The development of PCR test-systems for diagnostics of virus diseases

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Abstract: The results of the data obtained on the basis of the development of diagnostic test- system RT-PCR based on the hemagglutinin gene F / R for identifying equine influenza virus of strain H3N8, showed high sensitivity and specificity in working conditions. And RNA can be reliably detected in the researched virus strains. Viral fragment 200 b.p. was shown as the product in the result of the amplification, with a sensitivity of $10^{-9}TCD_{50/ml}$ and the high specificity of the reaction. Isolated RNA maintains its activity for 2-10 months at -18 ° C. Research work on the development of diagnostic test- system for the detection of HA gene, the gene of EIV, was hold on the basis of RSE RIBSP. In connection therewith, we are announcing our gratitude to all members of the laboratory of diagnostic and indication of viral infections.

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Keywords: EIV - equine influenza virus, AF - allantoic fluid, RT-PCR - polymerase chain reaction with reverse transcription, RNA – ribonucleic acid, cDNA - complementary DNA, TCD50/ml - designation of the infectious activity of the virus in tissue cytopathic dose.

Actuality

Industrial horse breeding is the most industrially developing field of agriculture, both in Kazakhstan and abroad. In our country, a large number of horses raises in individual farmsteads of citizens. Topical issues related to infectious diseases are very important for intensive horse breeding with a high concentration of livestock [1, 2, 3].

Equine influenza (*Latin - Grippus; Engl. - Equine influenza; influenza of horses)* - an acute contagious disease, characterized by short-term inconstant fever, catarrh of the mucous membranes of the upper respiratory tract, dry and painful cough [4, 5, 6, 7].

Nowadays epizootics of equine influenza are due to a virus of the subtype 2 – A/horse 2 / (H3N8). The virus of the first subtype A / horse 1 / (H7N7) is completely eliminated in horse population, or the infection is subclinical. In the late 80s, virus A /horse 2 / (H3N8) was divided into two "families": European and American type [8, 9, 10, 11, 12].

Nowadays, equine influenza virus (H3N8) belonging to the family of Orthomyxoviridae, type A is the most troubling.

Diagnosis of equine influenza virus includes clinical examination, isolation and identification of the virus in cell culture, antibody detection by various diagnostic methods (IFA, RN, HAI, RGA, CFT).

Despite the fact that veterinary practice has a large set of diagnostic sera and diagnosticums, it is still very important to improve the quality and standardization of diagnostic products for practical laboratories [13, 14, 15].

High pathogenicity of many viruses also makes experimental works of creating new diagnostic methods extremely difficult. Therefore, it is necessary to provide continuous development and improvement of biotechnological basis for accurate and timely diagnosis of infectious diseases [16, 17, 18, 19, 20].

This fact shows the necessity of developing more sensitive and specific diagnostic test- systems, especially for the detection of latent and persistent forms of equine influenza virus (H3N8).

In international practice there are two main approaches for improving infection diagnosis. These are the technology of the production of monoclonal antibodies (mAb) and polymerase chain reaction (PCR).

The effectiveness of the developing test - system based on RT-PCR is determined by the fact that its development will give an opportunity to carry out the identification of EIV in various biological samples effectively, if it occurs on the territory of the Republic of Kazakhstan. Thus, it helps to prevent skidding and elimination of influenza among horses. Carrying out a full diagnosis on EI will allow using the budget funds rationally and reduce the economic damage caused by the disease.

Objective of the research: The aim of this research work is to develop diagnostic test- systems for the detection of equine influenza of strain H3N8, based on RT-PCR.

Materials and Methods:

Materials:

Virus

- In the experimental work we used the strain of EIV A/horse /Otar/764/07 (H3N8).

The following strains of influenza virus were used to determine the sensitivity and specificity of RT-PCR:

- Strain A / horse 1/Kirghizia/74 (H7N7).
- Strain NIBRG-121XP H1N1 HCV.
- A/Astana/818/09 H1N1 HCV.
- Strain A / tern / South Africa/61 (N5N3).
- Strain A/chicken /Rostok/29 (N7N1).
- The normal horse serum.

