Receiving new perspective for biotechnology and virology cell cultures of fishes

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Receiving new perspective for biotechnology and virology cell cultures of fishes

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Abstract. Due to the increase in the population of the planet and the increasing need for proteinaceous food one of the most important sectors of agriculture - the aquaculture - is crucial for ensuring food security of the country. Control of viral diseases of fishes with use of biotechnology methods - such as cell cultures is necessary for development of the industry. The new lines of cells offered by authors from fabrics and bodies of the fishes which are industrially grown up in Russin Federation (sturgeons, a trout, a bull-trout), the technology of their receiving and also information on sensitivity to viruses will allow to solve a problem of timely diagnostics of viral infections of fishes and to provide an intensive development of aquaculture. The obtained cultures: SSF (VIEV) -1,2,3; OMG, STLE are incorporated in the cryobank and deposited in the Specialized Collection of Intermittent somatic cell cultures of agricultural and commercial animals RKKK (P) (SZHZ RAAS) at the FGBU of the FSC of the VIEV RAS and can be demanded by other scientific-research institutions for the solution of different tasks.

1. Introduction

The fish which is grown up in an aquaculture and also withdrawn from sea and freshwater natural reservoirs represents the main source of protein and necessary nutrients for the person, its usefulness for health gains the increasing recognition. Therefore, sustainable fishery and an aquaculture are crucial for ensuring food security of the country. The only way of ensuring food security of the country is the aquaculture - one of the most fast-growing branches making production of animal origin, providing nearly a half of all world consumption of fish. One of the reasons of control of development of this sector of agriculture are viral diseases of the grown-up fishes which control high-quality and timely diagnostics with use of cell cultures can provide.

Cell cultures of living organisms are an integral part of biotechnology, they are used to solve scientific problems of general biology, cytology, genetics, virology, immunology, infectious pathology and also preservations of genetic material of endangered species of animals. [1,2,3]. The in vitro cell culture is a dynamic system of interacting cells that are in constant reproduction, development, and dying out. Creation of optimal conditions for the vital activity of cells outside the body is one of the main problems of cellular biotechnology.

The development of methods for cultivating cells and the mastering of these methods by specialists of biofactories has made it possible to solve many problems of the production of viral vaccines and

diagnosticums. Biofactories, companies and research institutes of Russia produce a large number of biologics for agriculture, using primary cell cultures, subcultures, diploid strains, permanent cell lines, hybrid and genetically transformed animal cell cultures. In practical virological work, the most important is to study the sensitivity spectrum of cell cultures to viruses of different taxonomic groups.

Due to the production of single-layered gonad carp cells in our country for the first time in 1970, a virus was isolated from sick carp fish, and when studying the properties and role of the virus in the pathology of fish, it was established that the agent caused a disease, later called spring carp viremia (SVC) [4]. And in 2001, the virus of infectious pancreatic necrosis of salmon (IPN), a representative of the family Birnaviridae was detected for the first time in Russia, in the salmon fry derived from fertilized caviar imported from Norway [5].

In total, over 450 cell cultures from different types of fish tissues have been obtained in the world, of which there are less than 20 in Russia. Therefore, it is necessary to further improve cultivation methods, to obtain new cell cultures that are highly susceptible to viruses and to create new cellular systems based on hybridization and genetic transformation of cells.

Currently, in diagnostic studies and in the isolation of viruses, the main laboratory cell models are transplantable cell lines due to their undoubted convenience, stability, characterized properties and some other advantages. The creation of a reference stock of cells, the study of viability in long-term storage in liquid nitrogen, gives great advantages in research work, since cells are a valuable biological material necessary for the reproduction and accumulation of the virus, the creation of diagnostic systems. The acquisition of new cell lines and the study of the spectrum of susceptibility to viruses will solve the problem of timely diagnosis of viral infections of fish and ensure intensive development of aquaculture.

