UDC 619:578.831.2:636.3:57.082.26

DOI 10.29326/2304-196X-2019-2-29-35-40

TESTING SHEEP AND GOAT POX VIRUSES FOR THEIR REPRODUCTION IN PRIMARY AND SUBCULTURED CELLS

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SUMMARY

Results of tests of sheep and goat poxviruses for their reproduction in primary and subcultured cell cultures derived from lamb and goat kid kidneys and testicles are presented. Monolayer cultures were subcultured by 5 passages in plastic vials and infected with sheep and goat poxviruses. It was shown that production ARRIAH strain of sheep pox virus and ARRIAH 2003 strain of goat pox virus successfully propagated both in primary lamb and goat kid kidney and testicle cell cultures and lamb and goat kid kidney and testicle cell subcultures. Activity of sheep and goat poxviruses passaged 5 times was 5.5–6.0 lg $TCID_{50}/cm^3$. Taking into account that modern cell cultivation conditions allow primary trypsinized cell populations subcultivation up to 25–30th passage, subcultures together with continuous cell lines can be used for large-scale sheep and goat poxvirus production and research purposes.

Key words: sheep and goat kidney and testicle tissue subcultures, sheep poxvirus, goat poxvirus, cytopathic effect.

INTRODUCTION

Sheep pox and goat pox are viral diseases characterized by fever, formation of papules, or nodules, pustules (rarely), internal lesions (particularly in the lungs), often with a fatal outcome. Sheep and goat pox cause enzootic outbreaks in Africa, north of the equator – in the Middle East and in Asia [1]. Outbreaks of these diseases have recently occurred in some parts of Europe. The countries that reported outbreaks from 2010 to 2015 are Bulgaria, Chinese Taipei, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Greece and Russia. Recurrence of the disease was observed in Greece, Israel and Russia [13]. According to the World Organization for Animal Health (OIE), in 1996–2007, the diseases affected 55 countries, including 7 CIS countries. In Russia, sheep pox and goat pox were reported in 1994–2000 and 2002–2003 [3, 9, 10].

Sheep pox and goat pox result from infection by sheep poxvirus (SPPV) and goat poxvirus (GTPV) from the family *Poxviridae*, genus *Capripoxvirus* [14].

Although capripoxviruses are mainly host-specific, they do not possess strict specificity and are capable of infecting heterologous animal species. Some literature sources describe that SPPV and GTPV strains can cause disease in both sheep and goats under experimental conditions [11, 12].

The disease is characterized by high contagiosity and mortality, which reaches 5–10% in benign course of disease and approaches to 80–100% in case of concurrent infections [2].

Primary and subcultured cells, and continuous cell lines have been used for many years for the production of SPPV and GTPV vaccines and diagnostica. The use of trophovariants of cell cultures has its advantages and disadvantages. Thus, the continuous lines are easy to produce and are used for vaccine manufacturing, and primary and subcultured cells are preferable for producing the seed virus material and for diagnostic purposes [8].

In view of the above, it is reasonable to have several culture systems for growing viruses in scientific and production laboratories, which would complement and replace each other.

Results of tests of sheep and goat poxviruses for their reproduction in primary and subcultured cell cultures derived from sheep (*Ovis aries*) and goat (*Capra hircus*) kidneys and testicles are presented.

MATERIALS AND METHODS

Virus material. Production ARRIAH strain of sheep pox virus (infectivity titer of 5.5 ± 0.25 lg $TCID_{50}/cm^3$) and production ARRIAH 2003 strain of goat pox virus (infectivity titer of 6.0 ± 0.25 lg $TCID_{50}/cm^3$), prepared from YaDK-04 cell line (goat gonad continuous cell line) were used.

Cell culture. Viruses were cultivated in primary trypsinized cell culture, and in subcultures of goat kid kidney and testicle cell cultures of passages 2–5. Subcultures of goat kid kidney and testicle cell cultures of passages 2–5 prepared in 2006 and stored in liquid nitrogen were used in the study. The animals were 4–6 weeks old. Cell cultures were incubated at 37.0 ± 0.5 °C.

