DOI: 10.29326/2304-196X-2021-3-38-230-238

Studies of biological properties of continuous suspension BHK-21/SUSP/ARRIAH cell line

M. I. Doronin[1], M. N. Guseva[2], D. V. Mikhalishin[3], A. S. Sharypov[4], N. S. Mudrak[5], N. Ye. Kamalova[6], B. L. Manin[7]

[1–3, 5–7] FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

[4] FSBI "Central Scientific and Methodological Veterinary Laboratory" (FSBI CNMVL), Moscow, Russia

[1] *ORCID 0000-0002-4682-6559, e-mail: doronin@arriah.ru*

[2] *ORCID 0000-0002-3997-3390, e-mail: guseva_mn@arriah.ru*

[3] *ORCID 0000-0003-1718-1955, e-mail: mihalishindv@arriah.ru*

[4] *ORCID 0000-0002-1479-5479, e-mail: science@cnmvl.ru*

[5] *ORCID 0000-0002-9788-9197, e-mail: mudrak@arriah.ru*

[6] *ORCID 0000-0001-5671-2347, e-mail: kamalova@arriah.ru*

[7] *ORCID 0000-0002-5263-1491, e-mail: manin_bl@arriah.ru*

SUMMARY

The results of the studies of cytomorphological, karyological, cultural properties of continuous suspension BHK-21/SUSP/ARRIAH subline of newborn Syrian hamster kidney cells intended for foot-and-mouth disease, rabies, bovine parainfluenza-3, Aujeszky's disease virus reproduction, as well as for production of diagnostic veterinary biologicals are presented. When cultured in suspension, BHK-21/SUSP/ARRIAH cell subline undergoes selection towards hypoploidy: modal class is represented by cells with 41 chromosomes (32–40% of cells); the share of cells containing 40–42 chromosomes is 78–80%; the share of polyploids averages around 1%; the range of variation in the number of chromosomes is from 36 to 54. BHK-21/SUSP/ARRIAH cell subline cultured in suspension with cell seeding concentration of 0.6–0.8 million cells/cm^3 demonstrates growth rate of 6.67–11.00 and 96–99% cell viability. After 48 hours, G1-phase (diploid-2*n*) cells prevail in the cell population of the new subline (30.0–75.0% of cells); cells that undergo preparation for mitosis (S-phase) and mitosis (G2+M-phase) account for 3.0 to 20.0% of the entire population; the number of meganucleated and multinucleated cells (> 4*n*) at the beginning and at the end of the logarithmic phase increases to 2%. BHK-21/SUSP/ARRIAH cells recover rapidly after cryopreservation and demonstrate 95–99% viability and growth rate of 3.36–5.88 at passages 1 to 3 and 6.85–10.95 at passages 4 to 12. Continuous suspension BHK-21/SUSP/ARRIAH cell line ensures virus accumulation at the following titres: FMD virus – 7.30–8.00 lg $TCID_{50}$/cm^3, rabies virus – 7.25–8.00 lg $CCID_{50}$/cm^3, bovine parainflunza-3 virus – at least 6.00 lg $TCID_{50}$/cm^3, Aujeszky's disease virus – 7.50–7.80 lg $TCID_{50}$/cm^3.

Keywords: BHK-21/SUSP/ARRIAH cell line, biological properties of cell culture, foot-and-mouth disease, rabies, bovine parainflunza-3, Aujeszky's disease

Acknowledgements: The study was funded by the FGBI "ARRIAH" within the framework of "Veterinary Welfare" research work.

For citation: Doronin M. I., Guseva M. N., Mikhalishin D. V., Sharypov A. S., Mudrak N. S., Kamalova N. Ye., Manin B. L. Studies of biological properties of continuous suspension BHK-21/SUSP/ARRIAH cell line. *Veterinary Science Today.* 2021; 10 (3): 230–238. DOI: 10.29326/2304-196X-2021-3-38-230-238.

Conflict of interest: The authors declare no conflict of interest.

For correspondence: Maksim I. Doronin, Candidate of Science (Biology), Leading Researcher, Laboratory for FMD Prevention, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: doronin@arriah.ru.*

УДК 619:578.835.2:578.824.11:578.831.11:616.98:578.825.1:57.082.26

Исследование биологических свойств перевиваемой суспензионной линии клеток BHK-21/SUSP/ARRIAH

М. И. Доронин[1], М. Н. Гусева[2], Д. В. Михалишин[3], А. С. Шарыпов[4], Н. С. Мудрак[5], Н. Е. Камалова[6], Б. Л. Манин[7]

[1–3, 5–7] ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

[4] ФГБУ «Центральная научно-методическая ветеринарная лаборатория» (ФГБУ ЦНМВЛ), г. Москва, Россия

[1] *ORCID 0000-0002-4682-6559, e-mail: doronin@arriah.ru*

[2] *ORCID 0000-0002-3997-3390, e-mail: guseva_mn@arriah.ru*

[3] *ORCID 0000-0003-1718-1955, e-mail: mihalishindv@arriah.ru*

[4] *ORCID 0000-0002-1479-5479, e-mail: science@cnmvl.ru*

[5] *ORCID 0000-0002-9788-9197, e-mail: mudrak@arriah.ru*

[6] *ORCID 0000-0001-5671-2347, e-mail: kamalova@arriah.ru*

[7] *ORCID 0000-0002-5263-1491, e-mail: manin_bl@arriah.ru*

РЕЗЮМЕ

Представлены результаты изучения цитоморфологических, кариологических, культуральных характеристик перевиваемой суспензионной клеточной сублинии из почки новорожденного сирийского хомячка ВНК-21/SUSP/ARRIAH, предназначенной для репродукции вирусов ящура, бешенства, парагриппа-3 крупного рогатого скота, болезни Ауески, а также для изготовления диагностических ветеринарных биопрепаратов. Сублиния клеток ВНК-21/SUSP/ARRIAH при суспензионном культивировании проходит селекцию в направлении гипоплоидии: модальный класс соответствует 41 хромосоме (32–40% клеток); доля клеток с количеством хромосом 40–42 составляет 78–80%; доля полиплоидов – в среднем около 1%; пределы изменчивости хромосомного набора соответствуют диапазону от 36 до 54 хромосом. Клеточная сублиния ВНК-21/SUSP/ARRIAH при суспензионном культивировании с посевной концентрацией 0,6–0,8 млн кл./см^3 имеет кратность прироста 6,67–11,00 при жизнеспособности клеток 96–99%. В клеточном цикле популяции новой сублинии через 48 ч преобладает G1-фаза (диплоидная-2*n*), на которую приходится 30,0–75,0% клеток; в фазах подготовки к митозу (S-фаза) и митотического деления (G2+M-фаза) находится от 3,0 до 20,0% всей популяции; количество крупноядерных и многоядерных клеток (> 4*n*) в начале и конце стадии логарифмического роста увеличивается до 2%. Клетки сублинии ВНК-21/SUSP/ARRIAH быстро восстанавливаются после криоконсервирования с жизнеспособностью 95–99% и кратностью прироста 3,36–5,88 на первом – третьем пассажах и 6,85–10,95 – с четвертого по двенадцатый пассаж. Перевиваемая суспензионная линия клеток ВНК-21/SUSP/ARRIAH обеспечивает накопление вируса ящура в титрах 7,30–8,00 lg ТЦД$_{50}$/см^3, вируса бешенства – 7,25–8,00 lg ККИД$_{50}$/см^3, вируса парагриппа-3 крупного рогатого скота в титрах не менее 6,00 lg ТЦД$_{50}$/см^3, вируса болезни Ауески – 7,50–7,80 lg ТЦД$_{50}$/см^3.