At present, the improvement of cultivation technologies, the emergence of a large number of synthetic media and modern equipment has allowed the laboratory staff to continue research and to obtain a number of new interchangeable lines for diagnostic activities [6,7].

2. Obtaining and characterization of cell lines from Siberian sturgeon fin tissue

In experiments on the preparation of primary trypsinized cultures, fin tissue of two-year-old Siberian sturgeon (*Acipenser baeri*) with a mass of 0.5 kg was used. To disaggregate the tissues, a 0.25% solution of trypsin was used. Nutrient media: 199 with Hanks salts, basic and minimal NEED and DMEM with Earle salts, fetal calf serum (20%). Hanks solution with antibiotics: gentamicin, kanamycin, streptomycin and amphotericin B. Subculturing of the cells was carried out once in 3-4 weeks, a mixture of trypsin solutions (0.25%) and versene (0.02%) was used in various ratios. At the level of the fifth and older passages, cytological preparations were prepared: fixed in absolute alcohol and stained by Romanovsky-Giemsa. For cryopreservation of cultures, a cryoprotective medium consisting of 50% medium 199, 40% serum of cow fruit and 10% DMSO was used. Sterility of the cell lines was determined by sowing on the nutrient media of MPB, MPA, thioglycolic, Edward-Hayflick and modified dense BEEV. Particular attention was paid to decontamination of the tissue in the first stage of the work: the fins were rinsed in tap water until most of the mucus was removed, then immersed in 70 ° C for 5-10 seconds, then transferred to Hanks's solution with a high content of antibiotics: gentamicin (250 μg / ml), kanamycin (1000 μg / ml), streptomycin (1000 μg / ml) and amphotericin B (5 μg / ml). Wash in this solution for 5 minutes 5-7-fold, then crushed, kept in fresh solution - 1 hour, after - another 40 minutes.

The first fraction, after 10 minutes of trypsinization at room temperature, was removed. The main trypsinization was carried out on a magnetic stirrer at a temperature of 4 ° C for 15 hours, then 3 times for 2 hours at room temperature, and the suspensions were combined. At the end of this time, pieces of incompletely disaggregated tissue, with a volume of 1-2 mm3, remained. After centrifugation, the cells were resuspended in medium 199 with Hanks salts 500-700 kp / ml, pieces of non-disaggregated tissue were sown in separate vials.

During the first month of cultivation, the environment was changed weekly. Four weeks later, the first subculture was performed. The monolayer was rinsed with Hanks' solution and poured with a dispersing mixture of 1 part of trypsin and 3 parts of Versene for 15 minutes until the monolayer

"swelled," then drained, incubated for another 15 minutes before the cells began to peel off the substrate, but some of the cells after resuspension with fresh medium remained on glass. The ratio of 1: 2 for the cells removed, a growth medium was added to the vial with the remaining cells.

After the first subculturing of the cultures of fixed pieces of tissue with a mixture of 1 part of trypsin and 3 parts of versene, only the explants were exfoliated, which subsequently lost the ability to attach and were removed from the medium. The cells were separated with a mixture of 1 part of trypsin and 6 parts of versene. Coefficient of 1: 2, the monolayer was formed on the 5th-7th day.

After 10 days of incubation at a temperature of 22-23 ° C, a mixed monolayer was formed in the vials, represented by cells of the epithelioid-like and fibroblast-like type. Cells of attached explant pieces actively migrated forming growth zones from epithelial-like cells.

From the first to the fifth passage a monolayer of fibroblast-like cells did not exfoliate completely from the substrate, the detached cells transferred to new vials formed a monolayer consisting of elongated cells lying in bundles of fan shape at different angles to each other, with large oval nuclei containing 2-3 nucleolus, vacuoles of various sizes were visible in the cytoplasm of most cells. After the growth medium was added to the vials with the remaining cells, a monolayer of another pattern was formed, cells with a clearly expressed flow. By the sixth passage of culture stabilized, the vacuolization of the cytoplasm significantly decreased, a gradual decrease in the amount of serum (up to 10%) was started and the addition of antibiotics was stopped. The lines were named SSF (Siberian sturgeon fins, VIEV) -1 and SSF (VIEV) -2, respectively.

