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Abstract. One of widely used methods to detect rabies virus RNA is a reverse transcription chain polymerase reaction (RT-PCR) allowing to determine a diagnosis in 5 hours. Thus, we have elaborated the method that allows to pursue effective elicitation of RNA strains and isolates of rabies virus in pathological and clinical material as well as to decrease research timespan to 6 hours, to lower by 9.8 times the diagnostics prime cost, labor expenditures – by 40 times. The way includes performance of nested RT-PCR with oligonucleotide primers having definite nucleotide sequences and synthesized to conservative glycoprotein gene. Wherein RT-PCR is pursued in two rounds. In the case of positive reaction, fragment corresponding to the size in the first round – 755 bp, in the second round – 259 bp, is synthesized.

1. Introduction

Rabies – acute infectious disease of central nervous system, invading all homoiotherms, also including humans. The disease is caused by rabies virus (Rabiesvirus), Lissavirus genus representative, Rhabdoviridae family. Lissavirus genus combines seven genotypes. Genotype 1 is presented by rabies virus classical strains (rabiesvirus) which circulate all over the world. Genotypes 2-7 include Lissa-like rabies-related (non-rabies) viruses: Lagosbatvirus (genotype 2), Mokolavirus (genotype 3), Davenhagevirus (genotype 4), Europeanbatlyssavirus 1 (EBLV1) и 2 (EBLV2) (genotypes 5 and 6, correspondingly) and Australianbatlyssavirus (ABL) (genotype 7). Rabies virus (genotype 1) is maintained in nature by trans-species transfer almost across the board (except Australia and some islands) among Carnivora and Microchiroptera representatives [1-7].

Rabies virus genome is presented by sole single-helix linear molecule minus-RNA, consisted of 11932 nucleotides. Virion RNA of rhabdovirus does not possess an infectivity. 5 polypeptides (glycoprotein, matrix protein M, nucleoprotein N, phosphoprotein NS, reverse transcriptase L (RNA-dependent RNA-polymerase)) are found in rhabdovirus virions, three of these polypeptides (L, N, S,) are bound to nucleocapsid, and two ones (G, M) are part of lipoprotein capsule. Protein G is glycolyzed, it forms protuberances on virion surface, impels synthesis of virus-neutralizing antibodies and provides for immunity development. Nucleocapsid proteins N and NS have group-specific

antigenic determinant. Nucleocapsid proteins L and NS are transcriptase components. Genes are set in the following order: 3΄-N-NS-M-G-L-5 [8-13].

The most sensitive and authentic method for rabies diagnostics so far is a classical biological test on white mice with subsequent identification of virus antigen with fluorescent antibody method. Nevertheless, essential drawbacks of the bioprobe are the investigation long term (up to 30 days in negative cases), potential danger of disease agent exposure onto ambient environment as well as impossibility to research putrescent biomaterial. Besides, bioassay setting is not economizing, demands special vivarium unit and qualified personnel [14, 15, 16, 17].

2. Chain polymerase reaction foe detection rabies virus

One of widely used methods to detect rabies virus RNA is a reverse transcription chain polymerase reaction (RT-PCR) allowing to determine a diagnosis in 5 hours.

In most cases, RT-PCR is applied for strain differentiation of rabies virus. Besides, RT-PCR application is possible for intravital elicitation of virus RNA in saliva and biopsy materials of animal salivary gland, spinal fluid.

The most applicable from the view of bioprobe described disadvantages and accepted as a methodical prototype is the rabies virus RNA elicitation method based on RT-PCR, including virus RNA isolation from virus-containing suspension, oligonucleotide primers synthesis from nucleoprotein gene, virus RNA amplification during RT-PCR, specific identification of RT-PCR products with the help of dot blot analysis. While RNA isolation during RT-PCR is based on phenol-chloroform method allowing to isolate total RNA without extraneous impurities (brain phospholipids and other), causing inhibiting effect on RT-PCR.

Together, to isolate high-quality RNA with phenol-chloroform method the pursuing of low-temperature conditions is needed. At the same time, frequently used sorbent method of DNA isolation is simple in performance and does not demand the compliance with special temperature conditions. However, this method does not allow to obtain qualitive native RNA samples. Thereat, single-step RT-PCR sensitivity substantially declines. Besides, virus material quantity in the samples under research can be lower than detected by PCR as a result of storing conditions and clinical material transportation and other.

To determine low amounts of virus RNA in probe the scientific literature describes the application of PCR nested modification allowing to raise sensitivity by 10000 times.

Nested PCR constitutes amplification in reaction first round of cDNA fragment, which serves as a matrix for second round. Given method is worked out and applied towards a row of pathogens. Also, different PCR method modifications are employed in enteroviral infections diagnostics. Nevertheless, the nested RT-PCR method for rabies virus RNA detection was not used before.

In the research process, for the first time, we determined nucleotide sequences of oligonucleotide primers -for rabies virus RNA detection by nested RT-PCR method in two-round amplification (table 1).

Table 1. Nucleotide sequences of oligonucleotide primers for rabies virus RNA detection by nested RT-PCR method.

