Optimization of cultivation conditions for bovine adenovirus type 3

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Abstract. This article presents the data of studies on optimization of conditions for culturing the causative agent of bovine adenovirus infection on continuous cell culture lines. The work used the reference strain of bovine adenovirus subgroup 1 "Adeno III WBR - 1"; cell lines of newborn Syrian hamster kidney (BHK-21/13), calf kidney (Taurus-1), endothelium of coronary vessels of the embryo cow (KST), epithelium of the lung embryo of cattle (LEK) and Madin-Darby bovine kidney (MDBK). It was found that with a stationary cultivation method, the adenovirus strain quickly adapted to the continuous cell culture lines BHK-21/13, Taurus-1, KST and MDBK. Among the continuous cell cultures, the MDBK (0.0001 MOI/cell) demonstrated the highest sensitivity to infection with the adenovirus strain. The maximum accumulation of the adenovirus strain in the culture fluid was observed 24 hours after infection. Comparative assessment of the level of strain accumulation in the culture fluid obtained using the MDBK cell line after various methods of its cultivation showed that the maximum titer of the virus strain ((6.55±0.21) lg $TCID_{50}/cm^2$) was observed with the roller method of cell cultivation. The obtained results can be used to develop a test system for the purpose of identifying pathogens of adenovirus infection in cattle.

1 Introduction

Adenoviruses of farm animals are among the main viral pathogens affecting the respiratory and digestive organs and causing gastrointestinal and respiratory infections [1, 2], and are among the important etiologic agents of the complex of gastrointestinal and respiratory diseases of cattle [3, 4]. According to the World Organization for Animal Health, in recent decades, adenovirus infection of cattle has been detected in such countries as the United States of America, the Russian Federation, Japan, Great Britain, Bulgaria, Germany, Holland and Hungary [5].

Despite the fact that bovine adenoviruses (BAdV) belong to the genus *Mastadenovirus* (mammalian adenoviruses), are characterized by the ability to reproduce in cell cultures of

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bovine origin and the absence or presence of a complement-fixing antigen, there are 11 types of bovine adenovirus, among which BAdV-3 is the most virulent [6, 7]. Such a diversity of genetic variants of bovine adenovirus complicates diagnostic and further therapeutic and preventive measures. Comparison of data on the prevalence of gastrointestinal and respiratory diseases of cattle caused by the causative agent of adenovirus infection for the period 2022-2024 in the territories of various countries of the world revealed the lack of accurate data on the frequency of occurrence of this pathogen in cattle. There is also no convincing evidence regarding the predisposition of certain breeds to the occurrence of these diseases, which are caused by adenoviruses.

Currently, the creation and production of diagnostic test systems and vaccines against adenovirus infection of cattle is impossible without the selection of sensitive cell systems, optimal conditions for the accumulation of adenoviruses in them and an effective method for their cultivation [8]. Such approaches to culturing cell culture lines allow not only to increase the yield of viral material, but also the quality of the final product. At the same time, to select optimal conditions for culturing pathogens of viral infection on transplantable cell culture lines, it is necessary to take into account the seeding concentration of cells, the dose of infection and the duration of cultivation [9, 10].

The aim of this work was to find the most optimal conditions for cultivating the causative agent of adenovirus infection of cattle on transplantable cell culture lines to obtain the maximum yield of viral biomass with high antigenic activity

2 Materials and Methods

The object of the research was the reference strain of bovine adenovirus subgroup 1 "Adeno III WBR - 1", obtained from the state collection of microorganisms of the Federal State Budgetary Scientific Institution “Federal Center for Toxicological, Radiation and Biological Safety” (FSBSI “FCTRB-VNIVI”, Kazan, Russia).

The cell lines of newborn Syrian hamster kidney (BHK-21/13), calf kidney (Taurus-1), endothelium of coronary vessels of the embryo cow (KST), epithelium of the lung embryo of cattle (LEK) and Madin-Darby bovine kidney (MDBK) were obtained from the collection of cell cultures and nutrient media of the Department of Virology and Ultrastructural Research of FSBSI “FCTRB-VNIVI”, which are permanently stored in a biorepository with liquid nitrogen at minus 196 ºC.

The following nutrient media were used for cell culture growth: synthetic medium 199, GLA medium (0.5% lactalbumin hydrolysate solution), Eagle's medium MEM (pH 7.5-7.6) with the addition of 1% L-glutamine, 10% bovine serum and gentamicin at a concentration of 50 μg/ml. To maintain cell cultures after infection, the above-mentioned serum-free media were used. The monolayer of transplanted cell cultures was washed with Hanks' solution.

The sensitivity of continuous cell culture lines to adenovirus was assessed by the time of cytopathic effect (CPE), infectious activity of the virus, and the minimum multiplicity of infection (MOI) required to kill the bulk of the cells [11, 12].