At level 10, the culture passage was laid in a cryobank at the FSC VIEV. By the present moment more than 100 passages have passed through the culture and are permanent cell lines.

In the vials with explants, the monolayer was not formed. For subcultivation, vials were used in which the active growth zone around the pieces was more than 5-7 mm. As a result, a line of epithelial-like cells was obtained, to the fifth transition completely lost fibroblast-like and polygonal elements.

The most active growth was observed on medium 199 with 20% fetal bovine serum. The cells are small, epithelio-like, with large rounded nuclei, there are no vacuoles in the cytoplasm. After the first transition, the monolayer was formed after 20 days, with subsequent passages after 7-10 days, an increase in the seed density to 1: 1.5 did not lead to a faster formation of the monolayer, some cells were not attached, degenerated after some time in the medium. To date, the culture has passed 70 transitiones, the addition of antibiotics is discontinued, the amount of serum is 10%, the line is called SSF (VIEV) -3. When studying the lines obtained for the presence of bacteria, fungi and mycoplasmas, negative results were obtained.

The technique of cell cultivation is constantly changing, and methodical techniques are systematically modernized, however, everything is very individual in each case. Initially, in carrying out this work, we relied on the data of the authors who prepared two cell lines from a similar tissue [8], but received completely different results. Three cell lines differing in morphology are obtained: fibroblast-like cells arranged in fan-shaped bundles SSF (VIEV) -1, fibroblast-like - forming an annular pattern reminiscent of the fingerprints SSF (VIEV) -2 and epithelio-like SSF (VIEV) -3. It was found that the SSF (VIEV) -2 line differs from other variants in the presence of secretory activity, which is manifested an increase in the viscosity of the growth medium, which is especially noticeable in long-term culture without replacement by a supporting one.

The obtained lines retain stable temperature and growth characteristics and morphology over more than 400 transitiones and are widely used in the virological practice of our laboratory, the SSF-1 culture is protected by the RF patent (No. 2488632) [9].

3. Obtaining, a cytomorphological and karyological characteristic of a diploid strain of gonad cells of rainbow trout (Oncorhynchus mykiss)

In experiments on the production of primary cell cultures, gonads of the third stage of maturity from five female rainbow trout (Oncorhynchus mykiss) weighing 2.0-2.2 kg, grown in an aquarium laboratory were used.

For the initial washing of the material, sterile saline and Hens's solution with penicillin 500 units / ml, streptomycin 500 U / ml, enrofloxacin 25-30 μg / ml, and amphotericin B 2.5 μg / ml were used. To disaggregate the tissues, 0.25% trypsin solution was taken. Nutrient media: MEM needle on Erl salt with 25mM HEPES and DMEM on Earl salts, newborn calf serum, fetal calf serum.

In the early transitiones subcultivation was carried out once every 4-6 weeks, using a mixture of trypsin solutions (0.25%) and versene (0.02%) in various ratios, subsequently every 2-3 weeks.

At the level of the first, fifth and further (multiples of 5) transitiones, cytological (fixation and staining according to Romanovsky-Giemsa) and karyological preparations (according to Moorhead) were prepared.

For cryopreservation of the culture, a cryoprotective medium consisting of 50% culture medium, 40% fetal calf serum and 10% DMSO, a concentration of 3-4 ppm / ml was used.

Sterility was determined by inoculating the culture media of MPB, MPA, thioglycolic, Edward-Hayflick, and modified dense CVEV.

The gonads were completely washed in Hanks solution and saline solution with antibiotics 3-4 times for 5 minutes in each portion. Scrapped with scissors, kept in fresh solution 4 times for 10 minutes and filled with trypsin.

