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EXPERIMENTAL WORKS

Development and Verification of Real-Time PCR Assay for Identification of Viral Agents Causing Acute Respiratory Infections in Human Beings

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Abstract—A multiplex polymerase chain reaction (PCR) for identification of four viruses causing acute respiratory diseases in human beings was developed. The analytical sensitivity of developed RT-PCR for identification of adenovirus, respiratory–syncytial virus, flu viruses types A and B, and actual subtypes of type A flu virus (seasonal and pandemic variants H1N1, seasonal H3N2, and viruses of bird flu that are pathogenic to human beings H5 and H7) was 1×10^3 genome equivalents per milliliter. Diagnostic sensitivity for flu virus type A and B, and also subtypes H1 (seasonal H1N1, pandemic variant of H1N1 of year 2009), H3, H5 was 1×10^3–10^4 viral particles per milliliter. The method developed has high specificity and does not have positive signal in experiments with DNA/cDNA of human beings and viral DNA. We have studied 50 samples using the developed set. Etiology was defined in 33 samples.

Keywords: multiplex PCR, diagnosis of respiratory infections, flu virus, respiratory–syncytial virus, adenovirus, acute respiratory infection (ARI)

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INTRODUCTION

Acute respiratory infections are primary in terms of common infectious morbidity, being the main reason for temporary disability, leading to significant economic losses. According to official data provided by the Federal Service for Monitoring of Consumer Rights Protection and Human Welfare, 28265634 cases of acute respiratory infections of upper airways were officially registered in 2010 in the Russian Federation, including flu, while in 2011 31038446 cases were [18]. The real number of cases of acute respiratory infections may be greater, because some infected people do not ask for medical help and are not reported statistically.

Viruses of six families are the main etiology agents of human viral respiratory diseases: *Orthomyxoviridae* (flu virus), *Paramyxoviridae* (paraflu, metapneumovirus, respiratory-syncytial virus), *Picornaviridae* (enterovirus, rhinovirus), *Coronaviridae* (coronaviruses 229E, NKU1, NL63, OC43, SARS agent), *Adenoviridae* (adenovirus), and *Parvoviridae* (bokavirus). Because acute respiratory infections in human beings are caused by a wide range of infectious agents, differential diagnosis of them requires significant material, time, and labor. In connection with this, developing tools fir differential diagnosis of respiratory infections in the form of multiplex PCR is very relevant [8]. This work is devoted to development and validation of the set of reagents to detect genetic material of four viruses (flu viruses type A and B, respiratory-syncytial virus, and adenovirus), causing the most severe infections of human respiratory tract.

MATERIALS AND METHODS

Clinical samples. Clinical material (swabs from naso- and oro-pharynx) from 30 patients without any symptoms of ARI were used to conduct the study. The study was carried out following principles of consent and confidentiality in accordance with the "Basis of Legislation of the Russian Federation for the Protection of Citizens' Health" (Decree of the President of the Russian Federation of December 24, 1993, T2288; Federal Laws no. 30-FL from March 2, 1998 and no. 214-FL from December 20, 1999). Interviews with the subject and picking up of clinical material were conducted after receiving written informed consent. The procedure of the study was approved by the Ethical Committee of the State Research Center of Virology and Biotechnology VECTOR on December 9, 2011. Another 50 samples from patients with ARI symptoms obtained by Vector from 2009 to 2010 for

verification of the presence of genetic material of pandemic flu virus type A (H1N1) (2009) in accordance with an order from the Federal Service for Monitoring Consumer Rights Protection and Human Welfare of March 17, 2008, no. 88 "On Ways to Improve Monitoring Agents of Infectious and Parasitic Diseases" were studied.

