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REPRODUCTION CAPABILITY OF BOVINE LEUCOSIS FIELD ISOLATES IN SOME CELL CULTURES

Gorbatenko S. K., Stegniy M. Yu., Korneykov A. N.
National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine»,
Kharkiv, Ukrain, e-mail: leylab@vet.kharkov.ua

Summary. The article observes capability of cow embryo lung (CEL), calf coronary vessel (CVC), sheep kidney (SK), and calf kidney (CK) passaged cells cultures to reproduce bovine leukemia virus after integration of pathogen field isolates. The presence of pathogen proviral DNA was detected by molecular-genetic studies at different cultivation stages. Increasing of hemagglutinin titer in CVC, SK, CK, and CEL cell cultures was established in between 5–6 passages.

Keywords: leucosis, cattle, cells cultures, CVC, SK, CK, field isolates, reproduction capability

Kidney of a sheep embryo, chronically infected by bovine leukemia virus (FLK-BLV), is used in developing of specific disease prophylactic methods, as well as in manufacturing derivation of leucosis antigen that is a part of diagnostic kit for leukemia infected animals detection (Busol et al., 2000; Burba et al., 1996; Stegniy et al., 2011; Tsymbal et al., 1990). Solving the problem of practical veterinary medicine support with facilities for leukemia diagnosis involves searching of more effective ways of viral biomass accumulating in the technological scheme of drugs production. The quantitative detection of virus accumulating in culture medium is provided by its activity analysis after concentration (Stegniy et al., 2011; Tsymbal et al., 1990). The results of some authors (Syurin et al., 1998) obtain the possibility to draw a conclusion about presence of bovine leukemia virus pathogen in culture media by its capability to agglutinate the mice erythrocytes.

The purpose of our research is to integrate the BLV field isolates in separate cell cultures that are homology for correspondent animal species with the aim of pathogen adaptation, and accumulation of viral biomass for further practical use.

Materials and methods. CEL, CVC, SK, and SK-2 passaged cultures that are kept in cryobank of NSC 'IECVM' biotechnology laboratory has been taken for study. The cell cultures, derived from cryobank, were adapted to the nutritional medium in three passages. At that time, the morphofunctional characteristics of cultures had been studied. Three flasks of each culture were used for infection contamination. The preparation of infected material was begun from blood sampling, stabilized with 3.8% sodium citrate, in sterile-assembled blood collection systems. The cows infected with bovine leukemia virus (and positive by the results of immunodiffusion test) with clinical features of lymphoid malignancy (with haematological illness based on the results of the conducted pilot research) have been used as blood donors. The amount of leucocytes in blood of animals-donors at the time of sampling were 18.4–19.4 g/sm^3, the ratio of lymphocytes in leucocytic fraction was 87–92%. The leucoconcentrate was collected by providing of erythrocyte lysis using 0.83% ammonium chloride solution during 10 min at room temperature 20–22 °C.

The obtained material has been washed off ammonium and erythrocytes vestiges three times by physiological saline and with addition of bacteriostatic medications. The separate samples of leucoconcentrate were mixed. Working suspension was obtained by leucoconcentrate and physiological saline mixing. The method of supravital staining by 0.1% solution of trypan blue was used for accounting the saved cells. Working suspension contained 72,000 of live cells in 1 sm^3.

Leucoconcentrate was controlled for sterility according to DSSU 4483:2005.

The suspension was divided into two parts after cell account. The first part of native leucocytes was passaged into two flasks of each cell culture in a dose of 72,000 of live cells in 1 sm^3, one flask of each culture was left as a positive control. Nutritional culture media included 50% of Eagle medium and 50% of 199 medium without addition of blood serum. Plating was incubated at 37 °C for 48 h in the germinating apparatus.

 [1] Scientific mentor — Prof. A. P. Gerilovych

The second part of leucoconcentrate was divided into three parts.

The first part was subjected to osmotic lysis using distilled water at 3–4 °C for 30 min.

The second part was defrosted at (–20 … +20) °C.

The third part was mixed with Versene solution for preventing leucocytes adhesion. Then it was subjected to disintegration with intensity at 4.5 kHz, synchronization at 4 for 1 min (duration of each step was 30 s) in ice cooling regimen with the use of disintegrating machine UZDU-A.

All three parts were mixed in total capacity, where the general protein content was detected. The suspension was used for infection of one flask of each used cell culture with leucoconcentrate in a dose of 0.22% by general protein content in 1 sm^3 of culture medium. The infected cell cultures were incubated in culture medium with equal content of Eagle and 199 media without addition of blood serum for 48 h at 37 °C. The cattle blood serum was added to the culture medium in next passages, its ratio was slowly increased from 1–2% to 10%. The culture medium contained maximal concentration of cattle aglobuline blood serum at the sixth passage — 10%.

The control of antigen-production activity of cell cultures was provided using concentration of virus fluid after 3^{rd}, 5^{th}, 10^{th}, 12^{th}, 15^{th} passages for the purpose of antigen activity detection. At the same time, the part of virus fluid from separate passages was investigated for proviral DNA detection using molecular-genetic method (PCR).