The biological activity of the viral material was determined by microtitration using established YaDK-04 cell line.

Nutrient media and solutions. A mixture of semisynthetic nutrient medium and nutrient medium 199 at 3:1 with the addition of 10% bovine blood serum treated with lanthanides and with a pH of 7.0–7.2 was used as a growth medium for cell subcultures.

Semisynthetic nutrient medium with 2% bovine blood serum (pH 7.3–7.5) inactivated at 58 °C for 30 min was used

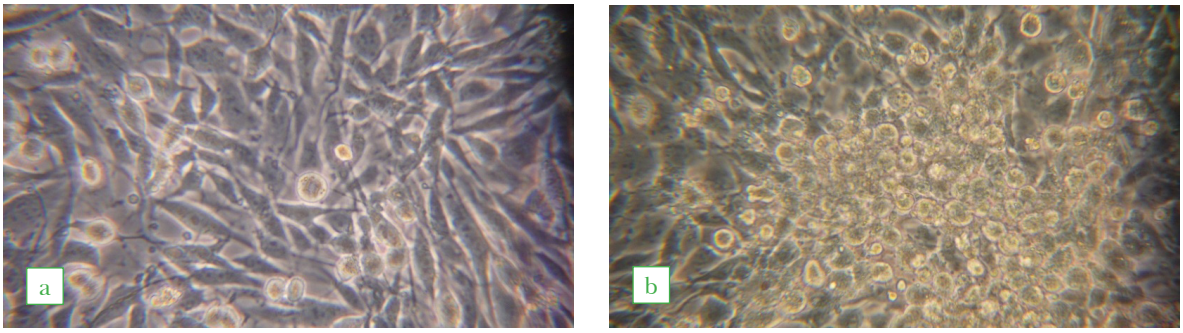


Fig. 1. YaDK-04 cell culture monolayer (a) and CPE after inoculation by sheep poxvirus and goat poxvirus (b)

as a maintenance medium for the virus. Before inoculation, the cell monolayer was washed with Hank's salt solution with a pH of 7.1–7.2. The pH correction of the medium during the cultivation of the virus was performed using 7.5% sodium bicarbonate solution.

To prevent bacterial growth, 100 units/ml kanamycin and 40 units/ml gentamicin were added to the nutrient medium and to Hank's solution.

Equipment. Laboratory cultivation of cells was carried out in Corning 25 cm^2 Growth Area Flasks.

Cytomorphologic changes in the culture after inoculation by the virus were daily studied and fixed in phase contrast by Olympus CKX-41 and Zeiss IM microscopes at magnifications of 100 and 400 times. All the Figures were made with magnification of ×400 in 48–72 h.

RESULTS AND DISCUSSION

In order to study the reproduction of SPPV and GTPV, cross-inoculation of primary and subcultured cells prepared from both natural hosts and closely related species was carried out by both viruses: SPPV for goats, and GTPV for sheep.

It should be noted that for the large-scale production of vaccines against sheep pox and goat pox, the established YaDK-04 cell line is normally used. The cytopathic effect (CPE) of these viruses on cells and the monolayer as a whole has been studied in detail [7]. The YaDK-04 cell line is highly productive (up to 30 thousand cells per cm^2). Normal morphology is represented mainly by homogeneous spindle-shaped and epithelial-like cells; dividing cells before mitosis take a spherical shape, and disjunctive chromosomes are visible (Fig. 1a). When the culture is exposed to SPPV and GTPV, the CPE pattern is the same: the bulk of the cells are de-adhesive, take a spherical shape and partially aggregate (Fig. 1b). At this stage, in 72 h, the cells are frozen and the virus with a high titer is subsequently prepared (see table).

Unlike the continuous YaDK-04 cell line, primary and subcultured cell cultures derived from lamb and goat kid kidneys and testicles are characterized by cell polymorphism and low productivity. On average, the productivity of primary and subcultured cell cultures is 3–4 times lower than that of continuous lines, but primary cell cultures are diploid and possess biological properties of donor organisms.