The first fraction, after 15 minutes of trypsinization, was removed, because it contained very few cells. Then fractional trypsinization was carried out, 4 times for 30 minutes, until tissue was completely disaggregated. Suspensions were collected in one vial, in which 10% of the serum of the newborn calves was added to inactivate trypsin. Cells were concentrated to 500 kl / ml, two media were used: Eagle MEM Ehler salts with 25 mM HEPES and DMEM, in each case 20% fetal bovine serum was added.

The cell suspensions were plated in the wells of the micropanel (Nunc) with the coverslips placed on the bottom: for a 6-well panel of 24x24 mm size, for a 12-hole 9x18mm, and left at temperatures of 15, 18 and 22 ° C.

Every two to three days of incubation, the micro-panels were examined. After 7 days a dense monolayer of mixed morphology was formed, so that fibroblast-like elements predominated at a temperature of 15 ° C, epithelio-like elements at 22 ° C.

For subculturing, the amount of epithelial cells taken from wells was maximal by a visual assesment. Glasses with cells were removed from the wells, rinsed with Hanks solution, transferred to new panels, filled with dispersing mixture 1h. trypsin and 3h. Versene, for 10 minutes, then drained, incubated, shaken, for another 10-15 minutes, until the cells completely separated from the glass. with a coefficient of 1: 3-5, the monolayer was formed after 7-10 days. The most active growth was observed in Eagle MEM medium on Earle salts with 25 mM HEPES and addition of 20% fetal bovine serum.

The first five transitiones were made in micro-panels with glasses. After formation of the monolayer, the most promising glasses, with growth sites of epithelial-like cells, were used for subcultivation, the rest for cytological analysis and karyological control of culture. When studying the culture for the presence of bacteria, fungi and mycoplasmas negative results were obtained.

Subsequently, the cells were transferred to culture mattresses, the amount of serum gradually, during 4-5 transitiones, was reduced to the traditional 10%, the addition of antibiotics was stopped. In the course of the work, it was noted that the cells obtained do not have such a high metabolic activity during the growth period, as transfused, even under very long storage (up to 3 months), there is no significant acidification of the medium, which indirectly indicates their diploid status.

The resulting strain was named OMG-Oncorhynchus mykiss gonade. At level 15 of the transitiones, the culture is laid in the cryobank at the FSC VIEV.The viability of cells after thawing is 80-85% when Trypan blue is stained on zero transitiones.

By now the culture has passed more than 180 transitiones. At this stage, disaggregation of the monolayer during subculturing is carried out with a mixture of trypsin 0.25% and versene 0.02% (1: 4), the cells are poured and left for 20 minutes, then the dispersing solution is drained, left for another 20 minutes before the separation of cells from the surface growth. Resuspended in Eagle MEM medium on Erl salts with 25mM HEPES with 10% embryonic serum without antibiotics, pH 7.4-7.6, 1: 3 sieving ratio. The sowing concentration is 250-300x103 cells / ml. The first day after subculturing the culture is

kept at 22oC, to form a monolayer, the next day the cells are transferred to storage at 15C, the frequency of 1 time 2 - 3 weeks.

Thus, a selection was made and a diploid cell strain was obtained from iridescent trout gonads having an even, dense monolayer of epithelial-like morphology cells, with large round or oval cores with 1-3 nucleoli. Cells are divided by bipolar mitosis. The karyotype corresponds to the species of trout. The modal class contains 60 chromosomes (2n = 60) and is 77%.

Diploid cultures have a number of advantages over primary cultures and constant cell lines. They are distinguished from the primary by the homogeneity of the population and the stability of the cultural and morphological properties, the absence of contamination, high sensitivity to viruses, the possibility of scaling, and distinguished from the transplantable by the lack of transformation and various kinds of contamination. Thus, the obtained diploid cell culture of gonads of rainbow trout OMG is a highly sensitive, environmentally friendly cellular system that ensures the absence of foreign contaminants in scientific research and biotechnological processes.