Ключевые слова: клеточная линия ВНК-21/SUSP/ARRIAH, биологические свойства культуры клеток, ящур, бешенство, парагрипп-3 крупного рогатого скота, болезнь Ауески

Благодарность: Работа выполнена за счет средств ФГБУ «ВНИИЗЖ» в рамках тематики научно-исследовательских работ «Ветеринарное благополучие».

Для цитирования: Доронин М. И., Гусева М. Н., Михалишин Д. В., Шарыпов А. С., Мудрак Н. С., Камалова Н. Е., Манин Б. Л. Исследование биологических свойств перевиваемой суспензионной линии клеток ВНК-21/SUSP/ARRIAH. *Ветеринария сегодня*. 2021; 10 (3): 230–238. DOI: 10.29326/2304-196X-2021-3-38-230-238.

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

Для корреспонденции: Доронин Максим Игоревич, кандидат биологических наук, ведущий научный сотрудник лаборатории профилактики ящура ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: doronin@arriah.ru.*

INTRODUCTION

Continuous suspension BHK-21 clone 13 line derived from newborn Syrian hamster kidney cells by M. Stoker and J. Macpherson in England in 1961 is one of the cell cultures most commonly used worldwide [1]. The results of the study were published by the authors in scientific journals, such as *Virology* (1962) and *Journal of the National Cancer Institute* (1963). Later, the following analogues of this cell culture were developed:

– BHK-21/13 – a subline of continuous newborn Syrian hamster kidney cells developed at the Institute of Poliomyelitis and Viral Encephalitis of the Academy of Medical Sciences of the USSR (Moscow) [2];

– BHK-21/2-17b – a continuous monolayer-suspension clone of newborn Syrian hamster kidney cells developed at the All-Union Foot-and-Mouth Disease Research Institute (Vladimir) [2, 3];

– BHK-21/13-02 – a continuous monolayer-suspension subline of newborn Syrian hamster kidney cells developed at the Federal State Enterprise "Shchelkovo Biocombinat" for FMD and rabies virus reproduction [4];

– BHK-21/13-13 – a continuous monolayer-suspension subline of newborn Syrian hamster kidney cells developed at the Federal State Enterprise "Shchelkovo Biocombinat" for reproduction of type A, O, C, Asia-1, SAT-1, SAT-2, SAT-3 FMDV and "Shchelkovo-51" strain of rabies virus used for production of virus vaccines against foot-and-mouth disease and rabies [5, 6].

Continuous cell lines used in biotechnology differ in respect of morphology, karyology, growth properties, cultivation requirements, maintenance methods and susceptibility to viruses [7, 8]. The available BHK-21 cell sublines are characterized by high growth rates and allow for FMD and rabies virus reproduction. Full-scale production of culture vaccines against FMD, rabies, bovine parainfluenza-3 and Aujeszky's disease requires the use of a suspension BHK-21 cell subline to ensure the accumulation of the viruses with high infectivity titres. BHK-21/13, BHK-21/2-17b, BHK-21/13-02 and BHK-21/13-13 cell lines allow for preparation of viruses with the following infectivity titres: FMD virus – not more than 7.00 lg TCID$_{50}$/cm^3, rabies virus – 7.00 lg CCID50/cm^3 [4, 5, 9, 10]. Data on the results of bovine parainfluenza-3 and Aujeszky's disease virus cultivation are not provided in the patents for the said BHK-21 cell sublines.

To respond to the production needs related to the reproduction of FMD, rabies, bovine parainfluenza-3 and Aujeszky's disease viruses with high infectivity titres, BHK-21/2-17b cell subline was developed at the All-Union Foot-and-Mouth Disease Research Institute by means of BHK-21 clone 13 cell culture selection, from which a new BHK-21/SUSP/ARRIAH subline was derived 30 years later by means of permanent cultivation in suspension. BHK-21/SUSP/ARRIAH cell subline was deposited in the Specialized Cell Culture Collection of the Center for Collective Use "Collection of Vertebrate Cell Cultures" of the Institute of Cytology of the Russian Academy of Sciences with No. RKKK(P) 797 D [11].

The study is aimed at examining the biological properties of continuous suspension BHK-21/SUSP/ARRIAH subline of newborn Syrian hamster kidney cells and evaluating the possibility of using it for animal virus reproduction.

MATERIALS AND METHODS

Cell cultivation in suspension was carried out using semi-continuous culture technique in glass and metal fermenters with the capacity of 40 to 2,000 l in separate cycles, 10–12 passages each. A culture growth medium containing 5% of bovine serum, blood protein hydrolysate at a concentration of 15–20 cm^3/dm^3, Hottinger's digest (2–10 cm^3/dm^3), 8 proteinogenic aminoacids, vitamins and mineral salts were used for cultivation. Cell seeding concentration was 0.6–0.8 million cells/cm^3. Every 12 hours, pH levels, live and dead cell concentrations, suspension sterility were measured. During cell reproduction, lactate was formed, and this contributed to pH decrease. Therefore, a 7.5% baking soda solution was added to the cell suspension and/or pH was adjusted by bubbling.

Cell morphology analysis. Cells were examined using phase contrast microscopy at different magnifications. In order to assess the nucleus-to-cytoplasm ratio, the suspension cells were treated with acridine orange. The acridine orange exposure resulted in bright greenish-yellow staining of nuclei visualized on the yellow background of cytoplasm. Trypan blue staining was used for cell viability determination [12].

Karyological analysis of cells was carried out using the technique described by P. S. Moorhead et al. [13] that allows for detection of metaphase chromosome. Log phase cells collected from the fermenters were transferred onto the solid substrate in the growth nutrient medium containing 0.001% of colchicine and incubated for 3–4 hours. Rounded metaphase cells were agglomerated by shaking and then concentrated by centrifugation of the suspension. The further process was carried out in centrifuge tubes as follows: 10 minutes – hypotonic treatment at 36 °C; 3 treatments with fixing solution, 10 minutes each, with centrifuging at 22–25 °C. The resulting suspension was applied to the chilled slides with a Pasteur pipette and stained with Giemsa's solution for 10–15 minutes. After the preparation had been prepared, 100 metaphase plates were photographed, the number of chromosomes in them was calculated using immersion microscopy at 90× magnification, and karyogram was plotted [14].

