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MOLECULAR AND BIOLOGICAL ANALYSIS OF RABIES VIRUS CIRCULATING IN THE REPUBLIC OF KAZAKHSTAN

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The spread of rabies among animals is one of the most important international criteria for assessing the biological and environmental safety of the human habitat. In the world, from 55 to 70 thousand people die from rabies every year, half of which are children, and up to 6.5 million people are subjected to post-exposure anti-rabies treatments

The analysis of nucleotide sequences will make it possible to find out the evolutionary history of the rabies virus circulating in the territory of the Republic of Kazakhstan, to identify the pathogen genovariants, to determine the time of divergence of different evolutionary branches of the rabies virus. Such an approach can improve the epidemiological surveillance of rabies infection, provide a better understanding of the dynamics of the spread of rabies and give a clear idea.

Materials of research. In order to exclude the possibility of infection of researchers, as well as compliance with the regulations in force in the Republic of Kazakhstan, all work with infected material was carried out in the RSE «National Center for Monitoring and Reference in Veterinary Medicine» of the Ministry of Agriculture of the Republic of Kazakhstan. The isolation of rabies virus RNA was carried out on the basis of the «National Reference Center for Veterinary Medicine». This organization is equipped with the latest devices and apparatus for laboratory research, and has a permit to work with pathogens of particularly dangerous diseases. Work with cDNA samples and further molecular biological analysis was carried out in the «National Center of Biotechnology» of the KN of the Ministry of Education and Science of the Republic of Kazakhstan.

The material for the project was brain samples from various types of agricultural, domestic and wild animals. Biological material was selected from animals with clinical signs characteristic of rabies. The formation of a collection of biological samples with virus-containing material includes the following stages: collection of biological material and its transportation to the laboratory; preparation of brain suspensions and their aliquation; freezing of at least two suspensions at a temperature of minus 70 ° C; use of one suspension for RNA isolation and diagnosis verification.

Methods of research

- The preparation of the suspension.
- Isolation of RNA
- Conducting reverse transcription.

— Electrophoretic analysis of amplification products
— Evaluation of the effectiveness and specificity of reverse transcription
— Real-time recording of PCR results
— Selection of primers for reverse transcription and sequencing of rabies virus
— The sequencing reaction
— The phylogenetic tree constructed.

Research results. In total, 145 materials from animals with a clinical picture characteristic of rabies were collected in 2020-2021.

At the first stage of the study, the collection of pathological material (the brain of animal rabies patients) was carried out from the territories of Kazakhstan in which cases of animal rabies were registered.

At the second stage of the study, a collection of cDNA samples isolated from a suspension of the brain of animals with a confirmed diagnosis of rabies was formed. To this end, in 2020, RNA extraction was carried out from 25 brain samples, in 23 of them the presence of rabies virus RNA was established by PCR. In 2021, RNA extraction was carried out from 120 brain samples, in 57 of them the presence of rabies virus RNA was established by PCR. For the production of reverse transcription and PCR amplification, the design and synthesis of primers were carried out, which were used as primers for the production of reverse transcription. The effectiveness and specificity of reverse transcription was tested on three samples by PCR and sequencing of amplified fragments. As a result, the complete nucleotide sequence of the rabies virus was obtained for one sample, and the nucleotide sequence of the N and G gene was obtained for one other sample.

To isolate RNA, a commercial set of «FBioNucleo» was used to isolate nucleic acids from biological material produced by FractalBio, Russia with the following steps: 400 µl of PG buffer was added from the set to 100 µl of the sample. Thoroughly mixed on the vortex. The tubes with the mixture were incubated for 5 minutes at room temperature, periodically shaking on the vortex. 500 µl of Ca buffer was added to the test tube. Thoroughly mixed. Incubated for 5 minutes at room temperature. Centrifugation was carried out for 5 minutes After centrifugation, using a pipette, 700 ml of spernatant was added to the centrifuge column attached to the kit, placed in a collecting tube, avoiding agitation and sediment transfer. Centrifugation was carried out for 60 seconds. To wash the nucleic acids, 450 µl of PE buffer was added to the centrifuge column and centrifugation was carried out for 60 s. We repeated the washing procedure again. Having removed the drain, an additional centrifugation was performed for 60 seconds to remove the remnants of the PE buffer from the membrane. The centrifuge column was transferred to a clean collecting tube. For the elution of nucleic acids, 100 µl of EB buffer or water was added and centrifugation was carried out for 1 minute. The resulting eluent in the collecting tube was used for reverse transcription (obtaining cDNA from matrix RNA).