Viral strains. The following strains were used in the work: flu type A: A/Aichi/2/1969(H3N2); seasonal variant of flu A/Novosibirsk/1/2009(H1N1); strains of pandemic virus flu A/Yekaterinburg/01/2009(H1N1)v, A/Tomsk/01/2009(H1N1)v, A/Novosibirsk/02/2009(H1N1)v, and A/Irkutsk/01/2009(H1N1)v; strains of highly virulent bird flu A/chicken/Kurgan/05/2005(H5N1) and A/garganey/Crimea/2027/2006(H7N8); and strains of flu virus type B: B/Chita/01/2010, B/Chita/02/2010, B/Chita/03/2010, B/Chita/04/2010, B/Chita/05/2010. The strain A/Aichi/2/1969(H3N2) was obtained from the Ivanovskii Institute of Virology of Russian Academy of Medical Sciences (Moscow), and the strain of bird flu A/chicken/Kurgan/05/2005(H5N1) was obtained from the Research Institute of Influenza of Russian Academy of Medical Sciences, St. Petersburg. The strains of seasonal and pandemic variants of flu of subtype H1N1, as well as the strain of flu virus A/garganey/Crimea/2027/2006(H7N8), were isolated at Vector. Strains of flu virus type B were obtained from the Center of Hygiene and Epidemiology of Chita oblast. The strain Long of respiratory–syncytial virus (no. VR-26) and the strain Gomen of adenovirus type 7 (no. VR-7) were obtained from the American Typical Collection of Cellular Cultures (ATCC).

The strains of flu virus had at least three passages on the MDCK cellular culture at Vector. The strain of respiratory-syncytial virus was obtained on transplantable cellular culture of kidney of green monkey Vero and the strain of adenovirus on transplantable culture of modified cells of human embryo kidney 293. The cells were obtained from the Department of Cellular Technologies of Vector. Cultivation of infected cellular cultures was conducted at 37°C in atmosphere containing 5% of CO_2. The cultivation medium DMEM (Biolot, Moscow) with addition of 2 mM per 1 L of glutamine (Sigma-Aldrich, United States) and antibiotics in the amount of penicillin 100 ME/mL and 100μ/mL of streptomycin was used for maintenance of cellular cultures.

Primers and probes. Nucleotide sequences obtained from the GenBank international database were used to select primers and probes. To equalize the obtained sequences, as well as for analysis and calculation of primers and probes and studying their specificities, the Vector NTI v. 9.0 family of computer programs (Informax, United States) and the internet database of the US National Center of Biotechnology Information (NCBI) were used in an online regime.

Positive control samples. Positive control samples were obtained by the method of TOPO-T/A cloning of a synthesized DNA fragment with viral insertion into the pCR2.1 plasmid (Invitrogen, United States) for transformation.

Detection of DNA concentration. The concentrations of plasmid and genomic DNA of human beings were detected using the Quant-iT DNA HS commercial reagent set (Invitrogen, United States) and QUIBIT fluorometer (Invitrogen, United States).

Obtaining the samples of flu virus and detection of physical titer. To detect the virus concentration of (physical titer), specimens prepared in the following way were used. On the fifth day of infection, cultural virus-containing fluid (CVF) was taken from cultural vessels. Further cellular debris was removed by centrifuging at 6000 g at 4°C for 10 min, and PEG was then added to subprecipitate fluid to a final concentration of 8% (mass/volume). The obtained suspension was mixed for 1 h at 4°C and then centrifuged for 20 min at 1000 g. The precipitate obtained was resuspended in STE-buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA), and then the suspension was applied to a gradient from 10–60% (mass/volume) sucrose solutions. Centrifuging was conducted for 4 h at 150000 g by the means of an Optima L-90K ultracentrifuge in a 90Ti rotor (Beckman Coulter, United States). Fractions containing a maximum amount of flu virions in correspondence with the results of hemagglutination reaction, were applied to prepare consecutive tenfold dilutions and determine the diagnostic sensitivity of the developed set of reagents.

Extraction of viral and human RNA/DNA, reaction of reverse transcription. Viral and human RNA/DNA were isolated from 100 μL of clinical material, CVF, whole blood. Isolation was done using the RIBO-prep commercial set of reagents (State Central Research Institute of Epidemiology, Moscow, Russia) in accordance with the manufacturer's instructions. The obtained suspension of CDNA was diluted twofold by 1× TE-buffer (to 40 μL). Control of isolation of nucleic acids and reverse transcription reaction was performed in an individual sample with addition of flu virus suspension of strain A/Tomsk/01/2009(H1N1)v.

