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Isolation of a new strain M-2020 of the camelpox virus (*Poxviridae: Orthopoxvirus: Camelpox virus*) in Republic of Kazakhstan and study of its reproduction in various biological systems

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Introduction. This article presents the results of isolation of camel smallpox virus (*Poxviridae: Orthopoxvirus: Camelpox virus*, CMLPV) and study of its reproductive properties on sensitive biological systems.
Material and methods. The epizootic strain M-96 of the virus as well as its attenuated variants KM-40 and KM-70 obtained by sequential passivation were used in the study. Isolation of the pathogen from suspension of biopsy specimens was performed on cell culture and in embryonated chicken eggs (ECEs). All experiments were performed with the number of replications ensuring obtaining reliable results.
Results. The CMLPV was isolated from the crusts and pox papules of the skin taken from sick camels (*Camelus bactrianus*) during an outbreak in various districts of the Mangistau region at the end of 2019. The signs of pathogen reproduction on chorio-allantoic membrane (CAM) were observed from 3 passages. The obtained virus caused formation of pathological changes on the CAM in the form of elevated dot or solid white formations separated from the surrounding tissue, with hemorrhagic foci in the center. The reproductive properties of the isolate on sensitive biological systems were determined in comparison with the epizootic CMLPV strain M-96, isolated earlier in the territory of Kazakhstan during the outbreak 23–24 years ago, as well as its attenuated variants. The isolated virus was given the conventional name M-2020.
Discussion. When studied in two sensitive cultivation systems (cell culture and ECEs), strain M-96 and its attenuated variants KM-40, KM-70, which were used in the experiments as a control, demonstrated high infectious activity with titer 4.75–6.75 lg $TCID_{50}/cm^3$, while for the examined isolate M-2020 of CMLPV had the significantly lower values (3.00–4.75 lg $TCID_{50}/cm^3$, $p > 0,05$).

Key words: *camelpox; virus; isolation; embryonated chicken eggs; cell culture*

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НАУЧНАЯ СТАТЬЯ
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Выделение нового штамма М-2020 вируса оспы верблюдов (*Poxviridae: Orthopoxvirus: Camelpox virus*) в Республике Казахстан и изучение его репродукции на различных биологических системах

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Введение. В данной работе представлены результаты выделения вируса оспы верблюдов (ОВ) (*Poxviridae: Orthopoxvirus: Camelpox virus*, CMLPV) и изучения его репродуктивных свойств на чувствительных биологических системах.
Материал и методы. В исследовании использованы эпизоотический штамм М-96 вируса, а также его аттенуированные варианты КМ-40 и КМ-70, полученные путём последовательного пассирования. Выделение возбудителя из суспензии биопсийных образцов осуществляли на культуре клеток и в развивающихся куриных эмбрионах (РКЭ). Все эксперименты проводили с числом повторности, обеспечивающим получение достоверных результатов.
Результаты. В серии экспериментов выделен вирус ОВ из корочек и соскобов с оспенными папулами кожи, полученных во время вспышки заболевания от больных верблюдов (*Camelus bactrianus*) из различных районов Мангистауской области Республики Казахстан в конце 2019 г. При этом признаки размножения возбудителя на хорион-аллантоисной оболочке (ХАО) отмечались с 3 пассажа. Полученный вирус вызывал формирование на ХАО патологических изменений в виде возвышающихся точечных или сплошных узелков белого цвета, ограниченных от окружающей ткани, с геморрагическими очагами в центре, в размере от 1,0 до 5,0 мм. Определены репродуктивные свойства изолята на чувствительных биологических системах в сравнении с эпизоотическим штаммом М-96 CMLPV, выделенным ранее на территории Казахстана во время вспышки ОВ 1996 г., а также его аттенуированными вариантами. Выделенному вирусу присвоено условное название М-2020.
Обсуждение. При исследовании в обеих чувствительных системах культивирования (клеточной культуре и РКЭ) штаммы М-96 и его аттенуированные варианты КМ-40, КМ-70, использованные в экспериментах в качестве контроля, продемонстрировали высокую инфекционную активность с титром 4,75–6,75 lg $ТЦД_{50}$/$см^3$, тогда как для исследуемого изолята вируса ОВ М-2020 указанная величина оказалась существенно ниже (3,00–4,75 lg $ТЦД_{50}$/$см^3$, $p > 0,05$).