Methods:

Search and analysis of nucleotide sequences.

Search and analysis of the nucleotide sequences of EIV were carried out on the website NCBI - Influenza Virus Resource (Information, Search and Analysis).

Analysis and multiple alignment of nucleotide sequences were carried out using an application software package "FastPCR", "Primer Express-v2_0" and "BioEdit".

Biological sampling.

Venous blood, body fluid and tissue samples were the materials for research. Venous blood in an amount of 1-1, 5 ml was collected in a sterile vial with a solution of sodium citrate. Mucus from oral and nasal cavities was collected in sterile disposable tubes with tightly fitting lids by standard procedure using sterile (disposable) equipment in an amount of 0, 1 – 1, 0 ml. Tissue samples of lungs, spleen, duodenum and lymph nodes were taken to a sterile (disposable) tube of 2-3 mm3 with a tight lid.

RNA isolation technique.

Isolation of RNA from biological samples was carried out using phenol-chloroform extraction method with lithium chloride.

1. Dissolve the tissue sample in a buffer D (4M of guanidine isothiocyanate, 30 mM of sodium citrate, 30 mM of β-mercaptoethanol, pH 7.0-7.5).

2. Place the tube on ice. Add one volume of phenol and mix. Add 1/5 volume of chloroform: isoamyl alcohol (24: 1) and mix the sample. Mix by vortexing another 4 times for 1 min. In the mixing, the tubes are incubated on ice.

3. Remove at maximum speed at 4° C for 30 min. The upper (aqueous) phase should be transferred to a new tube.

4. Add 1mcl of coprecipitant and 2 volumes of 96% of ethanol. Mix by vortexing and immediately unscrewed at the maximum speed at RT for 10 min.

5. Rinse precipitate with 80% of ethanol and air dry until no traces of alcohol. Does not dry much!

6. Dissolve precipitate in 100 ml of deionized water. Add an equal volume of 12 M LiCl and cool the solution for 30 min at -20° C.

7. Remove at maximum speed for 15 minutes at room temperature. Wash precipitate by 0,8 ml of 80% of ethanol.

8. The precipitate should be dried as described above and it should be dissolved in 40 mcl of deionized water, RNase free.

Concentration determination of the obtained RNA preparation.

RNA was diluted with water 10 times (180 mcl of water was added on 20 l of the DNA solution). Measure absorbance at 260 nm is carried out in a cuvet with optical path length of 1mm, using water as control. The concentration of RNA in mother solution (g/mcl) is found by multiplying the absorbance value at wave – length of 260 nm for K = 4 (K - molecular extinction coefficient). [RNA] = A260 – K.

Methods of reverse transcription by the enzyme M-MuLV Reverse Transcriptase.

1. Prepare a mixture of a total of 11 mcl, consisting of RNA and primers: a) RNA: total RNA – 0, 1-5 mcg; mRNA - 10 ng-0, 5 mcg; specific RNA – 0, 01 pg-0, 5 ng. b) primer: oligo (dT) – 0, 5 mcg; random hexamer – 0, 2 mcg; specific primer - 15-20 pM c) bring up to 11 mcl by deionized water.

2. Mix and incubate at 70 ° C for 5 minutes and rapidly transfer to ice.

3. Add the following components: a) 10x reaction buffer - 2 mcl; b) 10 mM dNTPs - 2 mcl; c) ribonuclease inhibitor - 20 units; d) deionized water to 19 mcl.

4. Incubate the mixture at 37° C for 5 minutes. If random hexamer is used, incubation should be carried out at 25° C for 5 minutes.

5. Add 200 units of M-MuLV Reverse Transcriptase, incubate the reaction mixture at 37-42° C for 60 minutes containing oligo (dT) and specific primer. If random hexamer is used, incubation should be carried out at 25° C for 60 minutes.

6. Stop the reaction by heating to 70°. Cool on ice.

Procedure of the polymerase chain reaction.

PCR for the detection of cDNA of equine influenza virus was carried out according to the reports developed by RGP RIBSP, by using the apparatus «Mastercycler epgratient S» (Eppendorf).