PCR- amplification. An amplification reaction was carried out in 30 μL of reaction mixture containing 1× Taq-buffer and 0.17 mM of each of deoxyribonucleoside triphosphates and 1.5 active units of Smart-Taq-DNA-polymerase (all reagents produced by Medigin Laboratory, Russia). Into the reaction mixture, there were added 6 μL of the studied sample and 6 μL of 1× TE buffer containing 102 copies of one (in the case of monovariant PCR) or all (in the case of multiplex PCR) of pCR2.1 recombinant plasmids with insertion of a virus-specific DNA fragment as a positive control sample and 6 μL of 1× TE-buffer as a negative control sample. The reaction was conducted

in accordance with the following temperature–time profiles: activation of Smart-Taq-DNA polymerase at 95°C, 5 min; DNA denaturation at 95°C, 10 s; annealing of primers at 51°C, 20 s; and DNA elongation at 72°C, 20 s. In the first 20 cycles of amplification, a fluorescent signal was not detected, while in the following 30 cycles the intensity of a fluorescent signal was detected in four channels—Green (FAM), Yellow (R6G), Orange (ROX), and Red (Cy5)—on the Rotor Gene 6000 (Corbet Research, Australia) and iQ5 (BioRad, United States) instruments. In addition, electrophoresis of PCR products was conducted in 2% agarose gel with the presence of ethidium bromide.

Verifying PCR- test systems. To further confirm the results, the clinical material was studied using the means commercial sets of reagents for amplification and identification of flu virus cDNA AmpliSens Flu virus A/B-FL (flu viruses A/B), AmpliSens Flu virus A-FL (flu virus A (H1N1, H3N2), AmpliSens Flu virus A/H1-swine-FL (pandemic variant of flu virus H1N1 (2009)), AmpliSens Flu virus A/H5N1-FL (highly pathogenic bird-flu virus H5N1), and AmpliSens ARI-screen-FL (ten agents of human ARI) (State Central Research Center of Epidemiology, Moscow, Russia).

RESULTS AND DISCUSSIONS

During the creation of set for real-time PCR allowing identifying etiological agents of human acute respiratory-virus diseases, we selected four viruses: flu viruses A and B, human adenovirus, and human respiratory–syncytial virus. In the process of the work, we added actual subtypes of flu A virus—seasonal variants of H1N1 and H3N2, and pandemic variant H1N1 (2009)—as well as subtypes H5 and H7 of bird flu virus infecting human beings [5]. Thus, this work is devoted to developing PCR to detect genetic material (RNA/DNA) of the listed viral pathogens causing respiratory diseases in human beings.

According to the literature data, it is known that the most conservative regions of genomes of selected viruses are located in gene *M* of flu viruses A and B and in the *hexon* gene of human adenovirus and the F-gene (fusion-protein gene) of human respiratory–syncytial virus. Subtyping of flu A virus is traditionally performed by the hemagglutinin gene (*HA* gene).

For typing flu viruses A and B, as well as for identification of seasonal flu virus A/H1N1; the pandemic variant of flu virus A/H1N1 (2009); and flu A virus of subtypes H3, H5, and H7 primers and probes recommended by the World Health Organization (WHO) were used. Oligonucleotides recommended by the WHO were redesigned in accordance with the data that we obtained.

Both full and fragmented nucleotide sequences of viral genomes were taken from the GenBank database, and they were analyzed in detail. In total in this work, we analyzed 1852 nucleotide sequences of the seventh segment (*M* gene) of the flu-virus genome (1446 nucleotide sequences of flu A and 406 sequences of flu B viruses). Calculating specific primers and probes for the seasonal flu virus A/H1N1; pandemic flu virus A/H1N1 (2009); flu virus A of subtypes H3, H5, H7 1220, 1185, 714, and 316; and 78 nucleotide sequences of the fourth segment of the flu virus (HA gene) were analyzed, respectively. For human respiratory–syncytial and human adenovirus, original primers and probes were selected, but their nucleotide sequences are to be published in future works due to forthcoming patenting. In the calculation, 1562 nucleotide sequences of the genome of respiratory–syncytial virus and 1393 nucleotide sequences of adenovirus of serotypes 3, 4, 7, 14, and 21 most often infecting the human respiratory tract [10, 15] were analyzed. Primers and probe for detection of respiratory–syncytial virus were selected inside the F gene and for adenovirus inside the hexon gene. Nucleotide sequences of primers and probes are presented in the table. The specificities of selected oligonucleotides was analyzed at the stage of calculations using the Nucleotide Blast NCBI program. According to the results of our investigations, the selected primers and probes had no homology with human DNA or with agents that could be observed in swabs from feces, nasopharynx, oropharynx, aspirate, and other clinical materials chosen for diagnostic studies, namely, with human coronaviruses types 229E, NL63, OC43, and NKU1; coronavirus associated with severe acute respiratory syndrome human herpes virus type 1–2; cytomegalovirus; human paraflu virus type 1–4; metapneumovirus; human rhinovirus types A–C; bacteria of the genus *Streptococcus*; and bacteria of the species *Mycoplama pneumonia*, *Chlamydophila pneumonia*, *Haemophilus flu*, and *Legionella pneumophila*.

