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PERMISSIVITY OF LAMB SYNOVIAL MEMBRANE CELL CULTURE FOR AKABANE DISEASE VIRUS

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Abstract

Presently, due to the variety and diversity of economic and tourist ties of Russia, episodes of accidental or maybe purposeful (i.e., biological terrorism) entry of exotic infectious pathogens including Akabane disease to the Russian Federation should not be excluded. Akabane disease is a viral transmissible infection. Its recurrent outbreaks are characterized by abortions, still or premature births, or malformations (e.g. arthrogryposis and/or hydrocephaly) for calves, lambs and kids. Akabane disease virus can persist both in animal body and in vitro (e.g., in continuous cell lines). A study of the sensitivity of African green monkey kidney cell line to Akabane virus carried out earlier showed that Akabane virus caused definite cytopathic changes resulting in cell rounding followed by cytolysis and detachment of the cell monolayer within 48 hours post infection. In this paper we first reported on the cytomorphological changes caused by Akabane virus in the primary lamb synovial membrane diploid cell culture (LSMCC) prepared according to an earlier developed procedure, and on a suitability of this culture for the virus accumulation in titers sufficient for study and making diagnosis. It has been formerly determined that the lamb synovial membrane cell culture is sensitive to small ruminant lentiviruses like caprine arthritis encephalitis virus or Visna-Maedi virus in sheep. LSMCC was prepared using a method for tissue explant culture. On day 4 post inoculation of the cell monolayer with Akabane virus the cytopathic effect appeared which manifested as formation of syncytia that grew larger due to their fusion on day 5 to 6. The Akabane virus activity was 6.0 ± 0.05 lg TCID₅₀/cm³ for strain V8935, and 5.0 ± 0.05 lg TCID₅₀/cm³ for strain P. As many as three passages and also the primary cell culture (after freezing) kept the virus-producing activity, and the Akabane virus retained its infective properties. The lamb synovial membrane cells can be re-cultured, and excessive diploid culture can be frozen to preserve and thawed as required. It is expedient to use a strain of diploid lamb synovial membrane cells deposited and patented earlier. One more advantage of the primary LSMCC as compared to monkey cell lines is that the latter ones may be a source of simian herpes B virus.

Keywords: lamb synovial membrane cell culture, Akabane disease, *Orthobunyavirus*

Due to the expansion of international economic relations and the development of tourism, accidental entry of exotic infectious pathogens to the territory of Russia is possible. Moreover, because of episodes of international terrorism, there is a real threat of biological terrorism, the focused introduction of the dangerous infections pathogens. These include the virus of Akabane disease, a vector-transmitted disease with the recurrent outbreaks followed by abortions, still or premature births, or malformations (arthrogryposis, hydrocephaly) in calves [1-4], lambs and goatlings [5]. Anti-Akabane virus disease antibodies have been found in buffalo, horses, camels, pigs, and monkeys [6-8]. Encephalitis effects of the Akabane disease virus have been described in mice, hamsters, guinea pigs, and chicken embryos [9, 10]. Virus has no pathological effects on the humans, however anti-Akabane virus antibodies have been found in a number of subjects in Japan [11]. Infectious agent belongs to Simbu serogroup of the genus *Orthobunyavirus*, family *Bunyaviridae*, which includes Aino, Chamond, and Schmollenberg diseases viruses [12].

Akabane disease virus is transmitted by ticks and mosquitoes which ensures the formation of stable natural foci in harsh environments and creates the possibility of expanding the range of vertebrate hosts. Long-term preservation of the viral population in susceptible vertebrates contributes to the rapid spread of the disease among wild and domestic animals in a favorable for mosquitoes climate period [13]. Akabane disease virus has been isolated from the animals in Japan [14, 15], Israel [16], Korea [17-19], Australia [20], Turkey [21], Cyprus [22], Syria [23], Sudan [24], and Kenya [25]. The widespread, high contagiousness and economic losses are the reasons for which the study and control of this pathogen are necessary. Continuous cell cultures are the main laboratory model for the study of animal viruses, and the primary culture is the model in the absence of continuous cell lines.

Akabane disease virus can persist both in animal and in continuous cultures in vitro. At various times, a number of foreign papers reported that hamster lung (HmLu-1), African green monkey kidney (Vero), newborn Syrian hamster kidney (BHK-21-W12), pig kidney (PK-15), rabbit kidney (RK-13), and fetal calf kidney (BEK) continuous cultures were sensitive to Akabane disease virus [26-28].