Cytometric analysis of the cell subline. The comparative analysis of cell cycle phases was carried out by means of cytometry [15, 16] (n = 22) using an Accuri C6 flow cytometer and the cell DNA detection kit "C6 Flow Cytometer Fluid Kit" (BD Accuri™, USA) according to the manufacturer's recommendations. DNA histograms were obtained for the subline cells 48 hours after subcultivation.

DNA eluates were analyzed in the cytometer using the programme "Analysis of cell cycle parameters and DNA content in live cells". The process lasted 2 hours; fluorescent signal was recorded. Cell distribution by G1/G0-, S- and G2/M-phases of the cell cycle was determined by measuring relative DNA content in the cells using DNA binding fluorescent dyes.

Cryopreservation of the cell suspension was carried out in 100 cm^3 vials using a cryomedium (a growth medium supplemented with 7–10% of dimethyl sulfoxide and 20% of fetal calf serum). The cell suspension was cooled to minus 70 °C at a cooling rate of 2 °C/min, to minus 150 °C – at 10 °C/min; then the vials were placed in liquid nitrogen at minus 196 °C. The cell suspension was thawed at 39–42 °C during 2 minutes.

For cell thawing, direct seeding method comprising the following steps was used: rapid thawing of the suspension at 37 °C using a water bath; mixing of 1.0 cm^3 of cells with 20 cm^3 of the culture growth medium containing 10% of calf serum (cell seeding concentration was 0.5–0.7 million cells/cm^3); incubation of cells for 12 hours with subsequent change of the medium in order to remove the cryopreservation agent.

Viruses. The following viruses were used: FMDV A/Turkey/2006, O/Saudi Arabia/2008, Asia-1/Tajikistan/2011 strains, rabies virus "ARRIAH" and "RV-97" strains, Aujeszky's disease virus "VK" and "K" strains, bovine parainfluenza-3 virus "VGNKI-4" strain.

RESULTS AND DISCUSSION

BHK-21/SUSP/ARRIAH cell morphology analysis in comparison with BHK-21/2-17b. Cell morphology of BHK-21/2-17b subline was studied at the time of patenting in 1986 (Fig. 1).

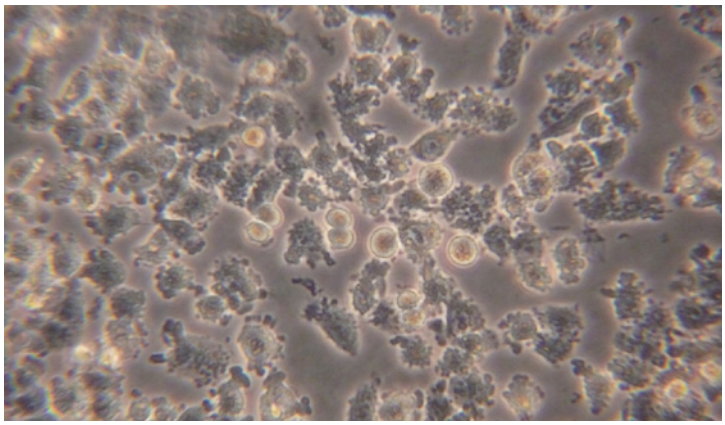

Fig. 1. Cell morphology of BHK-21/2-17b subline (passage 1, at the time of patenting in 1986; magnification 80×)

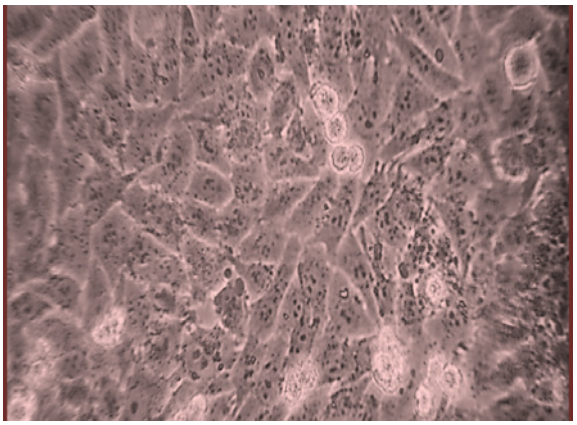

Fig. 2. Cell morphology of BHK-21/2-17b subline (passage 2; magnification 80×)

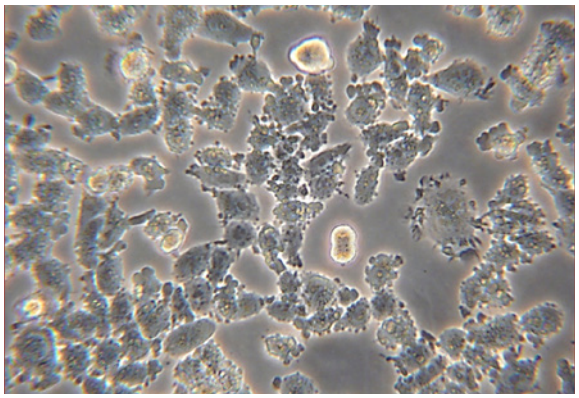

Fig. 3. Cell morphology of BHK-21/SUSP/ARRIAH subline (magnification 80×)

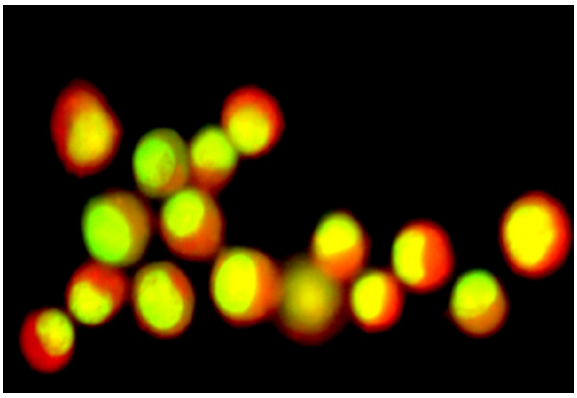

Fig. 4. Visualization of nuclei of suspension BHK-21/SUSP/ARRIAH subline cells stained with acridine orange

Morphologically, the cells were found to be amorphous; in other words, passage 1 cells seeded onto the substrate did not have a definite form, moved actively and attained a spherical form only during division. Passage 2 cells of this subline were fully adherent and attained an epithelioid form (Fig. 2). When all available substrate was covered with cells, some of the cells formed a suspension and demonstrated high proliferation rates (their growth rate was not less than 4–6).