Eight variants of sheep and goat poxviruses interaction with primary and subcultured cell cultures derived from sheep and goat kidneys and testicles were studied.

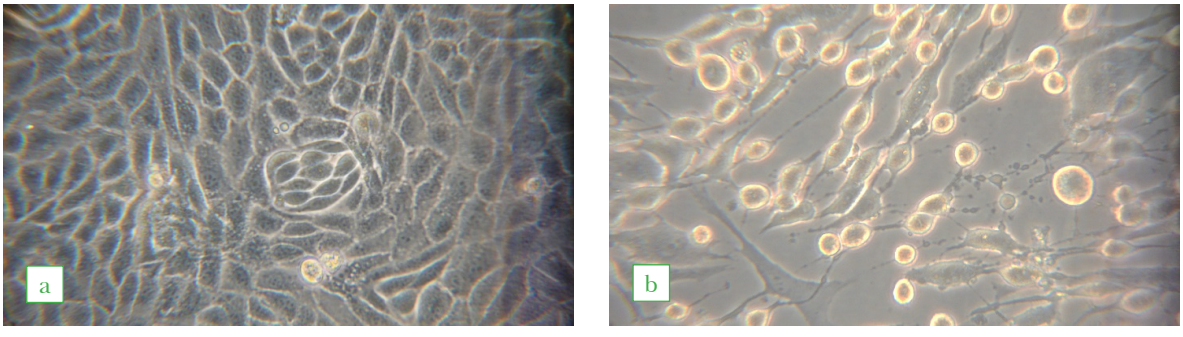
The morphology of the primary and subcultured sheep kidney cell cultures was characterized by the presence of large well-demarcated polymorphic cells (Fig. 2a). The culture had moderate glycolysis; the medium pH during the cultivation did not fall below 6.8.

Cytopathic effect of SPPV in primary sheep kidney cell culture started already in the first 24 h. In 72 h, the monolayer was totally affected. Almost all cells became de-adhesive (Fig. 2b). Strongly transformed large cells remained on the glass slide.

A similar transformation occurred with the subculture of the 5^{th} and 16^{th} passages. The virus titer is shown in the table.

Goat kidney cell subcultures were also characterized by polymorphism and large size (Fig. 3a), nuclei and nucleoli were well contrasted. Cytopathic effect of SPPV in this subculture differed from the previous variant. In the terminal stage of interaction, the monolayer was markedly sparse. Only part of the cells took a spherical shape. The remaining cells were characterized by high granularity and degradation of the cytoplasm and nuclei (Fig. 3b). After freezing, the virus titer was high (see table).

Fig. 2. Monolayer of primary sheep kidney cell culture (a) and CPE after inoculation by sheep poxvirus (b)



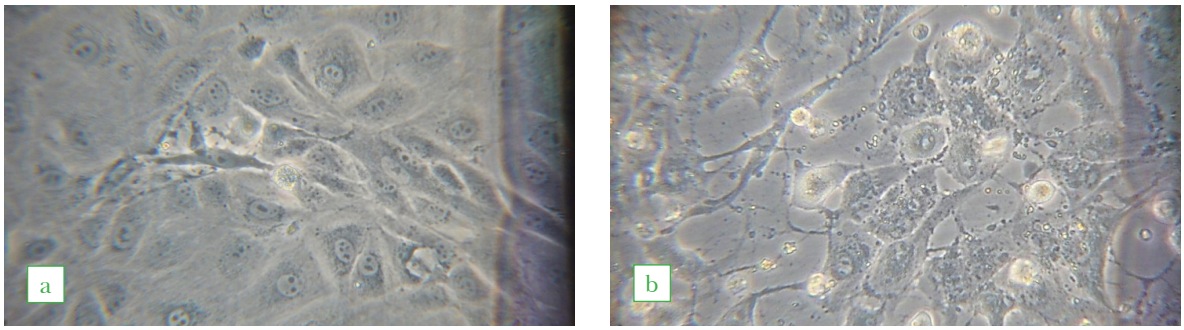


Fig. 3. Monolayer of subcultured goat kidney cells (a) and CPE after inoculation by sheep poxvirus (b)