Ключевые слова: *оспа верблюдов; вирус; выделение; куриные эмбрионы; культура клеток*

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Introduction

One of the sectors of livestock farming in desert and semi-desert areas in the Republic of Kazakhstan is camel breeding, having a special place in the agricultural industry, satisfying the population's demand for meat, milk, and wool. It makes a considerable contribution to development of such natural zones [1]. The present-day goal is to turn camel breeding into a highly profitable sector of livestock production. The goal can be achieved by increasing and maintaining the population of camels (*Camelus bactrianus)*, by taking effective measures and protecting it from infectious diseases, including camelpox (CMLP) causing a major economic impact [2]. In Kazakhstan, CMLP was repeatedly reported in the Mangistau and Atyrau (Guryev) Regions throughout 1930, in 1942–1943, 1965–1967, 1968–1969 [3], and 1996. During the outbreak in the Mangistau Region in 1996, 830 of 8,000 camels were infected, 43 of them died [4]. During that period, in the region, researchers from the Research Institute for Biological Safety Problems of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (RIBSP SC MES RK) isolated the epizootic M-96 strain of camelpox virus (*Poxviridae; Orthopoxvirus: Camelpox virus*, CMLPV) and studied its biological, morphological, physical, chemical, and genetic characteristics [5–9]. Later, the entire genome of the strain was sequenced and deposited to the GenBank database (No. AF438165.1) [10]. After the above outbreak, new cases of CMLP were reported in the Mangistau Region in summer 2019; the diagnosis was confirmed by laboratory tests performed by RIBSP researchers in December of the same year (unpublished data). At the end of 2019, the research institute received biomaterial collected from diseased animals from different areas of the Mangistau Region to isolate the virus and to study its replication properties using different biological systems and other strains available in the RIBSP collection of microorganisms. Such studies are instrumental for proper and efficient preventive measures; they also play a significant role in advancing the manufacturing of diagnostic assays and vaccines against especially dangerous infectious diseases of animals.

In this context, the aim of this study was to identify and explore replication properties of the CMLPV isolated during the disease outbreaks in the Mangistau Region in 2019.

Material and methods

The virus and pathological material. The study was performed on the epizootic M-96 strain of CMLPV, which was isolated from a diseased camel during the outbreak in the Mangistau Region in 1996; the study also included its attenuated variants KM-40 and KM-70, which were obtained through serial passages on sensitive biological systems. The study was focused on the pathological biological material (scabs and scrapings with pock papules), collected from sick animals during the CMLP outbreak from different areas of the Mangistau Region at the end of 2019. The region and sampling locations are shown in **Fig. 1**.

Biological systems of cell culture. The tests were conducted using trypsinized primary lamb kidney (LK) cell culture maintained in the growth medium containing 10% fetal bovine serum (FBS) as well as using 11–12-old embryonated chicken eggs (ECE). The eggs were purchased from epidemically and biologically safe poultry farms.

Isolation and cultivation of the virus on sensitive biological systems. The pathogen was isolated from the suspension of biopsy specimens using cell culture and in ECE in accordance with the protocols of the World Organization for Animal Health (formerly the Office Inter-

national des Épizooties (OIE), 2019) [11]. Prior to being infected, the embryos went through the organoleptic test by candling. After the embryo shells had been treated, we pierced the area above the air cell (air pocket) with a finely sharpened steel pin and made a 4–5-diameter hole with the help of forceps. Then, using sterile needles, we made incisions in 2–3 points on the membrane under the shell and applied the 0.2 cm^3 amount of viral material to them. The holes in the shells were sealed with tape; then ECEs were incubated in a vertical position at 37 ± 0.5°C and relative humidity (55 ± 5) % for 120 hrs.

For the control purpose, 2–3 embryos were not infected; their chorioallantoic membrane (CAM) was treated with 0.9% sodium chloride solution in the same amount. The candling examination was performed daily. The death of embryos within the first 48 hrs was considered non-specific. Starting from the third day of incubation, the dead ECEs were stored in a household refrigerator at (4 ± 2) °C till the completion of the test. After the incubation, the embryos that stayed alive during 120 hrs were also refrigerated at the same temperature for at least 18 hrs.