Plasmid constructions with an inserted corresponding virus-specific DNA fragment were used in the developed set as positive controls. The obtained plasmid structures (pIV-A, p-IV-B, pA/H1N1seas, pA/H1N1(2009)pand, pA/H3, pA/H5, pA/H7, pAdV, pPCB) were used for optimization of amplification-reaction conditions for each infectious agent separately (monoplex PCR). Experimentally optimal concentrations of Mg^{2+} (2.5 mM $MgCl_2$), direct and reverse primers (10–12 pM), and fluorescent DNA-probe (6–8 pM) were chosen for PCR in monovariant forms.

At the next stage of work, multiplex PCR conducted in three mixtures was optimized and verified (Fig. 1). The first mixture included four pairs of primers and four DNA probes; the second, three; and the third, two. The developed multiplex variant of PCR was also optimized according to the amount of Mg^{2+} ions (2.5 mM $MgCl_2$), probe (8 pM), and direct and reverse primers, the concentrations of which were

Primers and probes used in work

Viral agent	Reference sequence	Gene	Coordinates	Primers and probes 5' → 3'	T_m, °C
IV-A	CY037808.1	*M*	145–165 188–207 239–222	*AAGRCCAATMCTGTCACCTCT* *FAM-GTRTTCA CGCTCA CCGTGCC-BHQ1* *RCGYCTACGYTGCAGTCC*	51
IV-B	CY037432.1	*M*	19–39 49–65 111–93	*GACACRATTGCCTACYTGCTT* *R6G-GARGAYGGRGAARGCAAAGYAGA-BHQ2* *TCTTTYCCACCRAACCARC*	51
A/HIN1 seas.	CY002672.1	*HA*	213–231 256–239 279–263	*GCCCCMYTACAA7TGGGTA* *ROX-CCAHCCGGCAAYGCTGCA-BHQ2* *YCGCATTCTGGRTTYCCT*	51
A/H1N1 pand. 2009	GQ457514.1	*HA*	822–844 874–899 925–904	*GGAAAGARATRCTGRATCTGGTA* *Cy5-TGCAAYACAACTTGTCAGACAYCCR-BHQ2* *ATGGGAGRCTRGTRTTTATAGC*	51
2A/H3	CY000941.1	*HA*	1544–1569 1596–1574 1621–1599	*TRTACAGARAYGARGCATTRAACA* *FAM-TCAACRCCT7TGATYTGRRAYCG-BHQ1* *TAGRAYCCAATCYTTRTAYCCTG*	51
A/H5	DQ360835.1	*HA*	1547–1568 1604–1626 1654–1636	*GARARGAAATAARTGGRGTRAAATT* *R6G-TTTATTCHACWGTRRCRAGYTCCCTARYA-BHQ2* *RACCAGCYABCRTGATTGC*	51
A/H7	AB558258.1	*HA*	977–998 1051–1073 1102–1084	*CWACAGRAATGAARAAYGTYCC* *ROX-CTYTTYGGRGCRA7TGCTGG-BHQ2* *CRATGAGACCYTCCCAYCC*	51
RSV	–	*F*	–	To be published later	51
AdV	–	*hexon*	–	To be published later	52

higher than in the monoplex PCR variant (15–17 pM). The amplification reaction in the format "multiplex" was optimized for annealing of primers at a temperature 51°C.

The obtained plasmid constructions were also used to evaluate the effectiveness of real-time PCR, for the purposes of which successive tenfold dilutions of control plasmid DNA with virus-specific DNA insertion were prepared. The threshold of the method's sensitivity was 1×10^3 GE/mL (Figs. 2a–2c).

