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CHARACTERISTICS OF THE EQUINE RHINOPNEUMONIA VIRUS AK-2011

ANNOTATION

Equine rhinopneumonitis is an acute, highly contagious disease found virtually worldwide. The object of the studies was the AK-2011 strain isolated from the horses suffering from rhinopneumonitis during an outbreak of abortions. The viability of the AK-2011 strain was assessed using a continuous line of calf trachea cells, a continuous line of calf kidney cells, a continuous line of sheep kidney cells, a continuous line of bovine kidney cells, a continuous line of green monkey kidney cells, a continuous line of calf trachea cells, a primary trypsinized culture of horse kidney cells grown in tubes and flasks, and the AK-2011 laboratory strain of equine rhinopneumonitis virus with biological activity of 6.0 lg TCID50/cm3. Sequencing and polymerase chain reaction (PCR) analysis were performed. The virus isolated from the ORF68 gene in Kazakhstan appeared to be the most similar to the T-953 and 2222-03 strains isolated in the USA and Australia, respectively, in terms of phylogenetics. As to primary infections, cytopathic effects (CPE) induced by the AK-2011 virus strain (dilution 103) in calf trachea and horse kidney cell cultures were stable from the 1st to 10th passages, with biological activity of 5.75-6.00 lg TCID50/cm3. CPE caused by the virus were apparent on days 2-3, further developed intensively, and extended to 60-80% of the cell monolayer on days 5-7. In calf kidney, sheep kidney, green monkey kidney, and bovine kidney cell cultures, the same changes were observed 1-2 days later. The changes were slow, and by day 7-10 CPE extended to no more than 30-50% of the cell monolayer. An attenuated strain AK-2011 of equine rhinopneumonitis virus was obtained. It was considered a candidate for the manufacture of a vaccine for equine rhinopneumonitis. Thus, the attenuated strain AK-2011 of the equine rhinopneumonitis virus was characterized. He was considered a candidate for the manufacture of a vaccine against horse rhinopneumonitis.

Keywords: horses, rhinopneumonia, attenuated strain, transferable cell cultures, virus titer.

Introduction. The current global epizootic situation is characterized by cases of equine rhinopneumonitis registered worldwide both in domestic and wild horses. This disease causes concerns in many countries in Europe, South Asia, Africa, North and South Americas. In recent years, an increasing number of cases of equine rhinopneumonitis have been documented in a number of European countries, including the CIS countries [1-9]. Information on distribution of the disease on a global scale is important in the light of the fact that equine rhinopneumonitis is associated with significant economic damages. This disease is responsible for reproductive losses in mares, culling of genetically valuable animals, and expenditure on preventive veterinary care. Through being financially beneficial, international economic cooperation poses a significant risk of spreading various contagious diseases throughout Kazakhstan.

In particular, there is a risk of equine rhinopneumonitis due to the prevalence of this disease in the countries having economic ties with the Republic of Kazakhstan, as listed by the International Epizootic
Recently, Russian scientists have studied epizootiology of the disease and developed a viral vaccine based on the CB/69 strain, which is now in use. However, testing of this vaccine in herds of horses has shown its insufficient efficacy explained by the laborious vaccination scheme, which consists of two doses. Thus, there is a need for the development of a new vaccine which would have less toxic properties than the existing ones. The presented data indicate the relevance of the research aimed at prevention of infection. The aim of the study is to characterize the isolated equine rhinopneumonia virus AK-2011 for the use of creating vaccines.

**Materials and research methods.** To reach the study objectives, the AK-2011 strain was used, which had been isolated from horses with rhinopneumonitis in 2011 during an outbreak of abortions. The viability of the AK-2011 strain was assessed using a continuous line of calf trachea (CT) cells, a continuous line of calf kidney (CK-80) cells, a continuous line of sheep kidney (SK) cells, a continuous line of bovine kidney (MDBK) cells, a continuous line of green monkey kidney (Vero) cells, a continuous line of Syrian hamster kidney (BHK-21/13) cells, a primary trypsinized culture of horse kidney (HK) cells grown in tubes and flasks, and the AK-2011 laboratory strain of equine rhinopneumonitis virus with biological activity of 6.0 lg TCID50/cm². Strain susceptibility of primary and continuous cell lines was checked during 10 consecutive passages. Susceptibility of cell cultures to the equine rhinopneumonitis virus strain was examined at each passage by the time of appearance of CPE, the intensity of CPE development, and the virus titer at the end of cultivation. To determine the infective dose (multiplicity of infection), at which the highest virus titers occur, the cell cultures grown under the same growth conditions, over the same incubation time with the same inoculum concentration introduced, were divided into equal groups and each of them was infected with a varying dose of the virus. Incubation of the infected cell culture and replacement of the nutrient medium were carried out under equivalent conditions, in equivalent volumes and over the equivalent periods of time [15-17]. Enzyme-linked immunosorbent assay (ELISA) was applied to detect equine herpesvirus types 1 and 4 in equine serum and plasma [18]. Viral DNAs were isolated using the PureLink Microbiome DNA kit (Invitrogen) according to the manufacturer’s recommendations. DNA was converted to fragments of average size 400-450 bp. using the DNA Fragmentation kit (NEB, USA). Libraries for massive parallel sequencing were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, USA) according to the protocol provided by the manufacturer. The quality of the prepared libraries was checked on a Bioanalyzer 2100 (Agilent Technologies, Germany). Sequencing was performed using the MiSeq Reagent v.3 kit (Illumina, USA) on a next generation sequencing instrument MiSeq (Illumina, USA). Bioinformatic analysis of the sequences obtained was performed using Geneious 11.0 (Biomatters, New Zealand). Alignment and phylogenetic analysis of the sequenced genes with nucleotide sequences from GenBank were performed using MEGA 6.0 by the maximum likelihood method based on 500 samples, GTR model [19, 20].