The morphology of suspension BHK-21/SUSP/ARRIAH cells is shown in Figure 3. The morphology studies showed that the cells remained practically unchanged externally as compared with BHK-21/2-17b subline. Small cells of 8–10 μm in size prevailed in the population (up to 90%). It should be noted that, when passaged on the horizontal surface, the cells demonstrated low adhesion (the exceptions were polyploids) and moved intensively. Multiple mitotic divisions resulted in a significant increase in suspension cell concentration in the culture flask. Thus, the developed BHK-21/SUSP/ARRIAH cell subline is exclusively a suspension cell subline.

For nucleus-to-cytoplasm ratio assessment, 50 samples of the suspension of BHK-21/SUSP/ARRIAH cells grown in the glass and metal fermenters were collected and treated with acridine orange. The acridine orange exposure resulted in bright greenish-yellow staining of nuclei visualized on the yellow background of cytoplasm (Fig. 4).

Based on nucleus and cell diameter measurements, it was concluded that the new subline demonstrates a larger nucleus size in relation to cytoplasm and cell size. In most cases, the nucleus made up 60–80% of the cell volume.

Karyological analysis of BHK-21/SUSP/ARRIAH cells in comparison with BHK-21/2-17b. Chromosome preparations for the karyological analysis were prepared according to the technique described by P. S. Moorhead et al. [13]; then 100 metaphase plates were examined microscopically and microphotographed, the number of chromosomes was calculated, and karyogram was plotted.

Figure 5 shows BHK-21/2-17b cell population composition at the time of patenting in 1986. Multiple tests showed the stable predominance of 42-chromosome cell populations (28–40% of the entire population). The following chromosome numbers were detected: 36 chromosomes – in 1% of the populations, 37 chromosomes – in 2%, 38 chromosomes – in 9%, 39 chromosomes – in 3%, 40 chromosomes – in 8%, 41 chromosomes – in 27%, 42 chromosomes – in 40%, 43 chromosomes – in 7%, 44 chromosomes – in 2%, polyploids made up 1%.

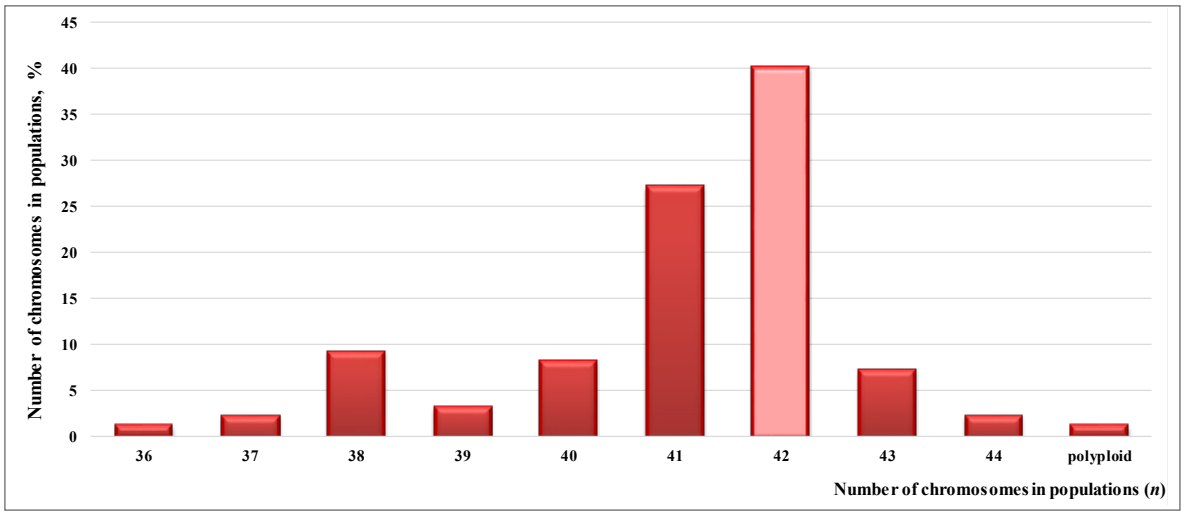

Fig. 5. Karyogram of BHK-21/2-17b subline cell population at the time of patenting (1986)