*Conducting reverse transcription***.** The required number of micro-tubes for reverse transcription was selected (1.5 ml). In a separate sterile test tube (1.5 ml),

the components included in the kit were mixed in the proportions indicated in the table 1 below:

Table 1 – Proportions of the components included in the kit

Component	Per 1 reaction (mcl)	For N reactions (mcl)
2X Mix	10 µl	10x N
Transcription of M-MLV	0.5 µl	0,5x N

Mixed, 10 ml of the mixture was precipitated into each microprobe. 10 ml of the mixture was transferred to each microprobe. 10 µl of each sample was added to micro-samples with the mixture. The reaction mixture was incubated in a Mastercycler amplifier manufactured by Eppendorf for 30 min. at +370C. The resulting cDNA was used for PCR. The reverse transcription reaction was performed using SuperScript IV Reverse Transcriptase (invitrogen) and synthesized primers.

*Electrophoretic analysis of amplification products***.** The analysis of amplified target DNA fragments was carried out by the method of separation of DNA fragments in 1.5% agarose gel, in the presence of an intercalating agent - ethidium bromide, which was used for the purpose of DNA visualization. Electrophoresis was performed in a PowerPac horizontal electrophoresis chamber using a Bio Rad Electrophoreticbath current source. A 1×TAE buffer was used as an electrode buffer. The results were documented using the Gel Doc documentation system (Bio-Rad), with the Quantity One software (Bio-Rad). The sizes of the molecules of the analyzed DNA samples were determined by comparing their electrophoretic mobility in the gel with the mobility of markers – a DNA fragment of a known molecular weight. «DNA Ladder 10kb» (Thermo Scientific, #SM1293, 100 – 10000 bp) was used as a marker of molecular weights.

*Evaluation of the effectiveness and specificity of reverse transcription***.** The effectiveness and specificity of reverse transcription was evaluated by the method of amplification of fragments of the rabies virus genome, followed by determination of the nucleotide sequence of the amplified fragments. The composition of the reaction mixture for PCR included: 1×Platinum II PCR buffer, 0.2 mM dNTP, 0.2 microns of direct and reverse primer, 1 Unit of Platinum II Taq Hot-Start DNA polymerase, 3 µl cDNA. The PCR amplification program included: prolonged denaturation of 94 ° C - 2 minutes; 10 cycles: 94 ° C - 15 seconds, 58 ° C - 30 seconds, 68 ° C - 1 minute; 25 cycles: 94 ° C - 15 seconds, 58 ° C - 15 seconds, 68 ° C - 1-minute, final elongation of 15 minutes at 68 ° C. PCR purification of the products was carried out using magnetic particles AMPure XP (Beckman Coulter). 12 µl of suspended magnetic particles were added to 20 µl of PCR reaction, pipetted 10 times by half volume and kept at room temperature for 5 minutes. After that, the test tubes were placed on a magnetic tripod and kept for 3 minutes, if the mixture did not become transparent, the time was increased by 3 minutes. The mixture was removed without affecting the magnetic particles. 70%

alcohols were washed twice and eluted in 20 µl of 10 mM TrisHCl (hH 8,0). Real-time polymerase chain reaction (PCR). Confirmation of the presence of the virus in the biological material was carried out by PCR.