**Results and their discussion.** Based on the viability, AK-2011 strain of equine rhinopneumonitis, isolated from the horses with equine rhinopneumonitis in 2011, belongs to the Herpesviridae family. The AK-2011 strain of the virus actively reproduces on the monolayer of the primary culture of lamb kidney cells and on the choriallantoic membrane (CAM) of 12-day-old embryonated chicken eggs (ECE) at 37 °C for 96-120 hours after inoculation. The CPE of the virus are manifested 72 hours post infection. The titer of the virus infectious activity in the culture of lamb kidney cells reaches 6.08 ± 0.17 lg TCID50/cm². 96 hours after ECE inoculation, plaques characteristic of camelpox start to form along the CAM. After 120 hours of cultivation in ECE, the virus titer reaches 6.12 ± 0.08 lg EID50/cm². In total, each cell culture was passaged 10 times. Biological activity of the virus was determined using the corresponding culture mentioned above and a primary trypsinized culture of horse kidney cell.
The presence and titer of the virus in the cell culture infected with virus dilutions was finally determined by titration of the culture suspensions in the culture of horse kidney cells. The results of the tests are presented in Figure 1.

Figure 1 – Virus passivation by titers

As can be seen from the data in Table 1 and Figure 1, during primary infection with AK-2011 strain of equine rhinopneumonitis virus (dilution $10^1$) CT and HK cell cultures were stable from the 1st to 10th passages, with biological activity of 5.75-6.00 lg TCID$_{50}$/cm$^2$. CPE caused by the virus were apparent on days 2-3, further developed intensively, and extended to 60-80% of the cell monolayer on days 5-7. In CK-80, SK, Vero, and MDBK cell cultures, similar changes were observed 1-2 days later. The changes were slow, and by day 7-10 CPE extended to no more than 30-50% of the cell monolayer. The above cell lines had less apparent and weak CPE at the second passage, and no CPE was seen at the third and fourth passages.

BHK-21 cell culture did not show pronounced CPE, except for weak focal cell modifications. The lesions gradually resolved with time and became poorly visible. There were no further experiments on this culture. The AK-2011 strain of equine rhinopneumonitis reproduces and accumulates in CT cell culture at a titer of 5.75 lg TCID$_{50}$/cm$^2$ and above (titer of cytopathic doses, causing cell damage in the cell monolayer in 50% of infected cell culture grown in flasks). The cytopathogenic effect of the virus was seen in the monolayer of infected cells 72-90 hours after inoculation and extended to more than 85% of the cell monolayer 110-144 hours after inoculation.

Thus, the results of the experiments showed that CT cell line and a primary trypsinized culture of HK cells were the most susceptible to the AK-2011 strain of equine rhinopneumonitis among the tested continuous cell lines. Virus reproduction in these cell cultures remains stable even after ten successive passages. The next study focused on the technological parameters of cultivation of the AK-2011 strain of equine rhinopneumonitis in a continuous line of CT cells. The results of the experiments are shown in Table 1 and Figure 2.
Table 1 – Cytopathogenic activity of the AK-2011 strain of equine rhinopneumonitis in CT cell culture by infectious doses