The CP virus titer in the obtained samples was measured by titration in the primary LK or ECE cell culture in accordance with the previously described method [9]. The titer was defined as the highest dilution causing cytopathic effect (CPE) displayed by 50% of the infected cell culture samples or development of plaques on ECE CAM. The Reed–Muench method was used to calculate the endpoint [12] expressed as lg $TCID_{50}/cm^3$ (TCID is tissue culture infectious dose) for the LK cell culture or as lg EID_{50}/cm^3 (EID is embryo infectious dose) for ECEs.

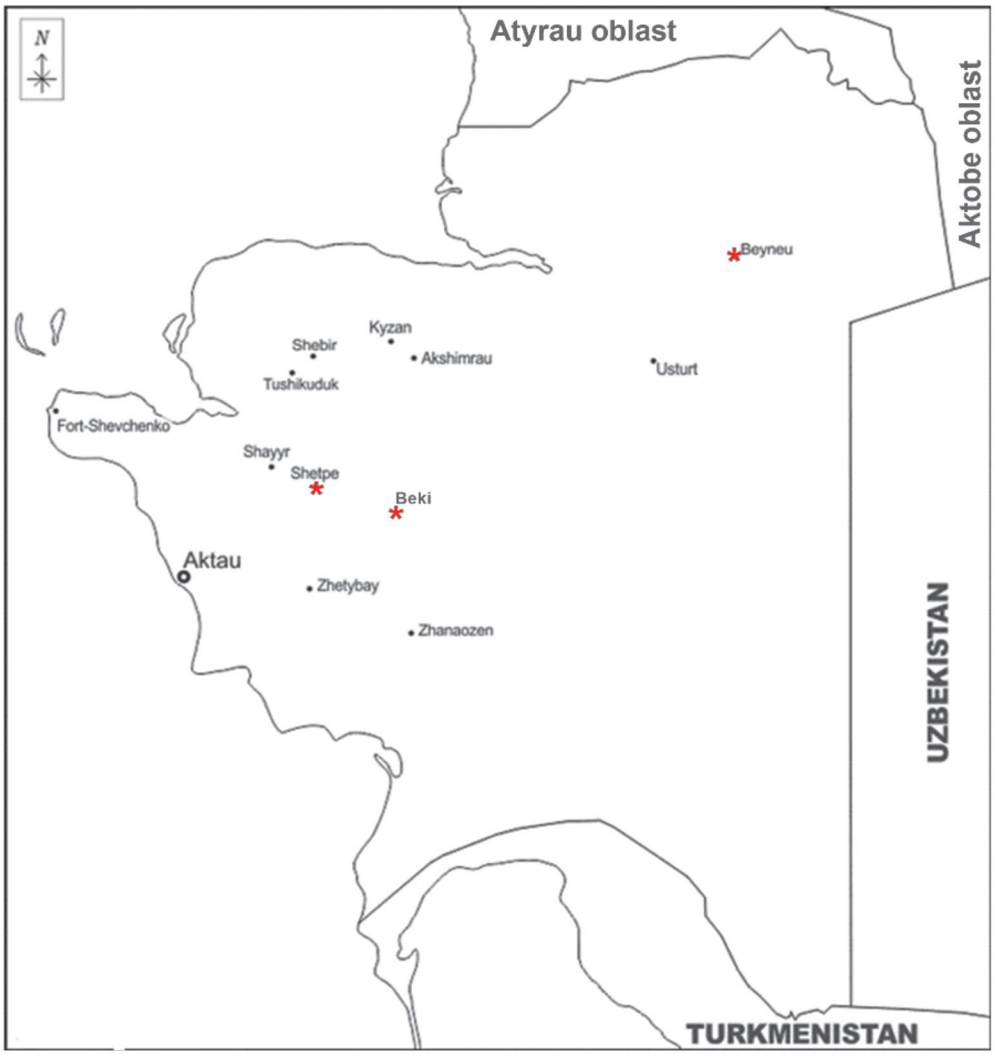


Fig. 1. Map and locations for sampling of the pathological material in Mangistau region.
Sampling location is indicated with red asterisks.
Рис. 1. Карта Мангистауской области и места отбора патологического биоматериала на территории.
Места отбора проб обозначены красными звездочками.

