial analysis for bovine viral diarrhea virus and bovine herpesvirus type 1 infections in the state of Paraíba, northeastern Brazil', *BMC Veterinary Research*, 14(1), p. 102. doi: 10.1186/s12917-018-1412-5.

Headley, S. A., Okano, W., Balbo, L. C., Marcasso, R. A., Oliveira, T. E., Alfieri, A. F., Negri Filho, L. C., Michelazzo, M. Z., Rodrigues, S. C., Baptista, A. L., Saut, J. P. E. and Alfieri, A. A. (2018) 'Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil', *Journal of Veterinary Diagnostic Investigation*, 30(2), pp. 249–251. doi: 10.1177/1040638717739945.

Hostnik, P., Černe, D., Mrkun, J., Starič, J. and Toplak, I. (2021) 'Review of infections with bovine herpesvirus 1 in Slovenia', *Frontiers in Veterinary Science*, 8, p. 676549. doi: 10.3389/fvets.2021.676549.

Hou, P., Wang, H., Zhao, G., He, C. and He, H. (2017) 'Rapid detection of infectious bovine rhinotracheitis virus using recombinase polymerase amplification assays', *BMC Veterinary Research*, 13(1), p. 386. doi: 10.1186/s12917-017-1284-0.

Kornieikov, O. M., Prokhoriatova, O. V., Kolchik, O. V., Oleshko, A. Yu., Borodai, N. I. and Al Jabari, M. (2019) 'The efficiency of different approaches to the prevention and control of Bovine Pneumoenteritis' [Efektyvnist riznykh pidkhodiv profilaktyky ta borotby z infektsiinymy pnevmoenterytamy VRKh], *Veterinary Medicine [Veterynarna medytsyna]*, 105, pp. 46–53. doi: 10.36016/VM-2019-105-9. [in Ukraine].

Krasochko, P. A., Borisovets, D. S., Krasochko, I. A., Krasochko, P. P., Tyapsha, Yu. I. and Dubanevich, O. V. (2016) *Guidelines for the Study of Cultural, Antigenic, Genetic Properties and Infectious Activity of Production and Epizootic Strains of Bovine Coronavirus [Metodicheskie rekomendatsii po izucheniyu kul'tural'nykh, antigennykh, geneticheskikh svoystv i infektsionnoy aktivnosti proizvodstvennykh i epizooticheskikh shtammov koronavirusa krupnogo rogatogo skota]*. Minsk: Institute of Experimental Veterinary named after S. N. Vyshelesskiy. Available at: http://old.bievm.by/gallery/%D0%BC%D0%B5%D1%82.%20%D1%80%D0%B5%D0%BA%D0%BE%D0%BC%D0%B5%D0%BD%D0%B4%D0%B0%D1%86%D0%B8%D0%B8%20%D0%BF%D0%BE%20%D0%B8%D0%B7%D1%83%D1%87%D0%B5%D0%BD%D0%B8%D1%8E%20%D0%BA%D1%83%D0%BB%D1%8C%D1%82.,%20%D0%B0%D0%BD%D1%82%D0%B8%D0%B3%D0%B5%D0%BD.,%20%20%D0%B3%D0%B5%D0%BD%D0%B5%D1%82%D0%B8%D1%87.%20%D1%81%D0%B2%D0%BE%D0%B9%D1%81%D1%82%D0%B2%20%D0%B8%20%D0%B8%D0%BD%D1%84.%20%D0%B0%D0%BA%D1%82%D0%B8%D0%B2%D0%BD%D0%BE%D1%81%D1%82%D0%B8%20%20%D0%BF%D1%80%D0%BE%D0%B8%D0%B7%D0%B2.%20%D0%B8%20%D1%8D%D0%BF%D0%B8%D0%B7%D0%BE%D0%BE%D1%82.%20%D1%88%D1%82%D0%B0%D0%BC%D0%BC%D0%BE%D0%B2%20%20%D0%BA%D0%BE%D1%80%D0%BE%D0%BD%D0%B0%D0%B2%D0%B8%D1%80%D1%83%D1%81%D0%B0%20%D0%BA%D1%80%D1%81.pdf. [in Russian].

Lakin, G. F. (1990) *Biometry [Biometriya]*. 4th ed. Moscow: Vysshaya shkola. ISBN 5060004716. [in Russian].

Maidana, S. S., Miño, S., Apostolo, R. M., De Stefano, G. A. and Romera, S. A. (2020) 'A new molecular method for the rapid subtyping of bovine herpesvirus 1 field isolates', *Journal of Veterinary Diagnostic Investigation*, 32(1), pp. 112–117. doi: 10.1177/1040638719898692.

d'Offay, J. M., Mock, R. E. and Fulton, R. W. (1993) 'Isolation and characterization of encephalitic bovine herpesvirus type 1 isolates from cattle in North America', *American Journal of Veterinary Research*, 54(4), pp. 534–539.

OIE (World Organisation for Animal Health) (2018) 'Chapter 3.4.11. Infectious Bovine Rhinotracheitis/Infectious Pustular Vulvovaginitis', in *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees)*. 8th ed. [version adopted in May 2017]. Paris: OIE, pp. 1139–1157. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.04.11_IBR_IPV.pdf.

Stegniy, B. T. and Gerilovych, A. P. (eds.) (2014) *Molecular Genetic Methods of Diagnostics in Veterinary Medicine and Biotechnology [Molekuliarno-henetychni metody diahnostyky u veterynarnii medytsyni ta biotekhnolohii]*. Kyiv: ST Druk. ISBN 9789662717143. [in Ukrainian].

Syurin, V. N., Belousova, R. V. and Fomina, N. V. (1984) *Veterinary Virology [Veterinarnaya virusologiya]*. Moscow: Kolos. [in Russian].

Teixeira, M. F. B., Esteves, P. A., Schmidt, C. S., Spilki, F. R., Silva, T. C., Dotta, M. A. and Roehe, P. M. (2001) 'A monoclonal blocking ELISA for the serological diagnosis of bovine herpesvirus type 1 (BHV-1) infections' [ELISA de bloqueio monoclonal para o diagnóstico sorológico de infecções pelo herpesvírus bovino tipo 1 (BHV-1)], *Brazilian Journal of Veterinary Research [Pesquisa Veterinária Brasileira]*, 21(1), pp. 33–37. doi: 10.1590/S0100-736X2001000100007. [in Portuguese].

Van Oirschot, J. T., Kaashoek, M. J., Maris-Veldhuis, M. A., Weerdmeester, K. and Rijsewijk, F. A. M. (1997) 'An enzyme-linked immunosorbent assay to detect antibodies against glycoprotein gE of bovine herpesvirus 1 allows differentiation between infected and vaccinated cattle', *Journal of Virological Methods*, 67(1), pp. 23–34. doi: 10.1016/S0166-0934(97)00073-6.

Weiss, M., Brum, M. C. S., Anziliero, D., Weiblen, R. and Flores, E. F. (2015) 'A glycoprotein E gene-deleted bovine herpesvirus 1 as a candidate vaccine strain', *Brazilian Journal of Medical and Biological Research*, 48(9), pp. 843–851. doi: 10.1590/1414-431x20154243.