In the Russian Federation, Akabane disease virus has been studied only at the All-Russian Scientific Research Institute of Veterinary Virology and Microbiology (VNIIVViM). The pathogen morphology has been described [29], a method of reverse transcription PCR (RT-PCR) was developed to detect Akabane virus genome [30], and the sensitivity of Vero [31] and African green monkey kidney CV-1 [32] cultures to the virus was studied.

Russian collections include continuous cell cultures sensitive to Akabane virus. Alternatively, however, it is important to have sensitive primary cell cultures (with a proof of their advantages or detection of disadvantages) which can be prepared in any equipped laboratory. A strain of lamb *Ovis aries* synovial membrane diploid cells has been patented and deposited in VNIIVViM [33] which is used to derive a primary culture. It is noted that this culture, and also its subcultures are stable in its biological characteristics [33] and sensitive in the early passages to small ruminant lentiviruses, such as caprine arthritis encephalitis virus and Visna-Maedi virus in sheep [33, 34].

In this paper, we first reported the cytomorphological changes caused by Akabane disease virus in lamb synovial membrane diploid cell culture prepared according to an earlier developed procedure [34]. This culture is suitable for the virus accumulation in titers sufficient for the study and diagnosis.

We evaluated primary lamb synovial membrane cell culture as a laboratory model for the accumulation and titration of Akabane disease virus.

Technique. Akabane disease virus (strains B8935 and P) was obtained from State VNIIVViM collection of microorganisms. Primary synovial membrane cell culture donor was a 3-day old lamb (grown in VNIIVViM experimental animals sector).

For cell culture, we used minimal Dulbecco's Modified Eagle's Medium (DMEM, HyClone Laboratories, Inc., USA) with a double amount of amino acids and vitamins, the fetal bovine serum (HyClone Laboratories, Inc., USA), Benzylpenicillin sodium salt (150 U/cm³) and gentamicin (100 µg/cm³). A mixture of 0.02 % Versene (Sigma, USA) and 0.25 % trypsin (Sigma, USA) at a ratio of 2:1 heated to 37 °C was applied for cell dispersion.

Cell culture was derived from a tissue explant [34]. Hock and wrist joints were collected aseptically, the skin was removed and the joints treated with 96 % alcohol for 15-30 seconds. The isolated synovial membrane was transferred into a Petri dish with nutrient DMEM medium containing 2 % fetal bovine serum

and the antibiotics, crushed mechanically into 1-2 mm fragments, then washed thrice with the medium of the same composition. Explants were placed into culture flasks with DMEM, 10 % fetal bovine serum and the antibiotics, and incubated at 38 °C, 90 % relative air humidity and 5 % CO₂. Lamb synovial membrane diploid cells resulted from passaging primary culture.

Virus containing culture liquid of the continuous CV-1 cell line (the infectious activity of $10^{4.0}$ lg TCID₅₀/cm³, a dilution of 1:100) was used for the lamb synovial membrane cell culture inoculation. The added culture liquid level was 3-4 mm above the cell monolayer. The inoculum was stored at -60 ± 0.5 °C before using. Akabane disease virus was cultured without absorption on cells and change of the culture medium using standard methods. Intact lamb synovial membrane cell culture was a control. Infected and intact lamb synovial membrane cell culture incubation continued for 8 days at 37 ± 0.5 °C under daily observation.

In Akabane disease virus titration performed in 3 replicates the lamb synovial membrane cells were cultured in 96-well polystyrene plates. A 150 µl aliquot of virus-containing DMEM diluted from 1:10 to 1:10 000 000 was added in each well. The plates were kept at 5 % CO₂ and 90 % relative humidity in an incubator. To compare sensitivity, Vero and CV-1 cultures from VNIIVViM Collection of Cell Cultures were used as well.

Statistical analysis included estimation of the mean and standard deviation.

Results. In continuous Vero [31] and CV-1 cultures (Fig. A, B), Akabane virus caused cytopathic changes resulting in cell rounding followed by cytolysis and detachment of the cell monolayer within 48 hours post infection. Cytomorphological transformation took place in lamb synovial membrane cell culture as well (see Fig., C-E).

In subculture, lamb synovial membrane cell population transformed from primary culture to cell subculture. At day 6, we observed extensive cell colonies which merged with each other turning into a confluent monolayer. The cells formed a clearly defined multidirectional chords typical of fibroblast-like cultures (see. Fig., C). Lamb synovial membrane cell culture was viable and not subjected to morphological changes.

Akabane disease virus was able to replicate in lamb synovial membrane cell culture without prior adaptation. In the infected culture, unlike the intact one, we found significant cytopathic changes. The culture retained its sensitivity to the Akabane disease virus up to 12 passages (the observation period).