Electron microscopy. Samples were prepared using the negative staining technique and 2% phosphotungstic acid (PTA) aqueous solution. A drop of the virus-containing material was placed in a well in the Teflon plate. A support grid stabilized with a carbon-coated film was applied to the drop. After 5–10-min adsorption, the grid was removed, and the excess liquid was wicked off with filter paper. The grid with the sample was transferred to a drop of pH 6.8 PTA solution for 1–2 min and then to a drop of pH 7.0 PTA solution for 5 min. After it had been stained and the excess stain had been removed, the sample was air-dried. The samples were examined with the JEM-100 CX JEOL electron microscope (Japan) at the accelerating voltage of 80 kV and magnification ×15,000–20,000.

Identification of antigenic relatedness. The antigen relatedness between the new isolate and previously isolated strain was assessed using the neutralization test and specific serum collected from camels immunized with the attenuated CMLPV strain KM-40. The results of the neutralization test were recorded for 7 days and assessed for presence or absence of virus CPE in the LK cell culture.

The statistical analysis of the data. All the tests were repeated as many times as required to obtain valid results. The statistical analysis included calculation of the arithmetic mean (X) and the root mean square error (m) using the GraphPad Prism v.9 program. The differences were considered statistically significant at the 95% confidence interval ($p \leq 0.05$).

Results

Isolation of camelpox virus in embryonated chicken eggs. The results of the tests performed *in vitro* using sensitive biological systems are presented in **Table 1**.

As is seen from **Table 1**, after ECE were opened and their CAM was examined, no pox nodules were detected in the 1st and 2nd passages. In sample 5, the signs of virus replication were detected, starting from the 3rd passage. In the first passages, lesions were poorly developed and were characterized by the presence of a very few circumscribed nodules rising above the surrounding surface at the point of inoculation. The visible pathological changes in CAM were observed starting from the 6th passage; the affected area was characterized by the presence of extensive lesions represented by elevated massive white formations with hemorrhagic foci. It should be noted that the virus was not isolated from other samples, as there

Table 1. Results of bioassays for the isolation of CMLPV on the ECEs by the method of serial passages

Таблица 1. Результаты биопроб по выделению вируса оспы верблюдов на развивающихся куриных эмбрионах методом последовательного пассажа

Bioassay number Номер биопробы	Name of the isolate and sampling date of the pathological material Наименование изолята и дата получения образца	Number of passages and passing results Количество пассажей и результаты пассирования					
		I	II	III	IV	V	VI
1	No. 1 dated December 12, 2019, from the crust, Shetpe district № 1 от 12.12.2019 г., из корочки, район Шетпе	–	–	–	–	–	–
2	No. 2 dated December 12, 2019, from the crust, Shetpe district № 2 от 12.12.2019 г., из корочки, район Шетпе	–	–	–	–	–	–
3	No. 3 dated December 12, 2019, from the crust, Shetpe district № 3, 1272 от 12.12.2019 г., из корочки, район Шетпе	–	–	–	–	–	–
4	No. 4 dated December 12, 2019, from the crust, Shetpe district № 4, 303405 от 12.12.2019 г., из корочки, район Шетпе	–	–	–	–	–	–
5	No. KZR506384619, dated December 12, 2019, from the crust, Beyneu district № KZR506384619, от 12.12.2019 г., из корочки, район Бейнеу	–	–	+	+	+	+
6	No. KZR506384620, dated December 12, 2019, from the crust, Beyneu district № KZR506384620, от 12.12.2019 г., из корочки, район Бейнеу	–	–	–	–	–	–
7	No. KZR506375943, dated December 12, 2019, from the crust, Beyneu district № KZR506375943, от 12.12.2019 г., из корочки, район Бейнеу	–	–	–	–	–	–
8	No. 06290618, dated December 12, 2019, from the crust, Beki district № 06290618, от 12.12.2019 г., из корочки, район Беки	–	–	–	–	–	–
9	No. 06302984, dated December 12, 2019, from the crust, Beki district № 06302984 от 12.12.2019 г., из корочки, район Беки	–	–	–	–	–	–
10	No. 06302710, dated December 12, 2019, from the crust, Beki district № 06302710 от 12.12.2019 г., из корочки, район Беки	–	–	–	–	–	–

Note. «–», no camelpox plaques on the chorio-allantoic membrane; «+», presence of camelpox plaques on the chorio-allantoic membrane.