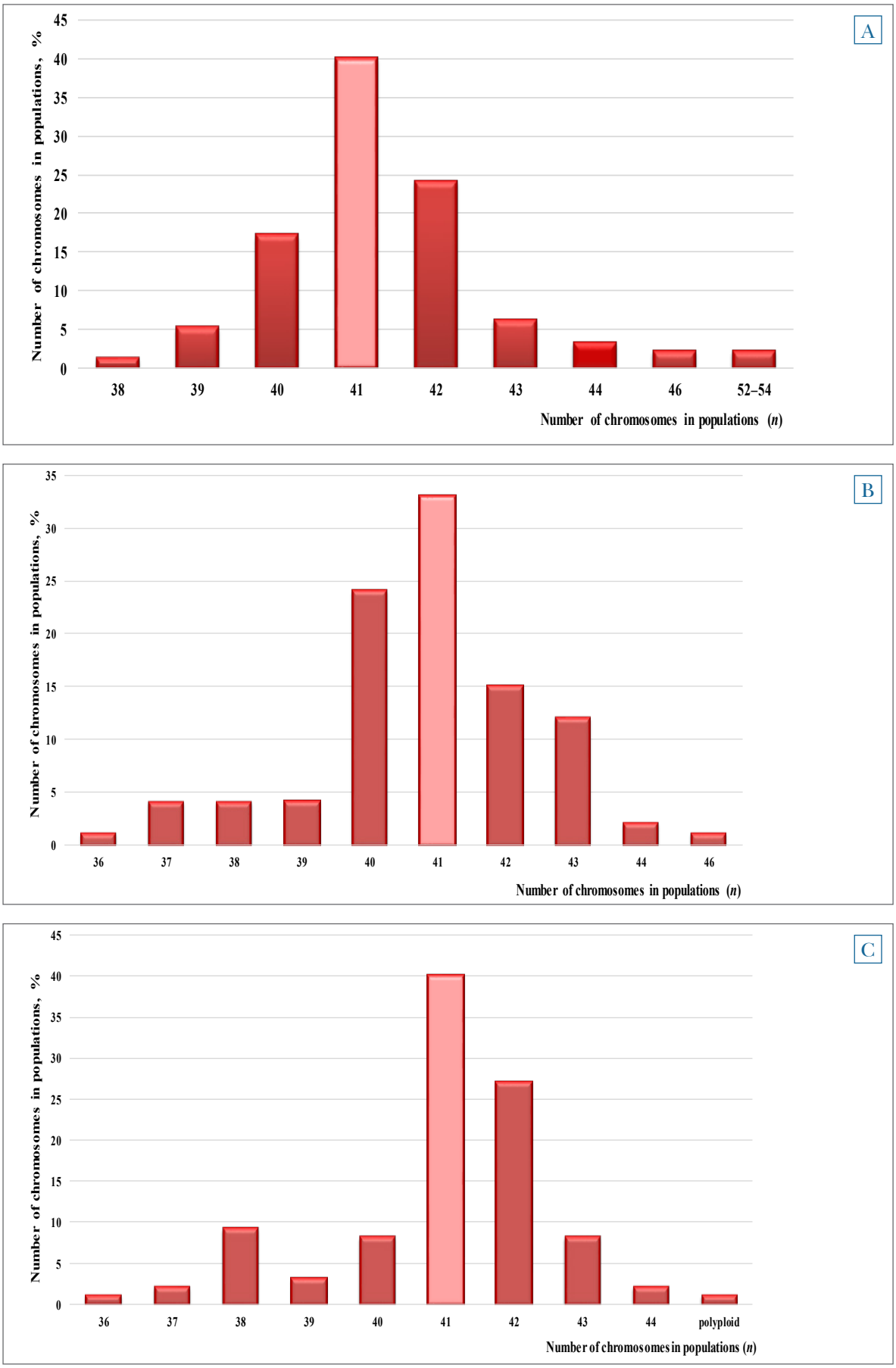

Fig. 6. Karyogram of BHK-21/SUSP/ARRIAH subline cells grown in 50 (A), 250 (B), 2000 dm³ (C) fermenters

During three decades, monolayer culture of these cells was not used in production. As a result of suspension cultivation in the growth medium during about 100 consecutive passages, BHK-21/2-17b cells transformed into a new cell subline, BHK-21/SUSP/ARRIAH. The populations of cells of this line grown in 40, 250 and 2000 dm3 fermenters were subjected to karyological analysis. Fifty samples from each of the cell suspensions prepared 48 hours after seeding were used for the tests. It was found that the cells had undergone some karyological changes under homogeneous culture conditions. The modal class of the new subline is represented by cells with 41 chromosomes (32–40% of the population). The share of cells containing 40–42 chromosomes is 78–80%, the share of polyploids averages around 1%. The range of variation in the number of chromosomes in BHK-21/SUSP/ARRIAH cells is from 36 to 54. The following chromosome numbers were detected: 36 chromosomes – in 0–1% of the populations, 37 chromosomes – in 0–4%, 38 chromosomes – in 1–9%, 39 chromosomes – in 3–5%, 40 chromosomes – in 8–24%, 41 chromosomes – in 33–40%, 42 chromosomes – in 15–27%, 43 chromosomes – in 6–12%, 44 chromosomes – in 2–3%, 46 chromosomes – in 0–2%, and 52–54 chromosomes – in 0–2% (Fig. 6).

Cytometric analysis of BHK-21/SUSP/ARRIAH cells in comparison with BHK-21/2-17b. DNA histograms presented in Figure 7 and data in Table 1 were obtained in the course of comparative analysis of cell cycle phases of the two sublines 48 hours after cell suspension subcultivation.

Based on the data obtained, it was found that apoptotic cells and cell debris made up 51.0–60.0% of BHK-21/2-17b cells, that is 5.0–31.0% more than the percentage for BHK-21/SUSP/ARRIAH subline (20.0–55.0%). Cells undergoing G1-phase (pre-synthesis phase) made up 30.0–75.0% of BHK-21/SUSP/ARRIAH subline cells and 28.0–70.3% of BHK-21/2-17b subline cells. This phase is characterized by cell preparation for chromosome duplication, intensive synthesis of polypeptides, an increase in the number of mitochondria and ribosomes. The share of G1-phase cells in the new subline was 2.0–4.7% more as compared with BHK-21/2-17b subline. Apparently, some of BHK-21/2-17b cells, in between mitotic M-phase and the beginning of G1-phase, enter apoptosis, and this subsequently has impact on the growth rate of the entire population.

Cells undergoing S-phase (synthesis phase) that involves cell DNA replication in many replicons and the beginning of centriole duplication in the cell centre make up 2.0–33.0% of BHK-21/SUSP/ARRIAH subline cells and

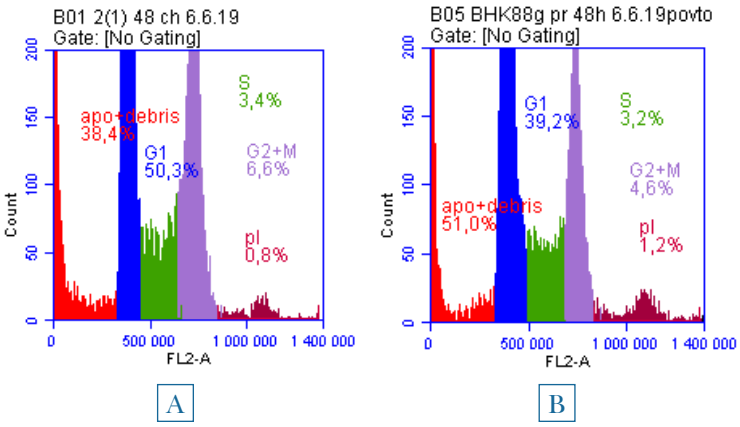

Fig. 7. DNA histogram of BHK-21/SUSP/ARRIAH (A) and BHK-21/2-17b (B) subline cells

Table 1
Comparative analysis of life cycles of BHK-21/SUSP/ARRIAH and BHK-21/2-17b subline cells based on cytometry data

Cell cycle phase	Percentage of cells undergoing the specified cell cycle phase, %		Difference in cell percentages, %
	BHK-21/SUSP/ARRIAH	BHK-21/2-17b	
Apoptosis	20.0–55.0	51.0–60.0	31.0–5.0
G1-phase	30.0–75.0	28.0–70.3	2.0–4.7
S-phase	2.0–33.0	1.5–30.0	0.5–3.0
G2+M-phase	3.0–20.0	2.6–18.0	0.4–2.0

Table 2
Evaluation of growth properties of suspension BHK-21/SUSP/ARRIAH subline cells in comparison with prototype cell sublines

Cell subline	Monolayer cultivation				Suspension cultivation		
	split ratio	monolayer formation time, days	monolayer survival time, days	number of cells per cultivation flask, mln*	growth rate	cell concentration, mln/cm^3	viable cells, %
BHK-21/13	1:3	2–3	3	40–45	maintained as suspension**		
BHK-21/2-17b	1:3	2–3	5	40–45	6.00–7.00	2.30–2.80	95–99
BHK-21/13-02	1:2–1:3	2–3	5	40–45	6.00–8.00	up to 4.00	95–99
BHK-21/13-13	1:2–1:3	2–3	5–6	40–45	6.00–8.00	2.40–4.00	95–98
BHK/SUSP/ARRIAH	low adhesion, amorphous cells with multiple cytoplasmic protrusions				6.67–11.00	4.00–5.50	96–99

* a cultivation flask with a growth surface area of 375 cm^2 is used;
** detailed information on suspension cultivation [6, 10] is not reflected.