The described strain of cells is protected by the patent of the Russian Federation No. 2495120 [10].

In experiments on sensitivity studies, a culture of OMG cells was used at 55-65 transitiones. Viruses were accumulated in reference cell lines, after complete destruction of the monolayer, a virus-containing culture fluid was used for titration in OMG cell culture. The infectious virus titer was determined by cytopathic action (CPD) in cell culture, in polystyrene micro-panels. The cytopathic effect was obtained by microscopy of infected cell cultures after 1, 3, 5, 7 days of incubation at the optimum temperature +15 °C for IPN, VHS (virus hemorrhagic septicemia), IHN (infectious hematopoietic tissue necrosis), SbSH (herpes sturgeon virus); plus 20 °C for SVC (spring carp viremia). The final registration of the CPD was carried out on the 10th day. The titer of the virus was determined by the method of Reed and Mench. Studies have shown that the OMG cell line is sensitive to six viruses of three taxonomic groups: Birnaviridae, Rhabdoviridae, Nerpesviridae - IPN, IHN, SVC, herpes carp koi (KH), SbSH, VHS.

The IPN virus was reproduced in the OMG culture from the first transitiones, with the symptoms of CPD observed one day earlier than in the reference culture, 24 hours after infection, and after 3-4 days - complete destruction of the monolayer. The first signs of degeneration were expressed in a cluster of dark cells with broken light, with subsequent separation from the substrate. The infectious titer was 1.5 times higher than in the reference culture.

On the sensitivity of detection and on the virus-producing ability of rhabdo-viruses VHSV and IHNV, the OMG cell line from the first transition exceeds by 0.5-1.25 order the reference line EPC and only the efficiency of detection of SVCV, a virus of the same family, even after three passages, is noticeably lower, by 1.5 orders. The first signs of CPD of rhabdoviruses on the OMG cell line appeared 72 hours later, and then increased. The complete destruction of the monolayer occurred on the 4th-5th day after infection. The character of the CPR of the viruses IHN, VHS, SVC was approximately the same and was expressed in cell rounding, pycnosis, and dilution of the monolayer.

When the culture of OMG cells was inoculated with herpesvirus of Siberian sturgeon, it was found that the virus was reproduced with the development of a typical CPD: the formation of simplasts, cellular debris and complete cell detachment from the substrate. However, the first signs of CPD and complete destruction of the monolayer were detected later (on the third day) than in the culture of WSSK-1, while the infectious titer was the same.

As a result of the experiments, it was established that the OMG cell line is particularly sensitive to salmonid birnavirus – IPNV [11].

4. Obtaining and biological properties of cell culture from troutta embryos (*Salmo truttae L.*)

In experiments on obtaining primary cultures of cells, fertilized caviar of trout trout (Salmo truttae L.) was used. The biomaterial was obtained from a fish farm that is free from infectious and invasive diseases, which is confirmed by the results of a seven-year monitoring (two or more times a year) conducted by the staff of the ichthyopathology laboratory of the FGBNU FNC of the VIEV RAS.

For the initial washing of the material, sterile saline and Hens's solution with penicillin 500 units / ml, streptomycin 500 U / ml, enrofloxacin 25-30 μg / ml, and amphotericin B 2.5 μg / ml were used. To

disaggregate the tissues, 0.25% trypsin solution was taken. Nutrient media: MEM needle on Erl salt with 25mM HEPES and DMEM on Earl salts, newborn calf serum, fetal calf serum.

In the early transitiones subcultivation was carried out once every 4-6 weeks, using a mixture of trypsin solutions (0.25%) and versene (0.02%) in various ratios, subsequently every 2-3 weeks.

At the level of the first and fifth transitiones, cytological (fixation and staining according to Romanovsky-Giemsa) and karyological preparations (according to Moorhead) were prepared.