Примечание. «–» – отсутствие оспенных бляшек на хорион-аллантоисной оболочке; «+» – наличие оспенных бляшек на хорион-аллантоисной оболочке.

were no pox nodules even during serial passaging until the 6th passage.

Thus, the studies on the biomaterial resulted in isolation of a CMLPV variant, which was tentatively named M-2020. After the biological and genetic characteristics of the isolate are thoroughly studied, it will be provided with the identification datasheet and will be deposited to the respective databases to be added to the collection strains as a virulent sample intended for testing the immunogenicity of vaccines and for further research.

Studying of replication properties of the M-2020 isolate using biological systems. The comparative study of replication properties of the M-2020 isolate versus other CMLPV strains was performed on the epizootic M-96 strain and its attenuated variants: KM-40 and KM-70. The tests were performed on ECEs.

The specificity of the virus-containing materials was measured by the presence of specific CPE in the monolayer of cell culture (**Fig. 2**) or by plaques detected on CAM of chicken embryos (**Fig. 3**), including the results of the electron microscopy (**Fig. 4**).

As is seen from **Fig. 1**, the CPE of the virus in the LK cell culture was characterized by focal damage of the monolayer, which developed light-refracting cytoplasm cell elements being of different shape (rounded, spindle-shaped, oval) and having clear outlines of the nuclear and plasma membranes. These cells were swollen and increased significantly in size compared to healthy cells. The dead cells were replaced by void areas. Pox plaques on CAM appeared 48–72 hour after the chicken embryos had been infected, keeping rapidly changing for another 72-96 hrs. In 96–120 hrs, in addition to plaques, there were secondary lesions represented by circumscribed white nodules ranging from 1.0 to 2.0–3.0 mm in size, scattered over the entire surface of the membrane, along blood vessels (**Fig. 3** *a*, *b*).

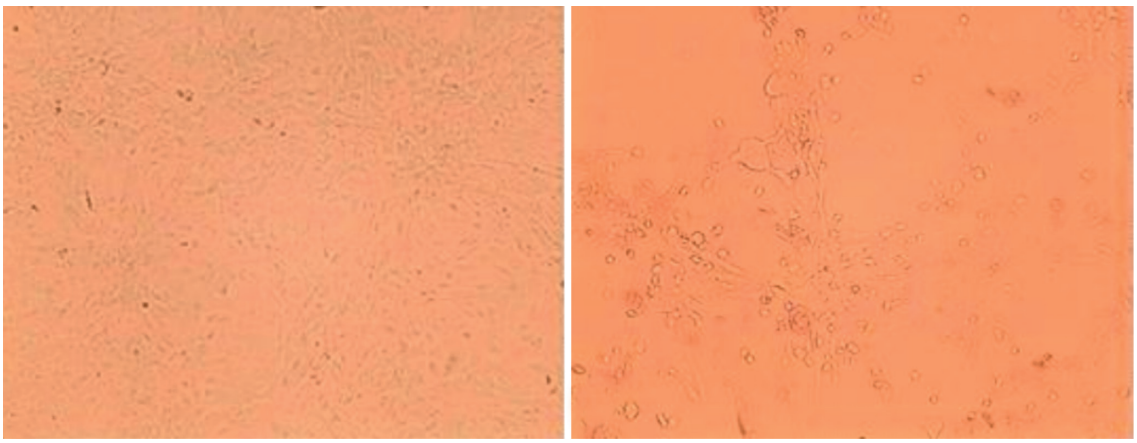
The results of the tests assessing replication properties of the M-2020 isolate compared with other strains of CMLPV by using sensitive biological systems are presented in **Table 2**.

The analysis of antigenic relatedness between the isolate and the attenuated strain of the CMLPV showed that in the neutralization test performed on the cell culture obtained from vaccinated animals, the specific serum in the 1 : 32 dilution completely neutralized the field isolate of the virulent pathogen in the dose of 200 lg $TCID_{50}/cm^3$.