The culture swabs has been investigated for leukemia virus presence using hemagglutination reaction with 1.5% suspension of mice erythrocytes. The suspension of erythrocytes opposite to physiological saline was used as negative control to exclude the spontaneous erythrocytes autoagglitination phenomena.

Isolation of total DNA was provided using commercial DNA kit 'Sorb-A' by firm 'Amplisens' (Moscow, Russian Federation), amplification was provided using commercial kit 'GenePak DNA PCR test' by firm 'Isogene Lab. Ltd' (Moscow, Russian Federation).

Electrophoresis assay was provided using electrophoresis kit by scientific-production organization 'Narvac' (Moscow, Russian Federation). The agarose concentration in gel was 1.5%, current electric intensity was 40 mA, voltage was 75 V.

Results. The visual estimation of cell cultures CVC, SK-2, CK, and CEL was provided before integration of native or lysed leucocytes. In all cases it showed that cell monolayer at 4–5 days of cultivating was uniform, separate peripheral cells were oblong and some of them were spherical, the regions without monolayer were particularly absent. Integration of lysed and native leucocytes from blood of animal, in which the clinical form of lympholeucosis was registered, was provided in mentioned cultures without cell monolayer violation. The visual estimation of experimental and control cell cultures was provided upon completing the second and following passages using inverted microscope at ×7.

The monolayer integrated by native leucocytes after the second passage in SK-2 cell culture was insufficient, a goodly proportion of cells was self-standing, separate parts of monolayer exfoliated and were present in culture fluid. The same cell cultures after integration of lysed leucocytes were marked by fulfill monolayer with good-visible cell borders via cell density. The last cells with normal morphology, separate from monolayer and with frank destruction have been rarely found. The control SK-2 cell culture showed density monolayer, the cells saved their normal morphology.

The CK cell culture, integrated with native leucocytes, was marked by well-visible monolayer with oblong cells. However, it was violated in some areas. Herewith, the separate cells with destructive changes were seen per visual field.

In turn, the CK cell culture, integrated with lysed leucocytes, was marked by uniform monolayer with good-visible cell borders. Cells with destructive changes have been rarely seen per visual field.

The monolayer of control CK cell cultures was scarcely no different from the monolayer of matrix initial passaged culture. It was uniform, mosaic, its cells had normal morphology, and cells that separate from monolayer were rarely seen per visual field.

The monolayer of CVC culture, integrated with both lysed and native leucocytes was marked by uniform fullness. The cells had normal morphology; destructive changes were rarely fixed among separate cells. The distinct destructive changes of control cell cultures monolayer were not found during visual estimating.

The destructive changes of CEL cell culture morphology after integration of native leucocytes were not found.

The monolayer cells were situated closely, with distinct borders. Vacuoles and oblong nucleuses were rarely occurred.

Similar passages estimating (monolayer structure, cells characteristics) was provided during 5–6 passages. It was fixed, that monolayer of all used cultures was marked by more clear areas with entirety violations with each subsequent passage. It fixed to the glass weaker and moved off easier. The number of cells with destructive violations increased. When estimating further passages, it was established the appearance of 'recovery' characteristics of cell cultures: the monolayer became more uniform, complete, the number of cells with destructive changes decreased.

In all cases when working with CVC, SK, CK, and CEL cell cultures, the syncytium formation was not observed. It only was noticed the appearance of separate cells with two or three nucleuses.

The culture fluid with cell monolayer was concentrated after the fifth passage according to the technology of leucosis antigen manufacturing. It was examined for activity in a standard immunodiffusion test with positive leukemic serum. Precipitin line on 48–72 h exposure was not observed.

The part of virus fluid of CVC and SK cell cultures was PCR-tested for proviral DNA detection after 4–14 passages in the NSC 'IECVM' laboratory of molecular biology. The results are shown in Table 1.

The results of the research conduct that samples number 2, 4, 5, 8, 9, 10 contain proviral DNA of bovine leukemia virus. The samples number 1, 3, 6, 7 has shown negative result.

Obtained results indicate the persistence of bovine leukemia virus to the 14th passage in CK cell culture, infected with native leucocytes; to the 12th passage in CK cell culture, infected with lysed leucocytes; and to the 4th passage in CVC cell culture, infected with lysed leucocytes.

Table 1 – Indication of proviral DNA in separate passages of cell cultures

№	Cell culture	Integrated leucocytes	Passage	Presence of BLV proviral DNA
1.	CVC	Control	4	–
2.	CVC	Lysed	4	+
3.	CVC	Lysed	6	+
4.	SK	Lysed	6	+
5.	SK	Lysed	13	+
6.	SK	Lysed	12	+
7.	SK	Lysed	14	–
8.	SK	Native	14	+
9.	SK	Lysed	13	–
10.	FLK-BLV	Control	14	+