In primary culture infected with Akabane virus the apparent cytopathic effect was manifested in cell rounding and formation of symplasts recorded 72 hours post-infection (see Fig., D). Some cells increased in sizes and were destroyed, and "windows" formation was followed by expansion of intercellular space. Probably, particles were released from the infected cells by endocytosis and cell lysis. Symplasts grew larger in 96-120 hours due to their fusion. After 120-144 hours, progressive detachment of cells from the walls occurred, then the most part of the monolayer was destroyed. These changes were not recorded in the intact culture.

At 80-90 % cytopathic effect in lamb synovial membrane cell culture in 96-120 hours (see Fig., E), virus containing culture fluid was frozen at -60 ± 0.5 °C for the release of the intracellular virus after thawing. Thawed culture fluid was titrated in 96-well polystyrene plates (Table). Thus, after three passages of the Akabane virus in lamb synovial membrane cell culture, virus activity was 5.0 ± 0.05 lg TCID₅₀/cm³ for strain P, and 6.0 ± 0.05 lg TCID₅₀/cm³ for strain V8935. Therefore, up to as many as three passages (observation period), primary cell culture retained its virus-producing activity, and the Akabane virus

retained its infective properties. Cytomorphological changes in lamb synovial membrane cell culture are a technical test to obtain qualitative results in the evaluation of Akabane disease virus activity by titration.

Cytopathic effect of the Akabane disease virus (strain B893) in continuous culture of African green monkey kidney cell line CV-1 and in primary lamb synovial membrane cell culture: A and B — intact and infected culture CV-1 72 hours after inoculation; C — intact primary lamb synovial membrane cell culture in 72 hours, D and E — infected primary lamb synovial membrane cell culture 72 and 96 hours after inoculation (light microscopy, magnification $\times 100$).

We compared Vero, CV-1 and lamb synovial membrane cell cultures sensitivity to Akabane disease virus and found virus activity which differed insignificantly. The obtained parameters were 5.85 ± 0.05 ; 5.8 ± 0.09 and 6.0 ± 0.05 lg TID₅₀/cm³, respectively.

Therefore, we can state that all the above cultures are suitable as a laboratory model for the Akabane disease virus research. However, the advantage of the primary diploid cell culture is that it is more sensitive, prepared in a special-

ized laboratory independently of available donor tissue, that is a referral to a culture museum is not required. The lamb synovial membrane cells can be re-cultured, and excessive diploid culture, like the passaged cells, can be frozen to preserve and thawed if necessary. It is expedient to use a strain of diploid lamb synovial membrane cells but not the primary culture, as the physiological state of diploid lines is better. Preference is usually given to continuous cell lines for the reasons of preservation of donor animals, reducing the cost and a possibility to control cell quality. However, in the case of monkey cells, for example, lack of these animals, their high cost and the fact that they are a source of potential infectious danger as carriers of herpes B virus should be considered.

Dynamics of Akabane disease virus cytopathic effect in primary lamb synovial membrane cell culture

Virus dilution	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Strain P								
1:10	—	—	+	+	+	+	+	+
1:100	—	—	—	+	+	+	+	+
1:1000	—	—	—	—	+	+	+	+
1:10 000	—	—	—	—	+	+	+	+
1:100 000	—	—	—	—	—	+	+	+
1:1 000 000	—	—	—	—	—	—	—	—
1:10 000 000	—	—	—	—	—	—	—	—
Strain B 8935								
1:10	—	—	+	+	+	+	+	+
1:100	—	—	+	+	+	+	+	+
1:1000	—	—	—	+	+	+	+	+
1:10 000	—	—	—	—	+	+	+	+
1:100 000	—	—	—	—	—	+	+	+
1:1 000 000	—	—	—	—	—	+	+	+
1:10 000 000	—	—	—	—	—	—	—	—

Note. «+» — effect, «—» — no effect. Titration was performed in three replicates.

Lamb synovial membrane cell culture is close to the cells of one of the animal species with natural susceptibility to the pathogen, so it can subsequently be used to obtain attenuation and live vaccine. Furthermore, the suitability of this culture for the primary isolation of the virus from pathological material should be studied. Note, primary cell cultures are more sensitive and are better suited for such purposes.

Thus, lamb synovial membrane cell culture, as permissive to Akabane disease virus, may be used, along with Vero and CV-1 cell lines, to produce the culture antigen for serological tests to diagnose the disease. Akabane virus causes a characteristic cytopathic effect in the infected monolayer of the above cultures, all of them can serve as a laboratory models for the study, accumulation and titration of the virus, and produced viral raw materials may be used in virology and molecular genetic studies. At the same time, the advantage of the proposed primary diploid cell culture is in its greater sensitivity and accessibility for independent preparation. It may be re-cultured and the excessive culture can be frozen to preserve and thawed as required.

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