Discussion

The isolation of a pathogen, being the main research method in classical virology, is of special significance for experimental studies. Pure culture of the isolated virus is important for scientists analyzing its phylogeny and evolution by studying biological, molecular, and genetic characteristics; it is a promising biological source that can be broadly used for development of diagnostic systems and in disease prevention. Pure culture is isolated with the help of sensitive biological systems (laboratory animal models, cell cultures, and chicken embryos) depending on the tropism of the studied infectious agent. Based on the data from literature sources, 11–12-day-old ECEs are an efficient system for primary isolation of CMLPV from pathological materials [11–13]. Therefore, we used CAM-infected chicken embryos for primary isolation of the infectious agent. The signs of virus replications were observed starting from the 3rd passage. The M-2020 isolate caused pathological changes on CAM, which were represented by elevated point or massive nodule-like lesions of white color, circumscribed and well-defined against the surrounding tissue, with hemorrhagic foci in the center. The similar descriptions could be found in works of other authors [3, 5, 14].

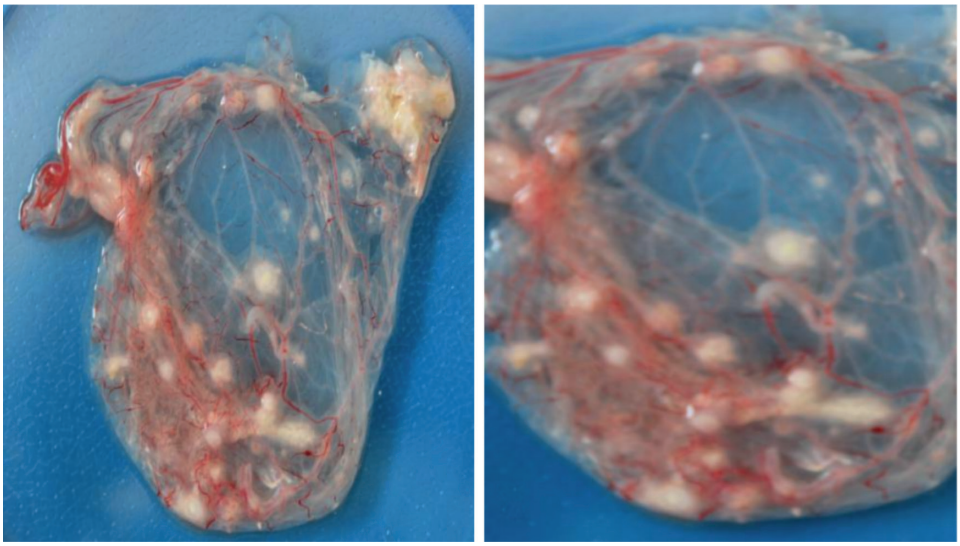
In the literature, there is information about successful CMLPV cultivation in naturally susceptible animals [3]. The examples are primary and secondary cell cultures prepared from lamb kidneys (LK), bovine kidneys (BK-80 or MDBK), camel fetal skin fibroblasts (CFS), chicken embryo



a/а *b/б*

Fig. 2. Light microscopy of the lamb kidney cells before and after infection with CMLPV: *a)*, non-infected lamb kidney cells (control on the 3rd day); *b)*, cytopathic effect of the virus in the cell culture (on the 3rd day after infection). Microphotograph (magnification ×20).

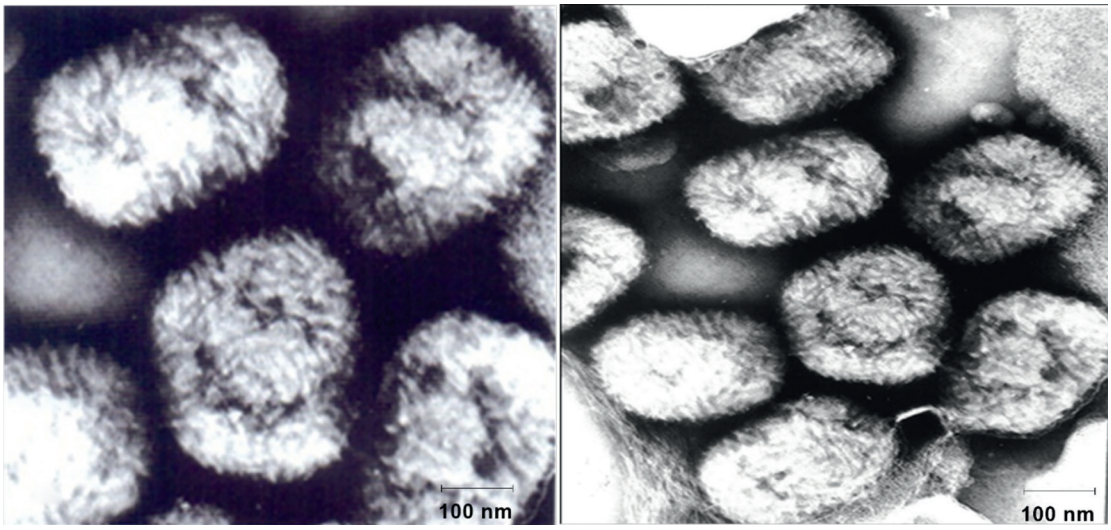
Рис. 2. Световая микроскопия культуры клеток почки ягнёнка до и после заражения вирусом оспы верблюдов: *а)* – неинфицированная клеточная культура (контроль на 3 сут); *б)* – цитопатическое действие вируса в культуре (на 3 сут после инфицирования). Микрофотография (увеличение ×20).