Title	Position	Gene	Sequence (5΄-3)
Outside primers for amplification first round			
fp_850_gp_rabv	4072-4096	G	TTAGACTTATGGATGGAACATGGGT
rp_850_gp_rabv	4805-4826	G	AGTGACTGACACCTCCCTCCCT
Internal primers for amplification second round			
fp_850_gp_rabv	4072-4096	G	TCAGACGAAATTGAGCACCTTGT
rp_850_gp_rabv	4805-4826	G	ACCTCCCCCCAACTCTTAAACA

Primers design was pursued by the comparison of nucleotide sequences of lyssaviruses different strains, deposited into international database GeneBank

(http:ncbi.nlm.nih.gov/GeneBank/GeneBankSearch.html) with the help of software package «VectorNTI 9.1». With this purpose, there were calculated and synthesized the oligonucleotide primers to glycoprotein gene region.

Primer final choice was based on following criteria: high index of similarity between the fragment and RNA of rabies virus various strains, high temperature of anneal (GC-method), consensus big length. Primer chemical synthesis was made by amidophosphite method on automatic synthesizer ASM-102U. Concentration of synthetic oligonucleotide primers in stock solution was defined by spectrophotometric method.

Characteristics of primer set and amplifiable genomic RNA region. Primers flank conservative region of rabies virus glycoprotein gene which DNA it does not have nucleotide palindromic repeats to and does not form prominent secondary structures, does not have prolonged G-C regions. Predicted melting temperatures for primers pairs were allied and constituted T_m=58˚C.

Thus, there were chosen specific to rabies virus the synthetic oligonucleotide primers being compliment to conservative domain of rabies virus genome of glycoprotein gene region.

Second variant of rabies virus RNA elicitation with the help of synthetic oligonucleotide primer set during RT-PCR was held in several steps.

Research first stage included rabies virus RNA isolation according to following scheme. Aseptic, in compliance with Russian State Standard 26075, samples of rabies virus antigen of brain origin (10% suspensions in physiological solution) and clinical material were collected by standard approach with the use of commercial kit “RIBO-sorb”.

On the second stage, there were performed RT-PCR reaction for RNA obtention in a standard way with the use of commercial kit “Reverta-L”. Tube, containing 10 µl of reaction mixture (buffer for RT and 6 µl of revertase from “Reverta-L” kit), was added with 10 µl of RNA-sample, all was cautiously mixed and incubated in thermostat at 37ºC for 60 minutes. Then, 20 µl of DNA-buffer was added, thoroughly mixed and applied for RT-PCR performance.

On the third stage, there was performed an amplification of rabies virus cDNA region which codes the glycoprotein gene. To set PCR a PCR-mixture-1 and a PCR-mixture-1a are dispensed by 5 µl into microtubes of 0.6 ml volume, are perfused with wax. The PCR-mixture-1 consists of primers solution, each in 1 picomole/µl concentration, and of triphosphates mixture, each of 0.2 mM final concentration. Prepared tubes were stored at +4ºC. The PCR-mixture-2 was mixed in separate microtube immediately before usage: 10xPCR buffer (60mMTris-HCl, 2.5mM$MgCl_2$, 25mMKCl, 10mM 2-mercapthoethanol, 0.1% Triton X-100) count by 1 µl and 9 µl of deionized sterile water per assay (assay quantity + 2 controls + 1), and TagF-DNA-polymerase with 5 un/µl activity was added till final concentration of 0.5 un/µl. Next, necessary number of tubes with PCR-mixture-1 was selected.

Amplification program, consisting of various thermal regimes, is presented in table 2.

Table 2. The amplification programs.

Temperature	time	Cycles quantity
95^0C	pause	
95^0C	15 min	1
95^0C	10 s	
58^0C	15 s	
72^0C	30 s	40
72^0C	2 min	1
10^0C	Storage	

By 10 µl of PCR-mixture-2 was introduced on the wax surface. By 1 mineral oil drop was added on the top for the PCR (approximately 25 µl). Under or on the oil, by 10 µl of researched cDNA was added into the tubes prepared for the PCR. Then, amplification control reactions were set: negative

control sample – 10 µl of TE-buffer was introduced into the tube; for positive control sample - 10 µl of rhabdovirus cDNA was introduced into the tube.

On the fourth stage, size of diagnostical PCR products was defined. RT-PCR products were analyzed by electrophoresis method in 1.7% agarose gel in standard Tris-borate buffer, pH 8.0 by standard methodology. Electrophoresis results were taken into account observing the gel in ultraviolet light with wavelength of 254 nm on "Transilluminator" device. Molecular weight marker is 100-3000 bp. RT-PCR result referred to positive if RT-PCR product was visualized as a luminous fragment corresponding to 755 bp.

Amplification second stage was held analogously to first one with PCR-mixture-1 application. Amplification products of first stage were used as a matrix. PCR-amplification product detection was pursued by electrophoresis method in agarose gel by afore-described scheme. While in positive assays, 259 bp pattern was visually registered.