<table>
<thead>
<tr>
<th>Virus dose, TCID&lt;sub&gt;50&lt;/sub&gt;/cell</th>
<th>Number of vessels (flasks), pieces</th>
<th>Period of CPE occurrence, hours</th>
<th>Period of maximum CPE development, days</th>
<th>CPE intensity, %</th>
<th>Virus titer, lg TCID&lt;sub&gt;50&lt;/sub&gt;/cm&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>30-48</td>
<td>4-5</td>
<td>80</td>
<td>6.05±0.09</td>
</tr>
<tr>
<td>0.5</td>
<td>15</td>
<td>48</td>
<td>5</td>
<td>80</td>
<td>6.25±0.02</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
<td>48-72</td>
<td>5-6</td>
<td>80</td>
<td>6.20±0.04</td>
</tr>
<tr>
<td>0.05</td>
<td>15</td>
<td>72</td>
<td>7</td>
<td>70-80</td>
<td>5.73±0.07</td>
</tr>
<tr>
<td>0.01</td>
<td>15</td>
<td>90</td>
<td>8-10</td>
<td>60-70</td>
<td>5.01±0.08</td>
</tr>
<tr>
<td>0.001</td>
<td>15</td>
<td>96</td>
<td>9-12</td>
<td>40-60</td>
<td>4.07±0.03</td>
</tr>
</tbody>
</table>

Multiple experiments have demonstrated that CPE induced by the virus in cell culture and the accumulated virus concentrations are largely dependent on the amount of the infecting virus. The virus predominantly caused CPE at doses ranging from 0.1 to 1 TCID<sub>50</sub>/cell. In this case, the cytopathogenic changes induced by the virus became detectable 30 to 72 hours post infection. During the following 48-72 hours, more than 80% of the monolayer cells were subjected to degeneration. The culture fluid harvested during this time interval contained a virus with a titer of 6.25 log TCID<sub>50</sub>/cm<sup>3</sup> and higher. In a cell culture inoculated with a lower dose of the virus, CPE became detectable 72 hours after inoculation, and the inoculated cell culture subsequently exhibited chronic viral lesions. It should be noted that at multiplicity of infection of 0.05 TCID<sub>50</sub>/cell or less CPE produced by the virus affected a significantly smaller number of the monolayer cells (70% or less of the cell monolayer when evaluated by eye) as compared with other multiplicities of infection. The titer of the virus in the culture fluid harvested from these flasks ranged from 4.07 to 5.73 log TCID<sub>50</sub>/cm<sup>3</sup>. Thus, the results of a series of the experiments have shown that in order to obtain a virus with a relatively high cytopathogenic activity from the AK-2011 strain of equine rhinopneumonitis, it is necessary to use a multiplicity of infection in the range of 0.1-0.5 TCID<sub>50</sub>/cell. The search of optimal cultivation conditions showed that the maximum virus accumulation in CT cell culture at an infectious dose of 0.1 to 0.5 TCID<sub>50</sub>/cm<sup>3</sup> incubated at 37 °C for 5-6 days enables obtaining a virus with biological activity of up to 6.25 ± 0.02 log TCID<sub>50</sub>/cm<sup>3</sup>. The results of the PCR analysis are shown in Figure 3.

Figure 2 – Indicators of the dose and titer of the virus.

Figure 3 – Electropherogram of PCR fragments obtained from multiplex

Note: 1, 2, 3, and 4 complementary DNA of rhinopneumonitis virus PCR with tested primers
Subsequently, the PCR product was cloned into the pGEM-T vector and sequenced using M13 primers. The resulting sequence was analyzed in BLAST (Figure 4). Phylogenetic analyses are presented in Figure 4.

As can be seen from the figure presented above, in terms of genome-wide characteristics, the virus appeared to be 99.9% similar to the RacL11 strain described in 1963 in Germany. The genome of the reference RacL11 strain consisted of 147,469 nucleotides; rhinopneumonitis virus had a series of deletions in the ORF67 protein-coding sequence. This deletion could be obtained artificially to attenuate the wild strain and develop a vaccine. As judged by the ORF68 gene which is generally accepted for exploring evolutionary relationships, the virus isolated in Kazakhstan appeared to be phylogenetically the closest to the T-953 and 2222-03 strains isolated in the USA and Australia, respectively. Thus, an attenuated AK-2011 strain of equine rhinopneumonitis virus was obtained which was regarded as a candidate for the manufacture of a vaccine against equine rhinopneumonitis. AK-2011 strain of equine rhinopneumonitis virus used to accumulate biomass of the pathogen was obtained by its reproducing in cell monolayer of CT-PEL in a titer of $10^{5.75}$, with subsequent storing under laboratory conditions.