a/а *b/б*

Fig. 3. Characteristic plaques on the chorioallantoic membrane of chicken embryos when infected with strains of the CMLPV: *a)*, after infection with strain KM-40; *b*), after infection with isolate M-2020. Native macropreparation.

Рис. 3. Характерные бляшки на хорион-аллантоисной оболочке куриных эмбрионов при инфицировании штаммами вируса оспы верблюдов; *а)* – при заражении штаммом КМ-40; *б)* – при заражении изолятом М-2020. Нативный макропрепарат.



a/а *b/б*

Fig. 4. Electron microscopy of the camelpox virions: *a)*, strain M-96; *b)*, isolate M-2020. Microphotograph, negative staining with 2% phosphotungstic acid solution, magnification ×150,000 (according to Kozhabergenov N.S.).

Рис. 4. Электронная микроскопия вирионов оспы верблюдов: *а)* – штамм М-96, *б)* – изолят М-2020. Микрофотография, негативное контрастирование 2% раствором фосфорно-вольфрамовой кислоты, увеличение ×150 000 (по Н.С. Кожабергенову).

fibroblasts (CEF), African green monkey kidney cells (Vero), baby hamster kidney cells (BHK-21), cervical tumor cells (HeLa) [5, 14–17]. There are also data on all species of laboratory animal models insusceptible to this pathogen, including birds (*Aves*) [3, 19]. Taking into account the above information, we decided to study replication properties of the Kazakhstani M-2020 isolate on LK and ECE culture; the isolate was compared with the epizootic M-96 strain of the CMLPV, which was isolated in Kazakhstan during the CMLP outbreak in 1996, and with its attenuated variants. It should be noted that the epizootic M-96 strain and its replication properties were thoroughly studied by Bulatov E.A. et al. [5]. They found that among 19 tested types of cell cultures and chicken embryos, the trypsinized primary LK cell cultures, the fetal lamb kidney (FLK) cells, continuous Vero cell lines, sheep kidney (SK) cells, and ECE are most susceptible to this strain. The virus was propagated in LK and FLK cultures with titers of 4.00–5.75 and 4.75–4.86, respectively; in the continuous Vero cell lines – with titers of 4.00–5.50, SK – 3.75–5.25 lg $TCID_{50}/cm^3$ and on ECE with titers ranging from 4.70 to 6.00 lg EID_{50}/cm^3. While in cell cultures prepared from Cameroon goat kidney cells

Table 2. Indicators of the biological activity of cultured and embryonic virus-containing suspensions of CMLPV strains

Таблица 2. Показатели биологической активности культуральных и эмбриональных вируссодержащих суспензий штаммов вируса оспы верблюдов