The sensitivity and specificity of the developed set were determined both in monovariant and "multiplex" formats. In the experiments with samples with a known concentration of the virus (physical titer), we detected the diagnostic sensitivity of flu viruses A and B, seasonal flu virus A/H1N1, pandemic flu virus of swine origin A/H1N1(2009), an flu virus A subtypes H3 and H5, which is 1×10^3–10^4 virions per milliliter (Fig. 2d) according to the experimental data. Thus, the sensitivity that was obtained of the developed set of reagents complies with the requirements for test systems recommended for use in clinical practice.

Th developed set of reagents was checked at the first stage using viral strains from the collection of Vector (flu type A: A/Aichi/2/1969(H3N2), A/Novosibirsk/1/2009(H1N1), A/Yekaterinburg/01/2009(H1N1)v, A/Tomsk/01/2009(H1N1)v, A/Novosibirsk/02/2009(H1N1)v, A/Irkutsk/01/2009(H1N1)v, A/chicken/Kurgan/05/2005(H5N1), A/garganey/Crimea/2027/2006(H7N8), strains of flu virus type B: B/Chita/01/2010, B/Chita/02/2010, B/Chita/03/2010, B/Chita/04/2010, B/Chita/05/2010; strain of respiratory-syncytial virus Long; strain of adenovirus type 7 Gomen). In all experiments, viral strains were identified and there were no nonspecific signals.

The specificity that we identified empirically is an important characteristic of the set that has been developed. This set does not have a positive signal when 3 μg of human genome DNA/cDNA are added to each mixture. A nonspecific signal was also absent in experiments on cDNA of coronavirus associated with

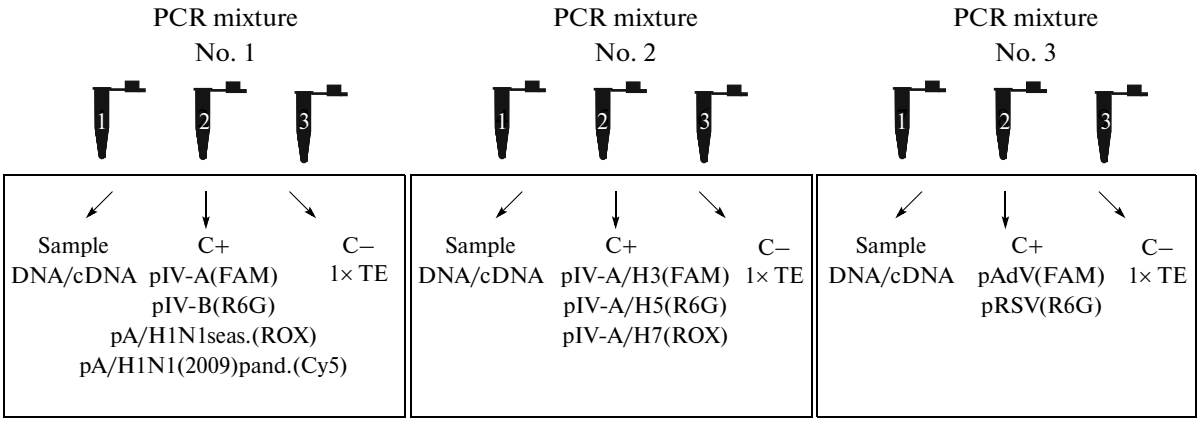

Fig. 1. Scheme of carrying out PCR in the "multiplex" format in a real-time regime using the developed set of reagents.