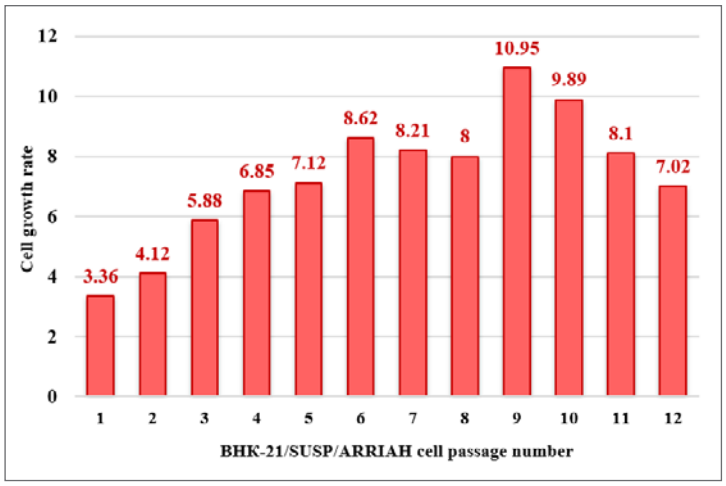

Fig. 8. Growth rate variations of BHK-21/SUSP/ARRIAH subline cells recultivated after cryopreservation (mean growth rate values are provided; n = 30, p < 0.005)

1.5–30.0% of prototype line cells, i.e. 0.5–3.0% less as compared with the cells of the new subline.

After 48 hours, cells undergoing G2-phase (post-synthesis phase) characterized by energy accumulation, protein accretion for mitosis and tetraploid DNA content attainment and cells undergoing M-phase (mitosis) account for 3.0–20% of BHK-21/SUSP/ARRIAH cells, which is 0.4–2.0% more than the percentage for BHK-21/2-17b cells (2.6–18%); this is indicative of a greater capacity of the cells of the new subline to continue division.

Thus, as a result of suspension cultivation in the growth medium with modified composition during about 100 consecutive passages, BHK-21/2-17b cells transformed into a new cell subline, BHK-21/SUSP/ARRIAH, in which, after 48 hours, cells undergoing G1-phase of the cell cycle prevail (30.0–75.0% of cells). Cells undergoing the phases of preparation for mitosis and mitosis (G2+M-phase) account for 3.0 to 20.0% of the entire population. The number of meganucleated and multinucleated cells (> 4*n*) at the beginning and at the end of the logarithmic phase increases to 2%. In other words, the basic population, apparently, underwent selection manifested as a 5.0–31.0% decrease in the number of apoptotic cells and cell debris amount and an increase in the number of cells undergoing G1-, S- and G2+M-phases that ensure cell growth by 2.0–4.7; 0.5–3.0 and 0.4–2.0%, respectively.

Table 3
Cultivation of FMD, rabies, bovine parainfluenza-3 and Aujeszky's disease viruses in suspension BHK-21/SUSP/ARRIAH cell subline ($p < 0.005$)

Cultivated viruses	Cytopathic effect, %	Virus infectivity titre	Number of tests
FMDV A/Turkey/06 strain	95–98	7.30 ± 0.13 lg $TCID_{50}/cm^3$	100
FMDV O/Saudi Arabia/08 strain	95–98	7.80 ± 0.23 lg $TCID_{50}/cm^3$	100
FMDV Asia-1/Tajikistan/11 strain	95–99	8.00 ± 0.21 lg $TCID_{50}/cm^3$	80
rabies virus "ARRIAH" strain	95–99	8.00 ± 0.13 lg $CCID_{50}/cm^3$	40
rabies virus "RV-97" strain	95–99	7.25 ± 0.20 lg $CCID_{50}/cm^3$	40
Aujeszky's disease virus "VK" strain	95–97	7.80 ± 0.15 lg $TCID_{50}/cm^3$	40
Aujeszky's disease virus "K" strain	95–97	7.50 ± 0.19 lg $TCID_{50}/cm^3$	40
bovine parainfluenza-3 virus "VGNKI-4" strain	95–98	6.00 ± 0.15 lg $TCID_{50}/cm^3$	50

Evaluation of growth properties of BHK-21/SUSP/ARRIAH subline cells in comparison with prototype sublines. BHK-21/SUSP/ARRIAH cells resulting from long-term suspension cultivation (100 consecutive passages) were tested for their growth properties in comparison with prototype BHK-21/13, BHK-21/2-17b, BHK-21/13-02 and BHK-21/13-13 sublines. The test results are presented in Table 2. These data show that BHK-21/SUSP/ARRIAH cells cultivated in suspension using semi-continuous culture technique (seeding concentration is 0.5–0.6 million cells/cm^3) reach a concentration of 4.0–5.5 million cells/cm^3 after 48 hours of cultivation under optimum conditions. Thus, they demonstrate growth rates of 6.67–11.00 and 96–99% viability. Suspension BHK-21/SUSP/ARRIAH cells grown in culture flasks under static conditions were characterized by low adhesion, amorphous form, multiple cytoplasmic protrusions (the protrusion dynamics spanned a period of several minutes).

Viability tests of BHK-21/SUSP/ARRIAH subline cells recultivated after cryoconservation. The number of living cells was determined in suspension after cryopreservation ($n = 10$). Based on the test results, it was found that the viability of cells after cryopreservation was 85–90% at passage 1 and increased to 96–99% starting from passage 2.

One-day proliferation delay was observed in depreserved cells grown in suspension during passage 1. Figure 8 shows BHK-21/SUSP/ARRIAH cell growth rates for 12 consecutive passages after cryopreservation. Proliferation rates at passages 1–3 varied from 3.36 ± 0.21 to 5.88 ± 0.15 ($p < 0.005$). Starting from passage 4, growth rates were from 6.85 ± 0.18 to 10.95 ± 0.31 ($p < 0.005$). It is considered that the normal growth rate for BHK-21 cells is 4 and above [9, 17]. In other words, BHK-21/SUSP/ARRIAH cell line attains the required cell growth rate starting from passage 2. The highest cell proliferation rate for the tested subline was observed at passage 9. It should be noted, that at passage 12 the cell growth rate was 7.02 and exceeded the lower normal value by 3.02. Thus, BHK-21/SUSP/ARRIAH cell subline is recultivated rapidly after cryopreservation and demonstrates high proliferation rates (up to 10.95 ± 0.31) and 95–99% cell viability.