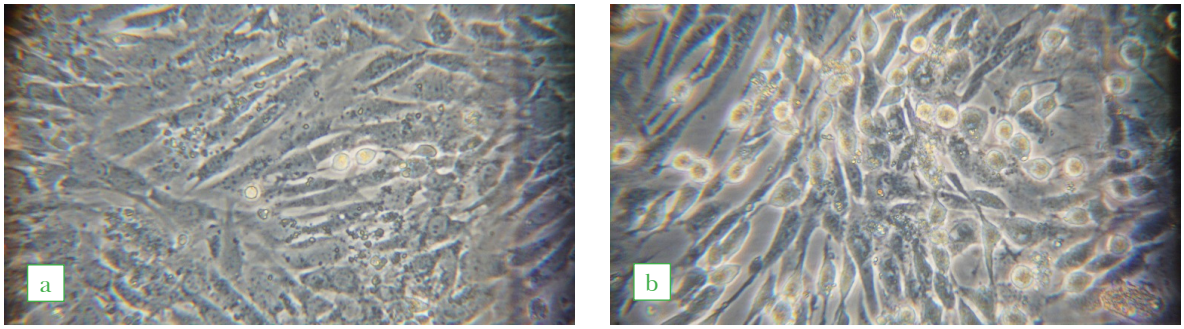


Fig. 4. Monolayer of sheep testicle cell culture (a) and CPE after inoculation by sheep poxvirus (b)

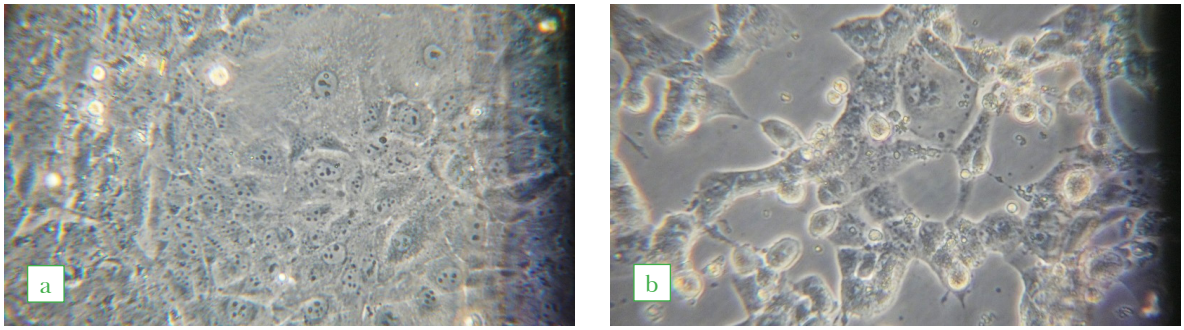


Fig. 5. Monolayer of subcultured goat kid testicle cells (a) and CPE after inoculation by sheep poxvirus (b)

Primary sheep testicle cell cultures and sheep testicle subcultures differed from those of kidney cell cultures by the predominance of spindle-shaped cells (Fig. 4a). They were also characterized by high granularity and the presence of extracellular matrix (connective proteins). Cytopathic effect of SPPV in sheep testicle cell cultures was similar to that in YaDK-04. The cells exfoliated, took a spherical shape, cell doublets were formed. The granularity increased in the remaining adhesive spindle-shaped cells (Fig. 4b).

Subcultures of goat kid testicles of passages 2–5 were similar in morphology to a culture in which epithelial-like cells with distinct nuclei and many well-contrasted nucleoli predominated (Fig. 5a). This fact indicates significant cell adhesion (binding with the substrate). After exposure to SPPV, the bulk of the cells became de-adhesive. Spherical cells formed doublets, and the remaining cells formed pseudosyncytia. The nuclear envelopes were no longer contrasted (Fig. 5b).