The reaction mixture for PCR carrying out comprises in 20 mcl: 2, 5 mcl of buffer for Taq DNA - polymerase (60 mM Tris-Hcl (pH 8,5 at 25 ° C), 1, 5 mM $MgCl_2$; 25 mM KCl; 10 mM 2-mercaptoethanol, 0, 1% Triton X-100; 2, 5 mcl 2, 5 mM dNTP (a mixture of deoxynucleoside triphosphates); 5 mcl of each primer at a concentration of 1 mM; 1, 25 units of active thermo stability Taq DNA -polymerase, the

remaining volume is deionized water. cDNA of 5 mcl was added to the mixture. All procedures for the preparation of the reaction mixture were carried out on ice.

Results of the research and conclusions.

The complete nucleotide sequences of viruses placed in the database of NCBI - Influenza Virus Resource (Information, Search and Analysis) were used for the design of oligonucleotide sequences that are specific for hemagglutinin gene of equine influenza type A of subtype H3. It was used about 1200 HA gene sequences of equine influenza viruses of subtype H3. We analyzed 600 sequences and 200 sequences - H3.

Comparative analysis of the sequences was carried out using computer application programs BioEdit, with the search of sites of HA gene, where the sequence of subtype H3 was conservative enough for creating specific oligonucleotides (Fig. 1).

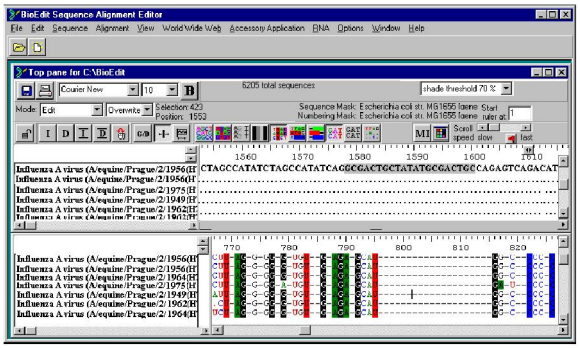

Figure 1. Alignment and determination of the location of the oligonucleotide in the areas of HA gene using a software package BioEdit version 5.0.6.

Thus, after pre-selection for subtype H3 IOL it was chosen only one pair of primers as the most suitable for setting RT-PCR, Table 1.

Table 1 - Main characteristics of the primer for the production of RT-PCR for equine influenza of subtype H7.

№	Name primer	Primer	Amplicon			
			Start	Length	Tm	GC%
1	H3/905F	gcatctccaacgacaagccat	905	21	57	52
2	H3/1108R	atcggaacccataccacccat	1108	21	57	52

With the help of it HA gene fragments of subtype H3 were successfully amplificated.

There were four different analysis layouts to develop optimal amplification function in PCR. Strain EIV A/horse /Otar/764 /07 (H3N8) was used as a matrix in dilutions of $10^{-1} – 10^{-7}$.

Diagram 1: Denaturation is at 95 ° C - 30 sec., annealing is at 57 ° C – 15 sec., synthesis is at 72 ° C – 45 sec. Then 35 cycles are according to the following scheme: denaturation is at 95 ° C – 30 sec., annealing is at 57 ° C – 15 sec., synthesis is at 72 ° C - 45 sec. Synthesis in the last cycle is at 72° C – 2 minutes. According to this scheme the sensitivity of RT-PCR was 10^{-6} $TCD_{50/ml}$.

Diagram 2: Denaturation is at 95° C - 30 sec., annealing is at 57° C – 20 seconds, synthesis is at 72° C - 45 sec. Then 40 cycles are according to the following scheme: denaturation is at 95° C – 3 sec., annealing is at 57 ° C - 30 sec., synthesis is at 72° C - 45 sec. Synthesis in the last cycle is at 72° C – 2 minutes. According to this scheme the sensitivity of RT-PCR was $10^{-9}TCD_{50/ml}$.