Reproduction of FMD, rabies, bovine parainfluenza-3 and Aujeszky's disease viruses in* BHK-*21/SUSP/ARRIAH subline cells in comparison with prototype sublines (based on literature data). To prepare virus-containing material for virus vaccine production, the following virus strains were reproduced in continuous suspension BHK-21/SUSP/ARRIAH cells: FMDV A/Turkey/2006 ($n = 100$), O/Saudi Arabia/08 ($n = 100$), Asia-1/Tajikistan/2011 ($n = 80$)

strains, rabies virus "ARRIAH" (n = 40) and "RV-97" (n = 40) strains, Aujeszky's disease virus "VK" (n = 40) and "K" (n = 40) strains, bovine parainfluenza-3 virus "VGNKI-4" (n = 50) strain (Table 3).

The comparative analysis based on the obtained results and literature data for prototype cell sublines [4, 5] demonstrated that FMDV caused 95–99% cytopathic effect (CPE) and showed infectivity titres of 7.30 ± 0.13 to 8.00 ± 0.21 lg $TCID_{50}/cm^3$ (n = 280), when cultivated in BHK-21/SUSP/ARRIAH cells, and up to 7.00 lg $TCID_{50}/cm^3$ in BHK-21/2-17b, BHK-21/13-02 and BHK-21/13-13 cell cultures.

Rabies virus caused 95–99% CPE and showed infectivity titres of 7.25 ± 0.20 to 8.00 ± 0.13 lg $CCID_{50}/cm^3$ (n = 80), when reproduced in BHK-21/SUSP/ARRIAH cell subline, 6.50 lg $CCID_{50}/cm^3$ in BHK-21/13-02 cell culture, up to 7.00 lg $CCID_{50}/cm^3$ in BHK-21/2-17b and BHK-21/13-13 cell cultures. Aujeszky's disease virus cultivated in BHK-21/SUSP/ARRIAH cell culture caused 95–97% CPE and was accumulated with infectivity titres of 7.50 ± 0.19 to 7.80 ± 0.15 lg $TCID_{50}/cm^3$ (n = 80); bovine parainfluenza-3 virus caused 95–98% CPE and showed infectivity titres of up to 6.00 ± 0.15 lg $TCID_{50}/cm^3$ (n = 50).

Thus, the cells of BHK-21/SUSP/ARRIAH subline developed for production of virus vaccines and diagnostic veterinary biologicals allow for FMD virus reproduction with titres of up to 8.00 ± 0.21 lg $TCID_{50}/cm^3$, i.e. 1.00 lg $TCID_{50}/cm^3$ higher in comparison with prototype sublines (not more than 7.00 lg $TCID_{50}/cm^3$). The proposed subline ensures rabies virus accumulation with titres of up to 8.00 ± 0.13 lg $CCID_{50}/cm^3$, i.e. 0.25–1.00 lg $CCID_{50}/cm^3$ higher in comparison with prototype lines (not more than 7.00 lg $CCID_{50}/cm^3$), as well as bovine parainfluenza-3 and Aujeszky's disease virus accumulation with titres of 6.00 ± 0.15 and up to 7.80 ± 0.15 lg $TCID_{50}/cm^3$, respectively.

CONCLUSION

The biological properties of continuous suspension BHK-21/SUSP/ARRIAH subline of newborn Syrian hamster kidney cells were studied, and the possibility of using it for foot-and-mouth disease, rabies, bovine parainfluenza-3, Aujeszky's disease virus reproduction was evaluated.

It was found that BHK-21/SUSP/ARRIAH cell subline, when cultured in suspension, undergoes selection towards hypoploidy: modal class is represented by cells with 41 chromosomes (32–40% of cells); the share of cells containing 40–42 chromosomes is 78–80%; the share of polyploids averages around 1%; the range of variation in the number of chromosomes is from 36 to 54.

It was detected that BHK-21/SUSP/ARRIAH cell subline cultivated in suspension using semi-continuous culture technique demonstrates exponential growth (proliferation rate decreases after 48 hours) with growth rates of 6.67–11.00 and 96–99% cell viability.

It was established that suspension BHK-21/SUSP/ARRIAH cells grown under static conditions are characterized by low adhesion, amorphous form, multiple cytoplasmic protrusions and during mitosis accumulate in the culture medium in suspension.

It was found that, after 48 hours, G1-phase cells prevail in the cell population of BHK-21/SUSP/ARRIAH subline (30.0–75.0% of cells); cells that undergo preparation for mitosis and mitosis (G2+M-phase) account for 3.0 to 20.0% of the entire population; the number of meganucleated and multinucleated cells (> 4n) at the beginning and at the end of the logarithmic phase increases to 2%.

Suspension BHK-21/SUSP/ARRIAH cells were found to recover rapidly after cryopreservation demonstrating 95–99% viability and growth rates of 3.36–5.88 (passages 1–3) and 6.85–10.95 (passages 4–12).

The study showed that suspension BHK-21/SUSP/ARRIAH cells allow for virus reproduction with the following titres:

– FMD virus – 7.30–8.00 lg $TCID_{50}/cm^3$;
– rabies virus – 7.25–8.00 lg $CCID_{50}/cm^3$;
– bovine parainflunza-3 virus – at least 6.00 lg $TCID_{50}/cm^3$;
– Aujeszky's disease virus – 7.50–7.80 lg $TCID_{50}/cm^3$.

REFERENCES

1. Stoker M., Macpherson I. Syrian hamster fibroblast cell line BHK-21 and its Derivatives. *Nature.* 1964; 203: 1355–1357. DOI: 10.1038/2031355A0.

2. Manin B. L., Mikhalishin V. V., Korpusova T. I., Stegniy B. T., Lavrik A. A. Biological tolerance parameters of production BHK-21 cell culture [Parametry biologicheskoj tolerantnosti proizvodstvennoj kletochnoj kul'tury BHK-21]. *Veterinary Medicine: Inter-Departmental Subject Scientific Collection*. Kharkiv; 2002; 80: 404–408. (in Russian)

3. Sokova V. V., Aliyeva L. A., Bondarenko A. F., Khudyakov G. A. BHK-21/2-17b line of newborn Syrian hamster kidney cells [Kletochnaya liniya pochki novorozhdennogo sirijskogo homyachka BHK-21/2-17b]. Certificate of Authorship No. 240289 USSR. All-Union Foot-and-Mouth Disease Research Institute; 1986. (in Russian)

4. Eliseev A. K., Melnik N. V., Zenov N. I., Litenkova I. Yu., Bogach V. N., Ivanov V. S., et al. BHK-21/13-02-transplantable monolayer-suspension subline of newborn syrian hamster kidney cells, intended for FMD virus and rabies virus reproduction. Patent No. 2614074 Russian Federation, Int. C12N 5/07 (2010.01). FKP "Shchelkovskij biokombinat". No. 2005116301. Date of filing: 31.05.2005. Date of publication: 22.03.2017. Bull. No. 9. (in Russian)