The cell culture was grown under standard conditions of a CO_2 incubator with a CO_2 concentration of 5% at a temperature of 37 °C. The cell culture seeding density was 200 thousand cells per milliliter of medium. After the formation of a cell monolayer and replacement of the complete growth medium with a supporting one, the three-day cell culture was infected with the virus strain at a dose of 5.0 lg TCD_{50}/cm^3.

Infected cell cultures were incubated at 37 °C and 5% CO_2 until cytopathic changes appeared. The cell culture without the virus, incubated under the same conditions, served as a control. The signs of CPE were determined visually every day, recording cellular changes that are characteristic of the virus (cell shape, monolayer condition were assessed). The degree of CPE was calculated using a conventional four-point scale and expressed as a

percentage of CPE: "-" (0% CPE), "+" (<25% CPE), "++" (from 25 to 50% CPE), "+++" (from 50 to 75% CPE), "++++" (from 75 to 100% CPE).

The infectious activity of adenovirus was determined by titrating the strain with a tenfold serial dilution followed by infection of the above-mentioned cell cultures [13]. After titrating the adenovirus on 96-well plates, they were used to infect a monolayer of cells at a rate of four wells for each dilution of the pathogen and incubated in a humidified atmosphere with a CO2 concentration of 5% at 37 °C for 24-96 hours, depending on the reproduction of the virus. The presence of the pathogen was assessed by the manifestation of the cytopathic effect of the virus in the culture. When 75% to 80% of the monolayer was affected by the virus, the former were frozen at minus 20 ºC. After thawing, the activity of the virus in the resulting suspensions was determined. The virus titer was calculated according to the generally accepted method of Reed and Mench (1938) and expressed as lg TCD_{50}/cm^3 [14].

To select the optimal conditions for culturing the strain, the following parameters were studied: the time of monolayer formation, the seeding content of cells, the infection dose, the time of CPE manifestation, the duration of cultivation, the number of cell and virus passages. The strain was cultivated in continuous cell cultures using the stationary and roller approaches [15].

Determination of cell concentration and viability was performed using the method of intravital staining with trypan blue [16]. For this purpose, equal volumes of cell suspension and 0.4% dye solution in fetal calf serum were mixed. Live and dead cells were counted using a Goryaev chamber on an optical microscope. Cell viability was calculated using the formula: $V = (a-b) \times 100\% / a$, where: a is the total number of cells; b is the number of dead cells.

The data obtained from three independent experiments were analyzed using the spreadsheet packages of the following software: Microsoft Office Excel 2013 (Windows, USA), Statistica 12.0 (StatSoft Inc., USA). The samples were assessed for normal distribution using the Shapiro-Wilk test and presented as arithmetic means and standard error of the mean (M±m). Differences in groups in mean values of the quantitative variable were determined using the one-way analysis of variance (ANOVA) method at a significance level of $p \leq 0.05$. Tukey's post hoc test was used for intergroup comparisons ($p \leq 0.05$).

3 Results and discussion

To select a cell model promising for creating diagnostic test systems, a study was conducted on the sensitivity of continuous cell culture lines to "Adeno III WBR - 1" strain. When choosing an effective biological model that ensures the reproduction of the virus with a high infectious titer, we relied on the species affiliation of a particular cell line, the tropism of the pathogen to the cells, as well as on the literature data on the use of cultures for these purposes. As cell systems, we used continuous cell lines LEK, Taurus-1, BHK-21/13, MDBK and KST, which are most often used for culturing various adenovirus strains. An analysis of the literature data showed that there is limited information regarding the sensitivity of various types of continuous cell cultures to bovine adenovirus strains.

Experiments to assess the sensitivity of cell lines showed that all cultures taken into the experiment were sensitive to varying degrees to “Adeno III WBR – 1” strain (Table 1).

Table 1. Sensitivity of mammalian cell lines to the strain of bovine adenovirus subgroup 1 “Adeno III WBR – 1”

Cell line	Multiplicity of infection, MOI/cell	Time of manifestation of CPE, h
LEK	0,001	48
Taurus-1	0,001	48

BHK-21/13	0,01	48
MDBK	0,0001	24
KST	0,1	72

Note: For each cell culture line, the minimum multiplicity of infection (MOI) values required to infect wells with growing cells are indicated. A lower MOI value indicates a higher sensitivity of the cell culture to virus infection

With the stationary method of culturing, the “Adeno III WBR – 1” virus strain quickly adapted to the continuous cell culture lines LEK, Taurus-1, BHK-21/13, MDBK and KST. The highest sensitivity among the continuous cell cultures for the Adeno III WBR – 1 adenovirus strain was MDBK (0.0001 MOI/cell). In this cell line, the manifestation of the CPE of adenovirus was detected 24 hours after infection. The other cell culture lines showed less pronounced sensitivity to the virus strain, the manifestation of the CPE of adenovirus occurred 48-72 hours after infection of the culture. The cell line KST turned out to be the most resistant to infection. The infectious titer of the adenovirus strain on the MDBK cell culture was (5.90±0.2) lg TCD_{50}/cm^3, which is 0.25, 0.5, 0.75 and 1.25 lg significantly higher ($p<0.05$) than on the BHK-21/13, LEK, Taurus-1, and KST cell cultures, respectively.