Diagram 3: Denaturation is at 95 ° C - 30 sec., annealing is at 57° C - 30 sec., synthesis is at 72° C - 45 sec. Then 35 cycles are according to the following scheme: denaturation is at 95° C – 30 sec., annealing is at 57° C - 30 sec., synthesis is at 72° C - 45 sec. Synthesis in the last cycle is at 72 ° C - 2 minutes. In this case, sensitivity was $10^{-7}TCD_{50/ml}$.

Diagram 4: Denaturation is at 95° C - 30 sec., annealing is at 57° C – 30 sec., synthesis is at 72° C – 45 sec. Then 35 cycles are according to the following scheme: denaturation is at 95° C – 30 sec., annealing is at 57° C – 40 seconds, synthesis is at 72° C – 45 sec. Synthesis in the last cycle is at 72° C – 2 minutes. In this case, sensitivity was 10^{-5} $TCD_{50/ml}$. Results of the research are presented in Table 2.

Table 2 - The sensitivity of RT-PCR with primers for hemagglutinin gene F/R

Diagram	Research results of RT-PCR					
	The dilutions of the virus, TCD 50/ml					
	10^{-1}–10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1	+	+	+	–	–	–
2	+	+	+	+	+	+
3	+	+	+	+	–	–
4	+	–	–	–	–	–
Note: The "+" - positive test, "-" - negative test.						

Results showed that the most optimal mode for carrying out RT-PCR is a diagram № 2, providing detection of RNA of equine influenza virus within dilution of $10^{-9}TCD_{50/ml}$ (Fig. 3).

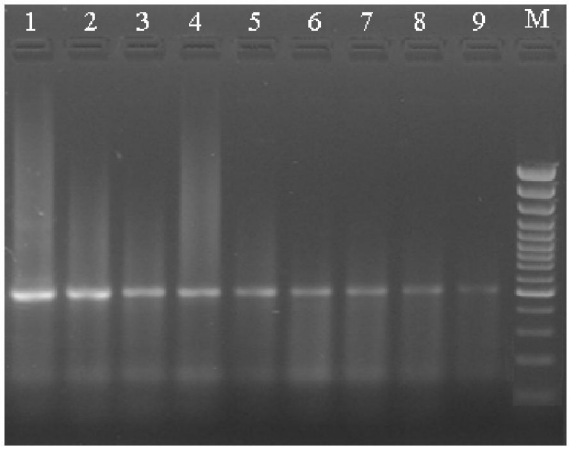

Fig. 3. The sensitivity of the test system based on RT-PCR for detecting equine influenza virus of subtype H3.

Tracks from 1 to 9 are the results of the analysis of dilutions of the samples containing the specimen EIV A/horse/Otar, M - molecular weight marker.

The sensitivity of the test for the detection of equine influenza virus of subtype H3 was higher than the sensitivity of the tests for the detection of A influenza virus of subtype H3 of commercial producers.

Virus titer $10^{-9}TCD_{50/ml}$ was the detection limit of the test- system for detection of equine influenza virus of subtype H3.This is probably due to the fact that the sequences of the primers contained degenerate nucleotides.

Thus, we have been worked optimal time for PCR comprising the following cycles: denaturation is at 95 ° C - 30 sec., annealing is at 57 ° C - 30 sec., synthesis is at 72 ° C - 45 sec. Then 35 cycles are according to the following scheme: denaturation is at 95 ° C - 30 sec., annealing is at 57 ° C - 30 sec., synthesis is at 72 ° C - 45 sec. Synthesis in the last cycle is at 72 ° C - 2 minutes.

The specificity of RT-PCR was determined according to the panel of control samples, previously characterized by virologic methods. The results are shown in Table 2.

Table 2 - Assessment of the specificity of RT-PCR for the detection of equine influenza virus of subtype H3.

Virus (strain, isolate)	Results
Strain A / horse /Otar/764/07 (H3N8);	+
Strain A / horse 1/Kirghizia/74 (H7N7);	–
Strain NIBRG-121XP H1N1 HCV;	–
A/Astana/818/09 H1N1 HCV;	–
Strain A / tern / South Africa/61 (N5N3);	–
Strain A/chicken /Rostok/29 (N7N1);	–
The normal horse serum	–
Note: «+» – positive result, «–» – negative result.	