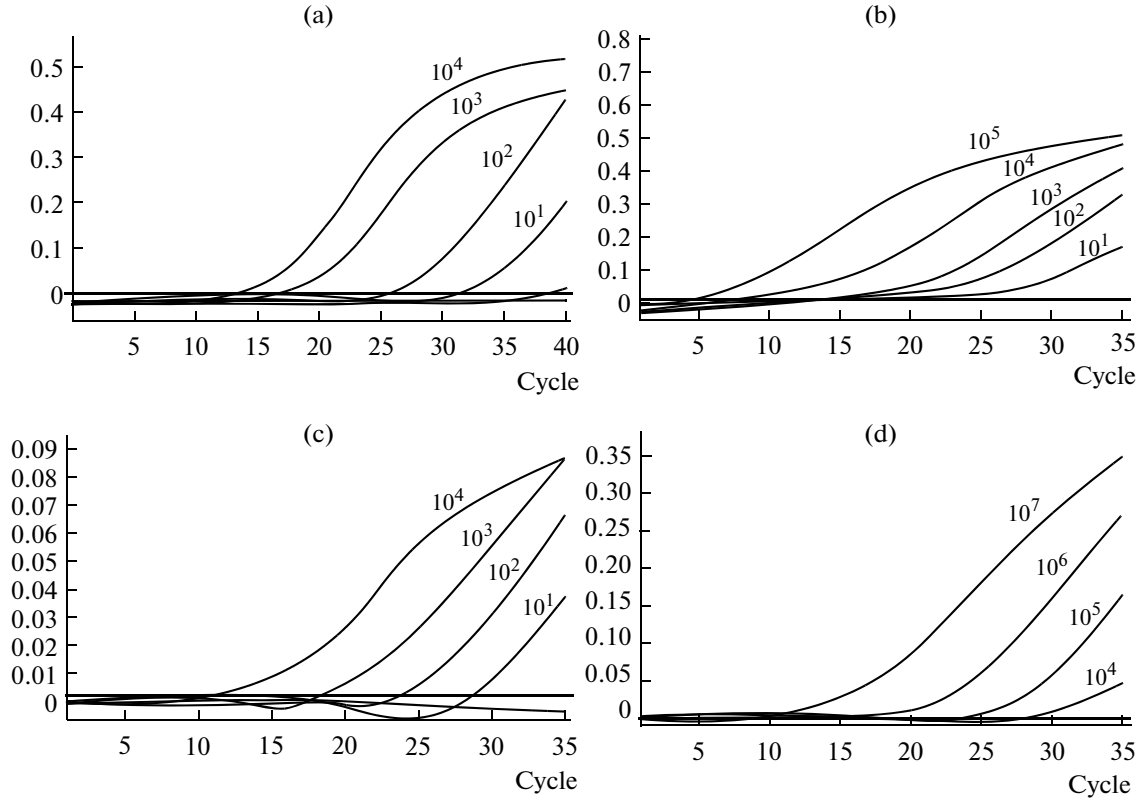

Fig. 2. Results of studying sensitivity by the real-time PCR method. The sensitivity of the method for (a) flu virus B, (b) adenovirus, an (c) respiratory-syncytial virus was 10 GE per reaction (10^3 GE/mL). The diagnostic sensitivity for (d) flu virus type A was 10^4 virions per mL.

Frankfurt severe acute respiratory syndrome strain, human herpes virus types 1–2, cytomegalovirus, and human enteroviruses types A–D. A nonspecific signal was not detected in any of the 15 samples containing enterovirus RNA. No specific counteractions with DNA/cDNA of normal microflora of the nasal cavity and oropharynx were found when studying 30 swabs of conditionally healthy donors with an absence of respiratory-disease symptoms.

Using this set, we have studied 50 samples obtained by Vector for experiments and isolation of the pandemic flu virus A/H1N1 (2009) (Fig. 3). In 29 of the studied samples, RNA of flu virus A was detected (58%), while RNA of flu virus B (8%) was found in 4. In