Subcultivation of primary trypsinized sheep kidney cells was performed in the course of the experiment. The culture not higher than the 5th passage was used for inoculation by the virus. The relative polymorphism of the culture was retained at this level (Fig. 6a). Cytopathic effect of SPPV in sheep kidney subculture was similar to the SPPV effect on sheep kidney (Fig. 2b). Less intensive degenerative changes were recorded, which consisted in

Table
Dynamics of sheep and goat poxviruses accumulation in primary and subcultured cells

Type of subculture or cell line	Virus titer, lg $TCID_{50}/cm^3$			
	SPPV		GTPV	
	Passage 1	Passage 5	Passage 1	Passage 5
Sheep kidney	5.5 ± 0.25	5.5 ± 0.25	5.0 ± 0.25	6.0 ± 0.25
Sheep testicle	5.0 ± 0.25	5.5 ± 0.25	5.5 ± 0.25	6.0 ± 0.25
Goat kid kidney	4.5 ± 0.25	6.0 ± 0.25	5.0 ± 0.25	6.5 ± 0.25
Goat kid testicles	5.0 ± 0.25	6.0 ± 0.25	5.0 ± 0.25	5.0 ± 0.25
YaDK-04	5.0 ± 0.25	5.5 ± 0.18	5.5 ± 0.25	6.0 ± 0.18

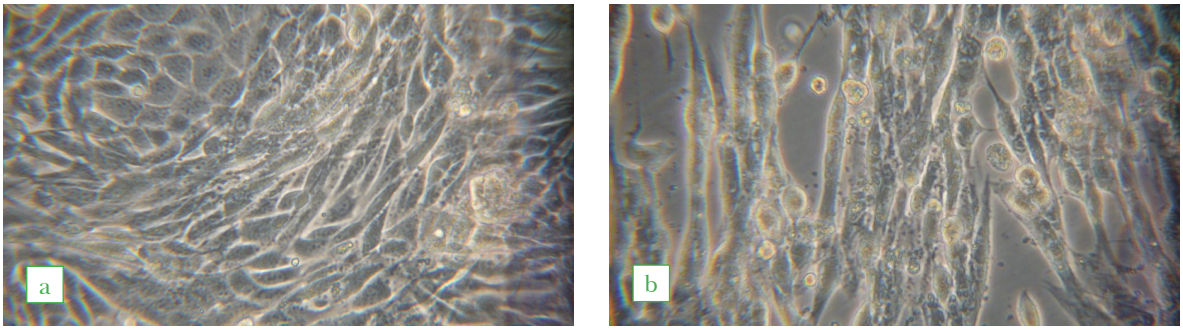


Fig. 6. Monolayer of subcultured sheep kidney cells (a) and CPE after inoculation by goat poxvirus (b)

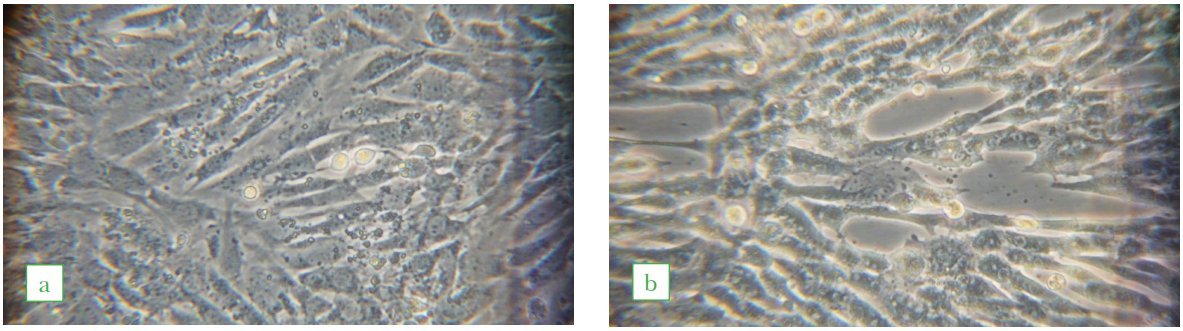


Fig. 7. Monolayer of subcultured sheep testicle cells (a) and CPE after inoculation by goat poxvirus (b)

the de-adhesion and formation of spherical aggregates, as well as in the retention of a significant number of cells on the glass slide. The remaining adhesive cells formed pseudosyncytium (Fig. 6b).