The data in Table 2 show that during RT-PCR carrying – out on RNA template of closely related equine influenza virus, according to strains A/horse 1 /Kirghizia/74 (H7N7); NIBRG-121XP H1N1 HCV; A/Astana/818/09 H1N1HCV; A/tern/South Africa/61(H5N3); A/chicken/Rostok/29 (H7N1); normal horse serum - negative results were shown. It proves the high specificity of the reaction (Fig. 2).

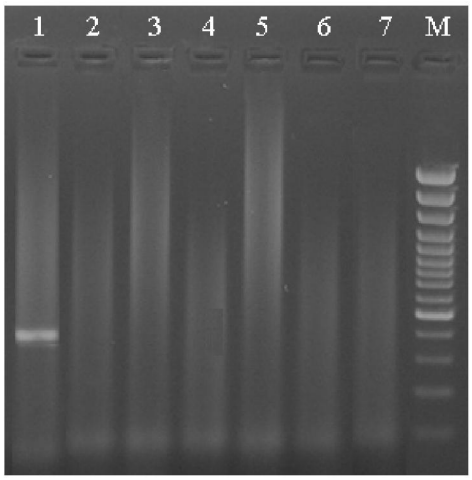

Fig. 2. The specificity of the test system based on RT-PCR for detecting equine influenza virus of subtype H3.

Tracks from 1 - Strain A / horse /Otar/764/07 (H3N8); 2 - Strain A / horse 1/Kirghizia/74 (H7N7); 3 - Strain NIBRG-121XP H1N1 HCV; 4 - A/Astana/818/09 H1N1 HCV; 5 - Strain A / tern / South Africa/61 (N5N3); 6 - Strain A/chicken /Rostok/29 (N7N1); 7 - The normal horse serum.

The specificity of the test system was determined by using program BLAST. In both cases the primers were sufficiently specific.

The effectiveness of RT-PCR in working conditions.

During the research work there was the testing of effectiveness of RT-PCR in working conditions with disease outbreaks of influenza virus of strain H3N8. The results are shown in Table 3.

Table 3 - Results of the detection of equine influenza virus of strain H3N8 by PCR in samples of biological material of various nature.

№	Biological material type	Number of samples	Number of positive samples
1	Lungs (of fallen animal)	7	3
2	Bronchus (of fallen animal)	7	3
3	Nasal discharge	15	12
4	Lymph glands (of fallen animal)	7	5
5	Spleen (of fallen animal)	7	4
6	Venous blood (of horses herd)	45	18
Total:		88	45

The data of Table 3 show that the virus is often detected in samples of venous blood (40, 0%), but it was very difficult to isolate it from the bodies of fallen animals. The virus isolation in the laboratory is possible if sampling of biological material is in the early stages of disease and in containers with transport medium chilled.

Summarizing the findings, we can conclude that developed and tested PCR with primers for hemagglutinin gene F / R of equine influenza virus of strain H3N8 has a high sensitivity and specificity and can be used effectively in complex diagnostic procedures, as well as serve as basic virologic method of diagnosis of equine influenza of strain H3N8.

Conclusion.

Thus, the results of the data showed that diagnostic test- system based on RT-PCR with primers, complementary to the highly conserved sequences of the hemagglutinin gene of F / R for identifying the equine influenza virus of strain H3N8, has a high sensitivity and specificity. And RNA can be reliably detected in the researched virus strains. Viral fragment 200 b.p. was synthesized in the result of the amplification of cDNA. The sensitivity of the reaction was $10^{-9}TCD_{50/ml}$. Isolated RNA, used as a positive control of RT-PCR, maintains its activity for 2-10 months at -18 ° C.

A test- system RT-PCR meets the requirements for sensitivity and specificity and can be recommended for using in the detection of equine influenza virus in any biological material and is suitable for further using in monitoring research.

Research work on the development of diagnostic test- system for the detection of HA gene, the gene of EIV, was hold on the basis of RSE RIBSP. In connection therewith, we are announcing our gratitude to all members of the laboratory of diagnostic and indication of viral infections.

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