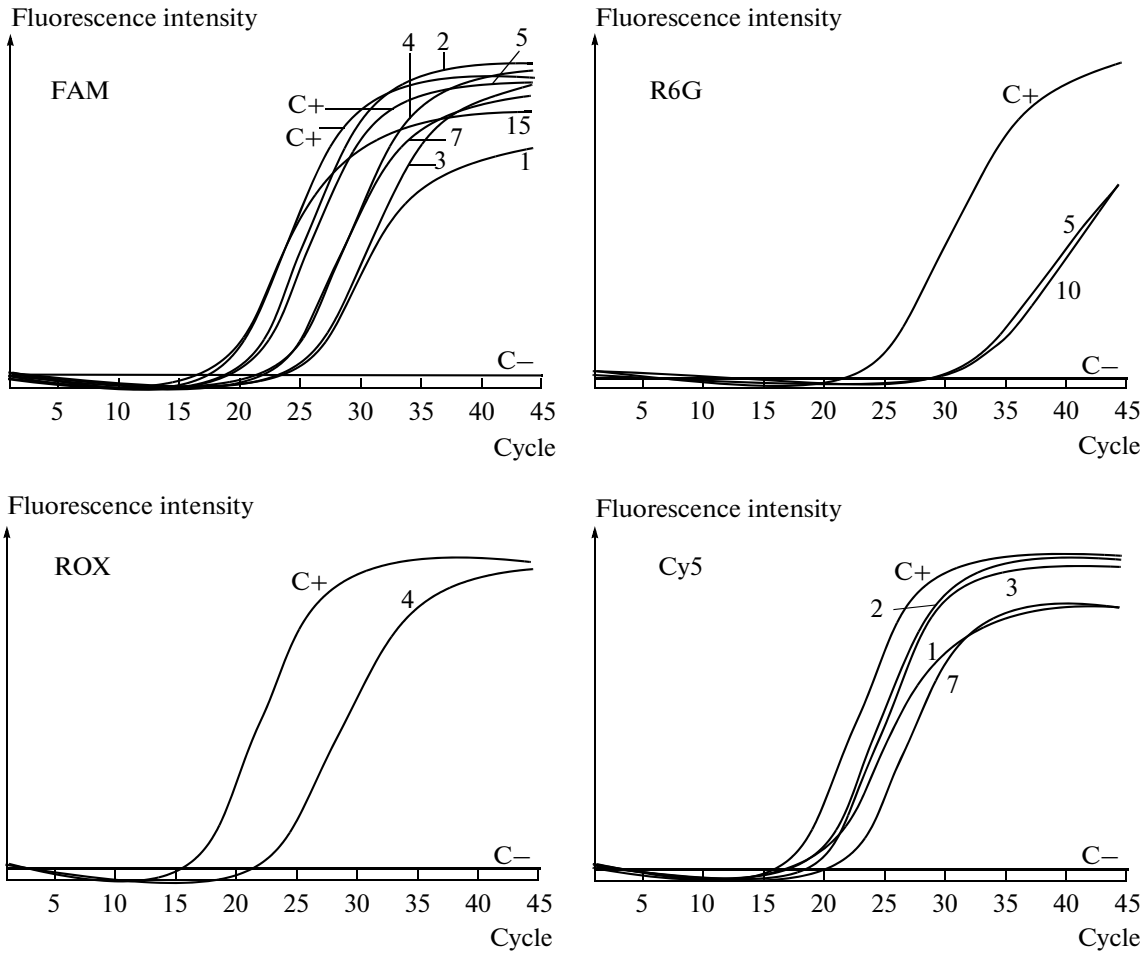

Fig. 3. Results of real-time PCR in clinical samples of patients with symptoms of ARI. In samples nos. 1, 2, 3, 4, 5, and 7, flu virus type A RNA was detected; in sample no. 15, RNA of flu virus type A/H3 was detected; in samples nos. 6 and 10, RNA of flu virus B was detected; in sample no. 4, RNA of seasonal variant of flu virus A/H1N1 was detected; and, in samples nos. 1, 2, 3, and 7, RNA of the pandemic variant of flu virus A/H1N1 was detected.

27 samples of 29 positive that were positive for flu virus A, pandemic flu virus A/H1N1 (2009) (93%) was detected; in individual samples. flu virus A subtypes H3 (3.5%) and H1 (3.5%) were detected. Genetic material of flu virus A subtype H5 and H7, respiratory-syncytial virus, and adenovirus were not detected in any of the samples that we studied. The etiological agent of the disease was not detected in 17 samples (34%). The obtained results in 100% of cases consisted of studying 50 samples of clinical material using a commercial set of reagents from the Federal Central Research Institute of Epidemiology.

Thus, we have developed a set of reagents for identification of four viral agents causing human respiratory diseases that allows identifying two types (A and B) and five actual subtypes of flu virus (seasonal variants H1N1 and H2N2, pandemic variant H1N1 (2009), and subtypes of bird H5 and H7 of flu virus). The diagnostic and analytic sensitivity of the set was evaluated. It was shown experimentally that the developed set of reagents has high specificity; however, there is still a need to extend the range of detected viral agents.

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