For cryopreservation of the culture, a cryoprotective medium consisting of 60% culture medium, 30% fetal bovine serum and 10% DMSO, a concentration of 3-4 ppm / ml was used.

Sterility was determined by inoculating the culture media of MPB, MPA, thioglycolic, Edward-Hayflick, and modified dense CVEV.

In the experiments we used caviar at the eye ocellus stage. Completely formed embryos were removed from the embryo sac with the help of scissors, and transferred for washing into saline solution with antibiotics 3-4 times for 5 minutes in each portion. Scrapped with scissors, kept in Hanks solution 4 times for 10 minutes and filled with trypsin.

Disaggregation of the tissue was carried out according to the technique repeatedly used by the authors, and it was well established in the preparation of cell cultures from sturgeon and rainbow trout tissues [12].

The resulting cell suspension in a concentration of 500-700 thousand cells / ml was plated in plastic bottles of 25 cm^3 (Nunc).

During the first month of cultivation, the environment was changed weekly. Four weeks later, the first subculture was performed. The monolayer was rinsed with Hanks' solution and poured with a dispersing mixture of 4 parts of trypsin and 1 part of Versene for 15 minutes, until the monolayer "swelled," then drained, incubated for another 15 minutes before the peeling of the cells from the substrate, the coefficient of re-entry was 1: 3. The monolayer, represented by cells of epithelioid and fibroblast-like types, was formed on 3-5 day. The most active growth was observed in Eagle MEM medium on Earle salts with 25 mM HEPES and addition of 20% fetal bovine serum. When studying the culture for the presence of bacteria, fungi and mycoplasmas negative results were obtained. Later, the number of serum was reduced to 15%, the addition of antibiotics was stopped.

The resulting strain was named STLE-Salmo truttae Linneus embrio. At level 8 of the transition, the culture is laid in the cryobank at the FSC VIEV. The viability of cells after thawing is 80-85% when Trypan blue is stained on zero passage.

By now, the culture has passed more than 150 passages. At this stage, disaggregation of the monolayer during subculturing is carried out with a mixture of trypsin 0.25% and versene 0.02% (2: 1), the cells are poured and left for 20 minutes, then the dispersing solution is drained, left for another 20 minutes before the separation of cells from the surface growth. Resuspend in Eagle MEM medium on Erl salts with 25mM HEPES with 10% embryonic serum without antibiotics, pH 7.4-7.6, 1: 3 sieving ratio. The sowing concentration is 250-300x103 cells / ml. Incubation between the resections at 15 ° C, the frequency of 1 time 2 - 3 weeks.

The culture is a diploid strain of cells from trout embryos having an even, dense monolayer of cells of epithelial-like morphology, with large nuclei of round or oval shape with 1-3 nucleoli. In some mattresses, cells form a loose monolayer consisting of cells of another type - large, polygonal with outgrowths and one nucleus. Cells are divided by bipolar mitosis. The karyotype corresponds to the species of trout. The modal class contains 68 chromosomes (2n = 60) and is 72% [13].

5. Conclusion

In total, more than 450 cell lines were obtained by researchers from different countries, but only a few dozens of them were deposited and stored in the American Type Culture Collection (ATCC) or the European Collection of Cultures (ECACC).

The Russian Collection of Cell Cultures (RKKK) was established in 1978 to collect and store cell lines, to certify and deposit them, and to create an information bank for cell line strains [14]. The bulk of the fish cell lines in Russia are deposited in one of the branches of the RCAC - Collections of

Intermittent somatic cells of agricultural and commercial animals (FHB) at the FGBNU FNC of the VIEV RAS and consists of 16 cell lines from eight fish species, some of which have been deposited for patenting purposes. Thanks to the continuation of scientific research work on obtaining fish cell cultures by the staff of the laboratory of ichthyopathology, the Collection constantly replenishes its funds.

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