To conduct studies on the adaptation of the bovine AB pathogen to transplantable cell cultures and further accumulation of viral material with a high titer value by selecting different cell culturing methods, we selected the bovine kidney cell line MDBK (Figure 1, Table 2). This cell line did not require long-term adaptation of "Adeno III WBR - 1" adenovirus strain.

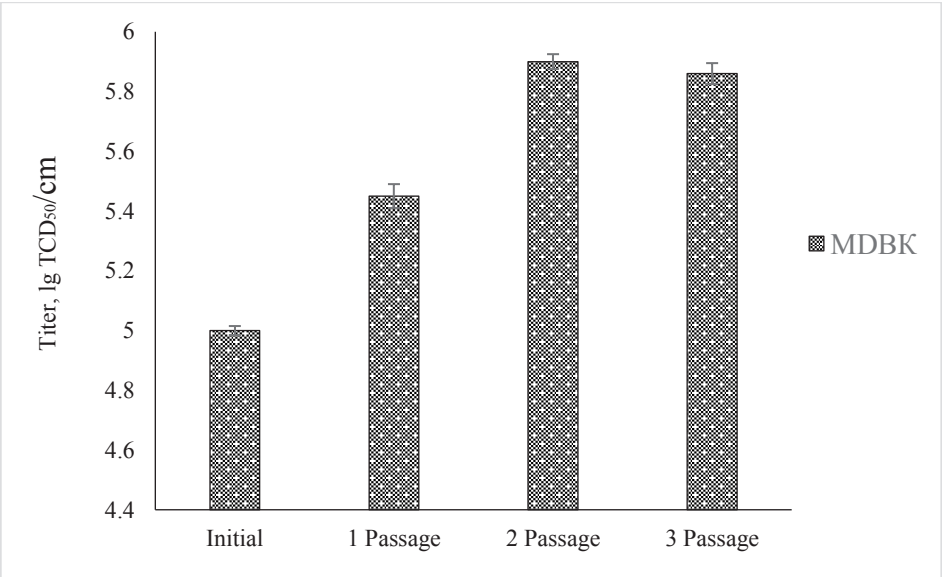

Fig. 1 The level of accumulation of "Adeno III WBR - 1" adenovirus strain in the stationary cell culture of MDBK

It was found that the viral titer index during sequential accumulation of "Adeno III WBR - 1" strain reached its maximum by the 2-3 passage (Table 2). During subsequent passaging, no reliable significant differences in the values of this index were found. The detected differences in the sensitivity of MDBK to the studied adenovirus strain during passage are probably due to a change in its reproduction, which is confirmed by the results we obtained for assessing the virus titer.

Observation of the morphology of the MDBK cell monolayer during the passage of the "Adeno III WBR - 1" virus strain showed a change in its structure depending on the time and passage of the culture. If after the first passage of the MDBK cell line, the formation of strands and inclusions, the appearance of granularity of the cytoplasm and erasure of intercellular boundaries were observed, then after the first two passages of the culture - rounding of the cells, the formation of cellular debris, conglomerates and extensive windows with subsequent lysis and destruction of the monolayer

Table 2. Results of the assessment of the infectious activity of "Adeno III WBR - 1" adenovirus strain in the culture fluid obtained using the MDBC cell line, using various cultivation methods

Cell cultivation methods	Volume of virus-containing fluid, cm^2	Virus cultivation time, hours	Virus titer, lg $TCID_{50}$/ cm^2 (M±m)
Stationary (culture flask, surface area 175 cm^2)	72	24	(5.90±0.15)*
Roller (roller bottle, surface area 850 cm^2)	448	24	(6.55±0.21)*

Notes: Differences between variants are statistically significant ($p < 0.05$)

The results of our studies are consistent with previously conducted and published data by other authors, which also show the advantage of the roller method of culturing cell cultures, namely, versatility, technological effectiveness in production and the possibility of rapid production of viral raw materials in large volumes [17, 18]. The specified method of culturing cell lines, according to literary data [17, 18], allows not only to increase the number of cells per unit volume of the liquid medium used and, accordingly, the yield of viral biomass by two times, but also to reduce the time for obtaining viral raw materials compared to the stationary method of culturing cultures.

4 Conclusions

Thus, the highest sensitivity among the transplantable cell cultures to infection with strain of bovine adenovirus subgroup 1 "Adeno III WBR - 1" was demonstrated by the line of bovine kidney cells MDBK. The optimal dose for infection of the MDBK cell line with the virus "Adeno III WBR - 1" strain was 0.0001 MOI/cell, while the maximum accumulation of adenovirus was detected 48 hours after infection - the infectious titer of the studied strain was equal to (5.90±0.15) lg $TCID_{50}/cm^2$. It was found that the maximum titer of infectious activity of the adenovirus strain was achieved with the roller cultivation method. The data obtained by us can be used to create diagnostic tools for adenovirus infection of cattle.

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