5. Eliseev A. K., Zenov N. I., Krasutkin S. N., Mel'nik N. V., Khajkina L. S., Litenkova I. Yu., et al. BHK-21/13-13-finite monolayer-suspension cell subline of kidney of new-born syrian hamster, intended for reproduction of foot-and-mouth disease virus and rabies virus. Patent No. 2553552 Russian Federation, Int. C12N 5/071 (2010.01). FKP "Shchelkovskij biokombinat". No. 2014131866/10. Date of filing: 01.08.2014. Date of publication: 20.06.2015. Bull. No. 17. (in Russian)

6. Russian Cell Culture Collection (RCCC) [Rossijskaya kollekciya kletochnyh kul'tur (RKKK)]: catalogue. Ed. by G. P. Pinayev, G. G. Polyanskaya. St. Petersburg; 2004. 315 p. (in Russian)

7. Bailey J. E., Ollis D. F. Biochemical Engineering Fundamentals. 2nd ed. New Delhi: McGraw-Hill, Inc.; 1986. 984 p.

8. Cell culture techniques [Metody kul'tivirovaniya kletok]: Proceedings of the Academy of Sciences of the USSR. Executive ed. G. P. Pinayev. L.: Nauka; 1988. 319 p. (in Russian)

9. Catalogue of Russian Cell Culture Collection [Katalog Rossijskoj kollekcii kletochnyh kul'tur]. Executive ed. G. P. Pinayev, G. G. Polyanskaya. Omsk: Omsk State Pedagogical University (OSPU); 1999. 429 p. (in Russian)

10. Yurkov S. G., Zuyev V. V., Sidorov S. I., Kushnir S. D., Smyslova N. Yu., Neverovskaya N. S., et al. Catalogue of Cell Culture Collection of the All-Russian Research Institute of Veterinary Virology and Microbiology (VNIIVViM) [Katalog kollekcii kletochnyh kul'tur VNIIVViM]. Pokrov; 2000; 60–62. eLIBRARY ID: 21632428. (in Russian)

11. Lozovoy D. A., Guseva M. N., Mikhalishin D. V., Doronin M. I., Manin B. L., Shishkova A. A., et al. BHK-21/SUSP/ARRIAH – continuous suspension subline of newborn syrian hamster kidney cells, intended for reproduction of foot-and-mouth disease viruses, rabies, parainfluenza-3, Aujeszky's disease in producing antiviral vaccines, as well as for making diagnostic and preventive veterinary biopreparations. Patent No. 2722671 Russian Federation, Int. C12N 5/10 (2006.01). FGBI "ARRIAH". No. 2019131190. Date of filing: 01.10.2019. Date of publication: 02.06.2020. Bull. No. 16. (in Russian)

12. Polyanskaya G. G. Karyotype variation patterns in cell cultures during long-term cultivation in different cell lines [Zakonomernosti kariotipicheskoj izmenchivosti v kletochnyh kul'turah pri dlitel'nom kul'tivirovanii v raznyh liniyah]: author's abstract of Candidate of Science (Biology) thesis. St. Petersburg; 2000. 38 p. (in Russian)

13. Moorhead P. S., Nowell P. C., Mellman W. J., Battips D. M., Hungerford D. A. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell. Res.* 1960; 20: 613–616. DOI: 10.1016/0014-4827(60)90138-5.

14. Mamayeva S. Ye. Chromosomal analysis of cultured cells [Hromosomnyj analiz kul'tiviruemyh kletok]. *In: Cell culture techniques*. L.: Nauka; 1987; 78–98. (in Russian)

15. Kudryavtsev I. V., Khaydukov S. V., Zurochka A. V., Chereshnev V. A. Flow cytometry in experimental biology [Protochnaya citometriya v eksperimental'noj biologii]. Yekaterinburg: Ural Branch of the Russian Academy of Sciences (UB RAS); 2012. 192 p. (in Russian)

16. Ormerod M. G., Tribukait B., Giaretti W. Consensus report of the task force on standardisation of DNA flow cytometry in clinical pathology. *Analytical Cellular Pathology*. 1998; 17 (2): 103–110. DOI: 10.1155/1998/842306.

17. Rais Y., Zviran A., Geula S., Gafni O., Chomsky E., Viukov S., et al. Deterministic direct reprogramming of somatic cells to pluripotency. *Nature*. 2013; 502 (7549): 65–70. DOI: 10.1038/nature12587.

Received on 12.03.2021
Approved for publication on 05.05.2021

INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Maksim I. Doronin, Candidate of Science (Biology), Leading Researcher, Laboratory for FMD Prevention, FGBI "ARRIAH", Vladimir, Russia.

Marina N. Guseva, Candidate of Science (Biology), Senior Researcher, Laboratory for FMD Prevention, FGBI "ARRIAH", Vladimir, Russia.

Dmitry V. Mikhalishin, Candidate of Science (Veterinary Medicine), Head of Laboratory for FMD Prevention, FGBI "ARRIAH", Vladimir, Russia.

Andrey S. Sharypov, Candidate of Science (Veterinary Medicine), Researcher, Department for Research Coordination, FSBI CNMVL, Moscow, Russia.

Natalia S. Mudrak, Doctor of Science (Biology), Chief Researcher, Reference Laboratory for Avian Viral Diseases, FGBI "ARRIAH", Vladimir, Russia.

Natalia Ye. Kamalova, Doctor of Science (Veterinary Medicine), Chief Researcher, Centre for Preclinical Studies, FGBI "ARRIAH", Vladimir, Russia.

Boris L. Manin, Candidate of Science (Biology), Leading Researcher, Sector for Cell Culture, Innovation Department, FGBI "ARRIAH", Vladimir, Russia.

Доронин Максим Игоревич, кандидат биологических наук, ведущий научный сотрудник лаборатории профилактики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Гусева Марина Николаевна, кандидат биологических наук, старший научный сотрудник лаборатории профилактики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Михалишин Дмитрий Валерьевич, кандидат ветеринарных наук, заведующий лабораторией профилактики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Шарыпов Андрей Сергеевич, кандидат ветеринарных наук, научный сотрудник отдела координации научно-исследовательских работ ФГБУ ЦНМВЛ, г. Москва, Россия.

Мудрак Наталья Станиславовна, доктор биологических наук, главный научный сотрудник референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Камалова Наталья Евгеньевна, доктор ветеринарных наук, главный научный сотрудник центра доклинических исследований ФГБУ «ВНИИЗЖ», г. Владимир, Россия.

Манин Борис Леонидович, кандидат биологических наук, ведущий научный сотрудник сектора культуры клеток отдела инноваций ФГБУ «ВНИИЗЖ», г. Владимир, Россия.