**Conclusions.** This paper explored the molecular and biological properties of the AK-2011 strain of equine rhinopneumonitis. The virus isolated from the ORF68 gene in Kazakhstan appeared to be the most similar to the T-953 and 2222-03 strains isolated in the USA and Australia, respectively, in terms of phylogenetics. An attenuated AK-2011 strain of equine rhinopneumonitis virus used for the manufacture of the vaccine was obtained by its reproducing in cell monolayer of CT-PEL in a titer of 105.75, with subsequent storing under laboratory conditions. AK-2011 strain of equine rhinopneumonitis virus used to accumulate biomass of the pathogen was obtained by its reproducing in cell monolayer of lamb testicle cells in a titer of $10^{5.75}$. During the experiments, cultivated cells were stored under laboratory conditions. Thus, the experiments described in this paper revealed the marine biological, molecular and genetic properties of an attenuated AK-2011 strain of equine rhinopneumonitis virus.

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**ТУЙИН**

Жылқы ринопневмониясы - жедел оңай тарапатын ауру, елімнен өзгерістер елдериңде көп тараған. Эмірет өсіндары түсік түсіріп келіп, ринопневмонияның ауырымдарының қалыптарының сөзін анықтаушы қойылмасының оқшауланған AK-2011 штаммы бөліді. AK-2011 штаммының өміршіндегі бізге келуі қажет, бұл бір қалып жасымдардың ауыспалы сызгы, қой бұйғы, ірі кара қалыптың бұйғы жасымдардың ауыспалы сызгы, сырқұлым көміркіштің бұйғы жасымдардың ауыспалы сызгы, пробиркалар және матрастраң өсірілген қалыптардың бұйғынан бастапқы триншілікзаран өсіндісі, биологиялық белсенділігі 6.0 IgTCD50/cm3 жылқылардың ринопневмония вируссының AK-2011 өзгеріштік штаммы, қоюнырлама және ППР-тәлдау жүргізілді. Қазақстанда ORF68 ген бойынша болған вирус түсініше АҚШ пен Австралияда болған 953 және 2222-03 штамдарының филогенетикалық қажының өсі болып шықып, бастапқы инфекциялық көзделі АҚШ-2011 штаммы вируссының цитопатиялық асері (101-сүйкі) ТТ және ПЛ жасымдарының дақылдырына біріншісі 10 пассажә дейін түсініше 5,75-6,00 LGTCD50/cm3 биологиялық белсенділігімен тұрақты болды. Вирустың ЦПД 2-3 тұлғақ корінді, болашақта каркының дамыды, 5-7 тұлғылық ол монокабат аймагының 60-80%-қа қамтыды. Pt-80, po,vero, mdvk жасуша дақылдырының дәл өзиндій әзергерістер 1-2 күннен кейін байалады. Әзергерістер бауу және тек 7-10 күнде CPD бар жасушалардың монокабатының ауданы 136
30-50% - дан аспады. Осылайша, жылық ринопневмониясы вирусының АК-2011 элсірекен штаммы сипатталды, ол кейіннен жылық ринопневмониясына карсы вакцина жасау үшін пайдаланылатын болады.

РЕЗЮМЕ

Ринопневмония лошадей - острая, легко передающаяся болезнь, широко распространена во многих странах мира. Объектами исследования служили штамм АК-2011, выделенный из пробы больных ринопневмонией лошадей при вспышке абортивов. Жизнеспособность штамма АК-2011 устанавливали на перевиваемых культурах клеток ТТ (трахея теленка), ПТ-80 (перевиваемая линия клеток почки теленка), ПО (почки овца), МДВК (перевиваемая линия клеток почки крупного рогатого скота), Vero (перевиваемая линия клеток почки зеленой мартышки), ВНК-21/13 (перевиваемая линия клеток почки сирийского хомячка), первично трипсинизированную культуру почек лошадей выращенных в пробирках и матрасах, лабораторный штамм АК-2011 вируса ринопневмонии лошадей с биологической активностью 6,0 lg ТЦД50/см3. Осуществляли секвенирование и ПЦР-анализ. Выделенный вирус в Казахстане по ORF68 гену оказался филогенетически наиболее близким со штаммами T-953 и 2222-03, выделенными в США и Австралии соответственно. При первичных заражениях цитопатическое действие (ЦПД) вируса штамма АК-2011 (разведение 101) в культурах клеток ТТ и ПЛ с первого до 10 пассажа были стабильными с биологической активностью 5,75-6,00 lg ТЦД50/см3, соответственно. ЦПД вируса проявлялось на 2-3 сутки, в дальнейшем развивалось интенсивно, на 5-7 сутки охватывало 60-80% площади монослоя. В культурах клеток ПТ-80, ПО, Vero, МДВК эти же изменения отмечались на 1-2 суток позже. Изменения происходили медленно и только на 7-10 сутки площадь монослоя клеток с ЦПД достигала не более 30-50%. Таким образом, был охарактеризован аттенуированный штамм АК-2011 вируса ринопневмонии лошадей, который в дальнейшем будут использованы для изготовления вакцины против ринопневмонии лошадей.