Table 2 – Indication of BLV in cell cultures using haemagglutination test

№ of the passage	Antigen titer dilutions in haemagglutination test							
	SK		CK		CVC		CEL	
	Lysate	Native leucocytes	Lysate	Native leucocytes	Lysate	Native leucocytes	Lysate	Native leucocytes
0.	1:128	1:256	1:128	1:256	1:128	1:256	1:128	1:256
1.	1:8	1:16	–	1:8	–	–	_	1:16
2.	–	–	–	–	–	–	-	-
3.	–	–	–	–	–	–	-	-
4.	–	–	–	–	–	–	-	-
5.	–	1:8	1:8	1:8	–	–	1:8	-
6.	1:8	1:16	1:32	1:64	–	1:32	1:8	1:16
7.	1:16	1:64	1:16	1:64	1:8	1:8	1:4	1:8
8.	1:64	1:128	1:64	1:128	1:16	1:32	-	1:4
9.	1:64	1:128	1:128	1:64	Not cultivated	Not cultivated	Not cultivated	Not cultivated
10.	1:64	1:32	1:128	1:128	Not cultivated	Not cultivated	Not cultivated	Not cultivated
11.	1:256	1:128	1:256	Not cultivated	Not cultivated	Not cultivated	Not cultivated	Not cultivated
12.	1:128	1:256	Not cultivated	Not cultivated	Not cultivated	Not cultivated	Not cultivated	Not cultivated

Unfortunately, setting the proportion of virus in culture fluid in separate passages using the results of leukemia antigen activity in immunodiffusion test failed. We provided investigation of culture virus fluid using haemagglutination reaction with mice erythrocytes in order to determine this question. The results of this study are shown in Table 2.

The table shows, that culture fluid, obtained from 5–6 passages, contains antigen, agglutinating mice erythrocytes in 1:8–1:64 titer. Following passages increase titers. Titers of SK and CK cultural fluids were 1:128–1:256 at 11th passage. The SK and CK cultures have been more sensitive to infection and more addiction to virus adaptation. The CVC and CEL cultures have been not so sensitive to infection; haemagglutination test had shown the antigen presence only in 1:16–1:32 titer.

The negative result has been obtained in all control samples. It gives the evidence of the autoagglutination absence.

Conclusions.

1. The results of molecular-genetic analyses for BLV proviral DNA presence has shown that DNA fixes in CVC passaged culture to 4th passage, in SK culture with native lymphocytes to 14th passage, and with lysed lymphocytes to 12th passage.

2. Increasing of antigen concentration in culture fluids of SK, CK, and CVC passage series has been shown in haemagglutination test with mice erythrocytes.

References

Burba, L. G., Valikhov, A. F., Shishkov, V. P., Kiselev, N. V. and Sorokina, A. A. (1996) 'Preparations for bovine leukemia virus diagnostics' [Preparaty dlya diagnostiki leykoza krupnogo rogatogo skota], Kursk biofactory. To the 100th anniversary of the biological industry in Russia [Kurskaya biofabrika. K 100-letiyu biologicheskoy promyshlennosti Rossii]. Kursk: GUIPP 'Kursk', pp. 451–478. [in Russian].

Busol, V. O., Miahkykh, N. V., Zdanevych, P. P., Shapovalova, O. V. and Tsymbal, V. I. (2000) 'Production of trail series of antileukemic vaccine and its examination in laboratory conditions' [Vyhotovlennia doslidnykh serii protyleikoznoi vaktsyny ta perevirka yii v laboratornykh umovakh], *Scientific Bulletin of the National Agrarian University [Naukovyi visnyk Natsionalnoho ahrarnoho universytetu]*, 28, pp. 120–122. [in Ukrainian].

DSSU [State Committee for Technical Regulation and Consumer Policy] (2005) DSTU 4483:2005. *Veterinary immunobiological preparations. Methods for determination of bacterial and fungous contamination [Preparaty veterynarni imunobiolohichni. Metody vyznachannia bakterialnoi i hrybkovoi kontaminatsii]*. Kyiv: Derzhspozhyvstandart Ukrainy.

Stegniy, B. T., Fisenko, S. A., Stegniy, M. Y, Dunaev, Y. K., Konovalov, A. V. and Babanin, N. I. (2011) 'Improvement of the technology of antigen for serological diagnosis of bovine leukemia in immunodiffusion test production' [Udoskonalennia tekhnolohii vyrobnytstva antyhenu dlia serolohichnoi diahnostyky leikozu velykoi rohatoi khudoby v reaktsii imunodyfuzii], *Veterinary Medicine [Veterynarna medytsyna]*, 95, pp. 227–229. Available at: http://jvm.kharkov.ua/sbornik/95/5_99.pdf. [in Ukrainian].

Syurin, V. N., Samuylenko, A. Y., Solov'ev, B. V. and Fomina, N. V. (1998) *Viral diseases of animals [Virusnye bolezni zhivotnykh]*. Moscow: VNITIBP. [in Russian].

Tsymbal, V. I., Kiprich, V. V., Busol, V. A., Shikov, V. P., Burba, L. G. and Valikhov, A. F. (1990) *A method of antigen derivation for bovine leukemia virus diagnostics in immunodiffusion test [Sposob polucheniya antigena dlya diagnostiki leykoza krupnogo rogatogo skota v reaktsii immunodiffuzii]*. USSR Author's Certificate no: SU1585951. [in Russian].