The effect of goat poxvirus on sheep testicle subculture (Fig. 7b) was similar to SPPV (Fig. 4b). The sufficient number of cells remain on the glass slide after CPE, and at the same time, significant morphological degenerative changes in the cytoplasm (vacuolization and granulation) indicate the terminal stage of the CPE. Nuclei and nucleoli are not contrasted due to cell compaction.

The subculture of goat kid kidney differs from other cultures in large epithelial-like cells with clear nuclei and nucleoli (Fig. 8a). The cytopathic effect of GTPV on goat kidney culture was similar to the SPPV (Fig. 3b), but not so intensive, and the retention of some undestroyed nuclei and the formation of pseudosyncytium was observed (Fig. 8b). It should be noted that the cytopathic effect of GTPV on goat kidney culture was prolonged – more than 80 h, and had the highest titer (6.5 ± 0.25 lg $TCID_{50}/cm^3$).

The effect of goat poxvirus on goat kid testicle subculture of passages 2–5 (Fig. 9b) was similar to the effect of the virus on sheep testicle cell culture (Fig. 7b). The specific nature of the CPE allows to state that the epithelial-like cells of the primary culture and subculture are firstly affected. Spindle-shaped cells gather in strands; at the end of the terminal stage (72–96 h), they also became de-adhesive and partially destroy.

2 types of monolayer destructions were detected based on analysis of morphological changes in cell cultures as a result of the CPE of SPPV and GTPV. The first type is associated with destruction of goat kid kidney subculture by both viruses (Fig. 3b, 8b). In this case, undestroyed epithelial-like cells accumulated in pseudosyncytium, and de-adhesive spherical cells of different sizes remain on the glass slide. This type of interaction differs from the control in morphology, but the titer of the resulting virus meets the production criteria. The second type of destruction is the classical one, which corresponds to the control points in the interaction of SPPV and GTPV with YaDK-04. As a result of this interaction, many cells became de-adhesive, partially aggregate. Spindle-shaped cells remaining on the glass slide lose their internal structure and are destroyed after freezing.

A very important characteristic has been demonstrated: the specific nature of the virus CPE persists during subculturing. The endpoint was the standard destruction of the sheep kidney subculture in the 5th passage of subculturing (Fig. 2b).

The biological activity of the viral materials determined by microtitration (see table), demonstrates that sheep and goat poxviruses are successfully reproduced in primary and subcultured cell cultures derived from lamb and goat kid kidneys and testicles. This data is consistent with the results of other researchers. So, V. N. Ivanyuschenkov et al. [5] used a primary tissue culture of sheep kidney to produce a virus vaccine against sheep pox. V. I. Diev et al. [4] also successfully used the primary tissue culture of sheep kidney when working with the vaccine strain of sheep pox virus. M. S. Kukushkina [6] reproduced sheep pox and goat pox viruses in a primary culture of sheep kidney with an activity of 4.75–5.0 lg $TCID_{50}$/ml.