Strain name Наименование штамма	Passage level Уровень пассажа	Onset of the CPE manifestation on plaques, day Начало проявления ЦПД на бляшках, сут	Cultivating period, days Срок культивирования, сут	Virus titer, lg $TCID_{50}/cm^3$ ($X \pm m$) Титр вируса, lg $ТЦД_{50}/см^3$ ($X \pm m$)	Virus titer, lg EID_{50}/cm^3 ($X \pm m$) Титр вируса, lg $ЭИД_{50}/см^3$ ($X \pm m$)
КМ-40	I	3	5	5,75 ± 0,14	5,83 ± 0,08
	II	3	5	5,83 ± 0,08	6,00 ± 0,14
	III	3	5	6,00 ± 0,25	6,75 ± 0,14
	IV	3	5	6,75 ± 0,25	6,50 ± 0,25
	V	3	4	6,50 ± 0,25	6,75 ± 0,08
КМ-70	I	3	5	5,75 ± 0,14	n.i. н.и.
	II	3	5	5,83 ± 0,08	n.i. н.и.
	III	3	5	6,00 ± 0,14	n.i. н.и.
	IV	3	5	6,75 ± 0,14	n.i. н.и.
	V	3	5	6,50 ± 0,08	n.i. н.и.
М-96	I	3	7	4,75 ± 0,08	5,00 ± 0,12
	II	3	7	4,81 ± 0,14	5,20 ± 0,17
	III	3	7	5,25 ± 0,13	5,50 ± 0,08
	IV	3	6	5,50 ± 0,08	5,75 ± 0,10
	V	3	5	5,50 ± 0,08	6,00 ± 0,08
М-2020	I	3	7	3,00 ± 0,11	3,50 ± 0,12
	II	3	7	3,00 ± 0,08	3,78 ± 0,11
	III	3	7	3,25 ± 0,12	4,00 ± 0,08
	IV	3	6	4,25 ± 0,08	4,50 ± 0,11
	V	3	6	4,50 ± 0,08	4,75 ± 0,08

Note. n.i, not investigated; TCID, tissue culture infectious dose; EID, embryo infectious dose.

Примечание. н.и. – не исследовано; ТЦД – тканевая цитопатическая доза; ЭИД – эмбриональная инфицирующая доза.

Table 3. Results of the assesment of the antigenic identity of isolated CMLPV with specific serum in the neutralization test

Таблица 3. Результаты определения антигенной идентичности выделенного вируса оспы верблюдов со специфической сывороткой в реакции нейтрализации

Сыворотка Serum	Dilution of specific and normal sera Разведение специфической и нормальной сыворотки						Титр антител в реакции нейтрализации Antibody titer in the neutralization test
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	
Specific Специфическая	- - - -	- - - -	- - - -	- - - -	- - - -	++++	1 : 32
Normal Нормальная	++++	++++	++++	++++	++++	++++	0

Note. «–», no cytopathic effect; «+», presence of cytopathic effect.

Примечание. «–» – отсутствие цитопатического действия; «+» – наличие цитопатического действия.

(CGK), Madin-Darby canine kidney cells (MDCK), versenized fetal porcine kidney cells (VFPK), and rabbit pancreatic cells (RP), the virus CPE was observed only in the first (titers 0.5–3.50 lg $TCID_{50}/cm^3$) passage, the above effect was observed in the first 2 passages (titers 0.25–5.50 lg $TCID_{50}/cm^3$) in fetal sheep lung (FSL) cell cultures, fetal sheep skin (FSS), and lamb testicular (LT) cell cultures; no virus CPE was recorded in the tests using RP, BK-80, bovine calf testicular (BCT) tissue, and bovine kidney (MDBK) cells. The strains (M-96 and its attenuated variants KMM-40, KM-70), which we used as control strains, demonstrated high infection activity with a titer 4.75–6.75 lg $TCID_{50}/cm^3$, while in the both culture systems, this parameter for the studied M-2020 isolate was significantly lower (3.00–4.75 lg $TCID_{50}/cm^3$, $p > 0.05$) as compared to other strains. The low biological activity of the isolated virus variant can be associated with such culture-specific factors as the minimum infective dose (MID), incubation temperature, culture techniques, the growth medium type, etc. [20].

Conclusion

The study resulted in the isolation of a pathogen, which was identified as CMLPV by using the neutralization test and electron microscopy. This isolate will be used in further studies and in vaccine development to evaluate vaccine protective efficacy through challenge infection of susceptible animals. The first stage was focused on studying of replication properties of the isolated virus variant. At present, its genetic characteristics are still being studied for the further preparation of the identification datasheet and depositing the virus variant as CMLPV M-2020 strain to the bank of pathogens of especially dangerous diseases in the republican collection of microorganisms.

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