In one of the variants for reaction sensitivity definition, control rabies virus (production strain "Ovechiy" GNKI) was titrated by method of tenfold dilutions till 107 LD_{50}/ml. Each dilution was researched by PCR. The sensitivity of worked-out RT-PCR constituted 1.3 $lgLD_{50}$/ml.

Experiment results on the definition of specificity of nested RT-PCR with the use of outside and internal primers to glycoprotein gen of rabies virus are shown in table 3.

Table 3. Specificity of nested RT-PCR with the use of outside and internal primers to glycoprotein gen of rabies virus.

DNA from biomaterial	Nested RT-PCR result	
	with outside primers (755 bp)	with internal primers (259 nbp)
Healthy fox brain tissue	negative	negative
Healthy dog brain tissue	negative	negative
Healthy cat brain tissue	negative	negative
Healthy sheep brain tissue	negative	negative
Healthy rabbit brain tissue	negative	negative
Escherichia coli	negative	negative
Mycobacteriumbovis, strain BCG	negative	negative
Mycobacteriumavium	negative	negative
Brucellaabortus	negative	negative
Bacillus anthracis, strain 55	negative	negative
Aujeszky disease strain VGNKI	negative	negative
Staphylococcus aureus	negative	negative
Rabies virus isolate 13991	positive	positive
Rabies virus isolate 3001	positive	positive
Rabies virus isolate 329	positive	positive
Rabies virus isolate 925	positive	positive
Rabies virus isolate 2228	positive	positive
Rabies virus isolate 139	positive	positive
Production rabies virus, strain "Ovechiy" GNKI	positive	Positive
Standard rabies virus CVS	positive	positive
Rabies virus vaccinal strain "Zhshelkovo-51"	positive	positive
Rabies virus vaccinal strain "Vnukovo-32"	positive	positive
Rabies virus vaccinal strain ERA 0/333	positive	positive

Basing on the research results the positive reaction takes place in two rounds of nested RT-PCR with application of outside and internal primers to glycoprotein gene of rabies virus with cDNA of epizootic isolates of rabies virus (№№13991, 3001, 329, 925, 2228, 139), standard rabies virus CVS, production strain "Ovechiy" GNKI and vaccinal strains – "Vnukovo-32". "Zhshelkovo-51" and ERA 0/333 of rabies virus. At the same time, the reactions with negative controls were not registered – DNA from brain tissue of healthy animals (fox, cat, dog, sheep and rabbit), as well as with heterological controls – DNA of various microorganisms that testifies on specificity of nested RT-PCR with the use of worked-out set of oligonucleotide primers to glycoprotein gene of rabies virus.

Lacrimal fluid and saliva of diseased one with hydrophobia clinical features, epizootic isolates (№ 36, 40, 258 and 5359), extracted within RT, isolate № 36, extracted in Smolensk region, and also production strain "Ovechiy" GNKI of rabies virus and standard rabies virus, CVS strain, are identified with the help of outside primers fp _850_gp _rabv and rp __850_ gp _rabv to glycoprotein gene of rabies virus which amplify glycoprotein gene region in 755 bp. Wherein, the reaction was absent with saliva and lacrimal fluid samples from healthy human and in negative control sample that says about the specificity of nested RT-PCR method.

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Thus, offered nested RT-PCR approach possesses specificity while elicitation of RNA strains and isolates of rabies virus. Pointed method allows to reveal rabies virus RNA in investigated samples (pathological material from animals diseased with rabies, culture broth of vaccinal strains of rabies virus, clinical material - lacrimal fluid and saliva of rabies patient).

Primers approbation is embodied with the use of rabies virus isolates (11 assays), extracted from various kinds of wild and house animals on the territory of Tatarstan republic and Smolensk region of Russian Federation, 3 vaccinal, productional and standard strains of rabies varies as well as clinical material (2 assays). It has been shown that found primers application for RNA virus indication in pathological and clinical material provides for DNA fragments synthesis of calculated sizes (external 755 bp and internal 259 bp) in nested RT-PCR conditions.

Advantages of elaborated method comparing to classical bioassay on white mice are presented in table 4.

Table 4. Efficiency comparative evaluation of white mice and nested RT-PCR samples in rabies sample research.

Indicator	White mice bioassay	Nested RT-PCR
One assay research running time, days	from 7 to 30	0.4
One assay prime cost, Russian rubles	6651	680
Necessity of rabies virus elicitation approval by method of fluorescent antibody (MFA)	yes	no

3. Conclusion

Thus, we have elaborated the method that allows to pursue effective elicitation of RNA strains and isolates of rabies virus in pathological and clinical material as well as to decrease research timespan to 6 hours, to lower by 9.8 times the diagnostics prime cost, labor expenditures – by 40 times. The way includes performance of nested RT-PCR with oligonucleotide primers having definite nucleotide sequences and synthesized to conservative glycoprotein gene. Wherein RT-PCR is pursued in two rounds. In the case of positive reaction, fragment corresponding to the size in the first round – 755 bp, in the second round – 259 bp, is synthesized.

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