Subcultures of primary cells derived from lamb kidneys and testicles can be used for large-scale production of viruses, but mostly they are used by the researches as an intermediate stage in the cultivation of viruses or during the primary isolation of viruses from pathological materials. In some cases, good recovery of the viruses in cell subculture was observed, and in some cases their replication gradually decreased. V. A. Sergeyev [8] after analyzing a large number of studies on the growth of animal viruses in continuous lines and in cell subcultures, concluded that cells in continuous cell lines are more stable in their properties

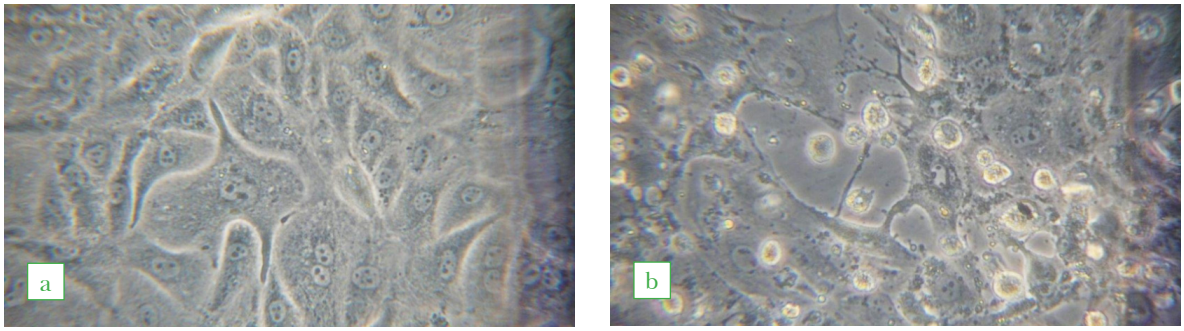


Fig. 8. Monolayer of subcultured goat kidney cells (a) and CPE after inoculation by goat poxvirus (b)

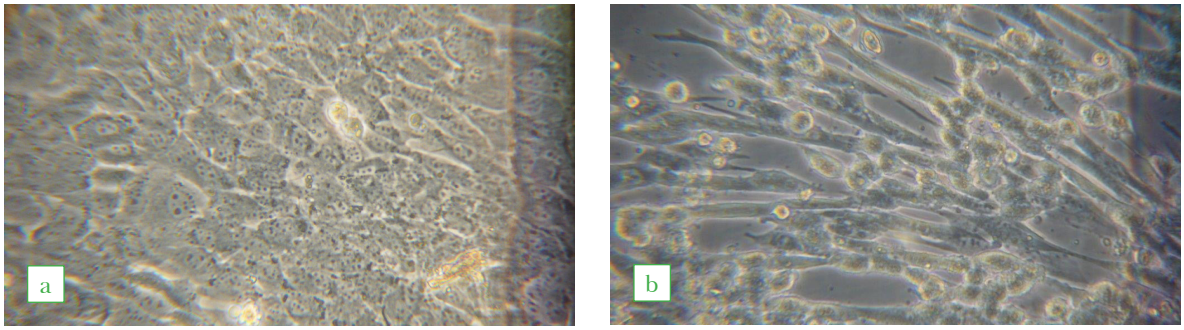


Fig. 9. Monolayer of subcultured goat kid testicle cells (a) and CPE after inoculation by goat poxvirus (b)

than the cells of subculture, and he suggested that it was associated with more intensive metabolism.

Results of tests of sheep and goat poxviruses for their reproduction in subculture of lamb and goat kid kidneys and testicles presented in this study demonstrated that these viruses accumulated in tested subcultures in significant quantities and accumulation level was not reduced within 5 passages (observation period), the infectious activity of the viruses during this period increased by 0.5–1.0 lg $TCID_{50}/cm^3$.

CONCLUSION

Study of sheep and goat poxviruses reproduction in primary and subcultured cell cultures derived from lamb and goat kid kidneys and testicles are presented. The production ARRIAH strain of sheep pox virus and ARRIAH 2003 strain of goat pox virus successfully propagated both in primary lamb and goat kid kidney and testicle cell cultures and in subsequent 5 passages of lamb and goat kid kidney and testicle cell subcultures. The results of the use of cell subculture for growing the viruses will be used to stabilize the production of vaccines against sheep pox and goat pox, which are of current importance.

Conflict of interests. The authors claim no conflict of interests.

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Submitted on: 20.03.19
Approved for publication: 24.04.19