Research progress: The required number of micro-samples for PCR (0.2-0.5 ml) was selected, taking into account control samples. In a separate sterile test tube (1.5 ml), the PCR and S-Tag polymerase included in the Mix were mixed, in proportions in the table 2 below:

Table 2 – Proportions of the PCR and S-Tag polymerase included in the Mix

Component	Per 1 reaction (mcl)	For N reactions (mcl)
PCR Mix	15 µl	10x N
S-Tag polymerase	0.5 µl	0,5x N

Next, the prepared mixture was mixed on a vortex and drops were precipitated by short-term centrifugation (2-3 seconds). After the preparation of the mixture, 15 microliters were transferred to each microprobe for PCR. They were added to the first micro-sample with a PCR mixture of 10 µl of a negative control sample (CS-). In the following micro-samples, 10 microliters of the samples were added. 10 µl of the control sample (CS+) was added to the last microprobe. The test tubes were placed in the Rotor-Gene 6000 amplifier (Qiagen) and the amplification program was launched in the table 3 below:

Table 3 – Amplification program in the Rotor-Gene 6000 amplifier (Qiagen)

Temperature	**Time (sec)**	**Number of cycles**
95^0C	180	1
60^0C	30	40
95^0C	10	

After the completion of all 40 amplification cycles, the results of the studies were taken into account.

Real-time recording of PCR results. For PCR, a Rotor-Gene 6000 (Qiagen) amplifier was used with the launch of the following amplification program, according to the instructions for using the test system in the table 4 below.

Table 4 - PCR conditions

Temperature	Time (sec)	Number of cycles
95^0C	180	1
60^0C	30	40
95^0C	10	40

The analysis of the results was carried out via the R6G channel, indicating the accumulation of the cDNA amplification product of the rabies virus. A signal indicating the accumulation of an internal control amplification product was recorded via the FAM channel. Ct values of the samples were checked in the table 5 below.

Table 5 - Interpretation of results

FAM Channel	R6G Channel	Result
<35	<35	Rabies virus RNA detected
<35	> 40	Rabies virus RNA was not detected

*Selection of primers for reverse transcription and sequencing of rabies virus***.** The selection of primers was carried out on the most homologous regions identified by the alignment of genome-wide sequences of the rabies virus. The selection and verification of specific primers was carried out using FastPCR programs and the Primer Blast (NCBI) web resource (https://www.ncbi.nlm.nih.gov/tools/primer-blast /). When selecting primers, the main parameters were taken into account: the close annealing temperature of the direct and reverse primers, the length of the primers from 18-25 p.n, the low probability of the formation of secondary structures. When selecting primers, the genome was divided into 12 equal sections with an overlap area of at least 100 bp.

The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions, followed by the separation of fragments on an automatic genetic analyzer 3730xl DNA Analyzer (Applide Biosystems).

The phylogenetic tree was constructed in MEGA X using the Maximum Likelihood method and the T92 + G model (Tamura 3-parameter model with gamma distributed), the stability of the obtained phylogenetic trees was evaluated by the bootstrap method (500 Replicas).

As a result of comparing 10 field isolates of rabies virus strains by the nucleotide sequences of the N-gene fragment, a dendrogram was constructed reflecting the phylogenetic relationships of rabies virus strains and isolates in the East Kazakhstan region.

According to the results of the study of antigenic properties, all the studied field isolates of the rabies virus belonged to the first genotype.

Conclusions. In total, in 2020, material was collected from 25 animals with a clinical picture characteristic of rabies. Of these, 23 positive and 2 negative results.

In total, 120 materials from animals with a clinical picture characteristic of rabies were collected in 2021. Of these, 57 positive and 63 negative results.

In total, 145 materials from animals with a clinical picture characteristic of rabies were collected in 2020-2021. Of these, 80 positive and 65 negative results.

As a result, analyzing the phylogenetic tree rabies virus strains isolated on the territory of East Kazakhstan region, they were divided into two groups: Eurasian

and Asian. All the studied field isolates of the rabies virus belonged to the first genotype.

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