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Development of real-time RT-PCR for N2 subtype avian influenza RNA-virus detection

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SUMMARY
Currently, N2 subtype avian influenza (AI) virus actively circulates in domestic and wild bird populations and is regularly detected in China, other Asian countries and Russia, particularly in combination with H9 hemagglutinin. Therefore, a method for rapid detection of the said infectious agent is urgently required. Data on oligonucleotide primer selection and reverse transcription real-time polymerase chain reaction condition optimization for N2 AI virus detection are presented in the paper. Modified primers and probe proposed by B. Hoffmann in 2006 as well as original primers and probes with the viruses available in the Laboratory working collection and selected during testing were assessed for N2 neuraminidase gene fragment amplification. Optimal concentrations of real-time RT-PCR master mix components and temperature-time mode were determined. Various combinations of primers were tested against ten N2 avian influenza virus isolates that genetically differed from each other in N gene. Nine viruses were isolated from birds in the Russian Federation regions and classified to different genetic groups. The real-time RT-PCR assay was tested for its specificity using AI virus isolates of different neuraminidase subtypes (H5N8, H3N6, H4N6, H5N1, H10N7) as well as samples containing other RNA-viruses: Newcastle disease virus, infectious bronchitis virus and infectious bursal disease virus. As a result of the testing, real-time RT-PCR conditions providing high sensitivity and specificity of the assay were selected and optimized.

Key words: avian influenza virus, real-time RT-PCR, optimization, N2 neuraminidase subtype, sensitivity, specificity.

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Разработка метода ОТ-ПЦР в режиме реального времени для выявления РНК вируса гриппа птиц подтипа N2

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РЕЗЮМЕ

В настоящее время вирус гриппа птиц подтипа N2 активно циркулирует в популяциях домашних и диких птиц, и его регулярно выявляют в Китае, других странах Азии и России, особенно в комбинации с гемагглютинином подтипа H9. Поэтому применение метода для быстрого обнаружения данного инфекционного агента крайне необходимо. В представленной работе приводятся данные по выбору олигонуклеотидных праймеров и оптимизации условий постановки полимеразной цепной реакции с обратной транскрипцией в режиме реального времени для выявления вируса гриппа птиц подтипа N2. Для амплификации фрагмента гена нейраминидазы подтипа N2 были апробированы предложенные в 2016 году B. Hoffmann праймеры и зонд в модификации, а также выбранные в ходе исследования оригинальные праймеры и зонды с вирусами, имеющимися в рабочей коллекции лаборатории. В ходе работы определены оптимальные концентрации компонентов реакционной смеси для проведения полимеразной цепной реакции с обратной транскрипцией в режиме реального времени и температурно-временной режим. Разные комбинации праймеров тестировали на десяти изолятах вируса гриппа птиц подтипа N2, генетически отличающихся друг от друга по гену N. Девять вирусов выделены от птиц из регионов Российской Федерации и относятся к различным генетическим группам. Специфичность метода проверяли методом полимеразной цепной реакции с обратной транскрипцией в режиме реального времени с использованием изолятов вируса гриппа птиц с другим подтипом нейраминидазы (H5N8, H3N6, H4N6, H5N1, H10N7), а также проб, содержащих РНК вирусов ньюкаслской болезни, инфекционного бронхита кур и инфекционной бурсальной болезни. В результате проведенных исследований были подобраны и оптимизированы условия постановки полимеразной цепной реакции с обратной транскрипцией в режиме реального времени, которые обеспечивают высокую чувствительность и специфичность метода.

Ключевые слова: вирус гриппа птиц, ОТ-ПЦР-РВ, оптимизация, подтип нейраминидазы N2, чувствительность, специфичность.

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INTRODUCTION

Avian influenza (AI) is an acute infectious disease caused by the virus of *Orthomyxoviridae* family having segmented negative-sense RNA genome and belonging to *Influenzavirus* A genus [1]. Segmented genome of avian influenza virus allows for its reassortment.

Current classification of avian influenza A viruses is based on antigenic properties of their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA).

Neuraminidase accounts for 20% of all surface glycoproteins. There may be single NA spikes or local NA clusters surrounded by HA on the virion surface [2]. Neuraminidase plays an important role in AIV infection: it facilitates the virion penetration into respiratory epithelial cells, optimizes HA fusogenic activity, enables release of new virions and prevents their aggregation on the host cell surface.

To date, 16 HA and 9 NA subtypes of the virus in various combinations have been isolated from birds. However, two new HA and NA subtypes of the virus, H17N10 and H18N11, isolated from bats were reported in 2012 and 2013 [3, 4].

In the FGBI "ARRRIAH" Reference Laboratory for Avian Viral Diseases reverse transcription real-time polymerase chain reaction (real-time RT-PCR) with M-gene-targeted primer system is initially used for diagnostic tests aimed at detection of type A avian influenza virus (AIV) in pathological material samples [5–7]. The samples tested positive with M-targeted real-time RT-PCR should be examined for virus H5 and H7 subtypes since the AI viruses of the said subtypes can be highly pathogenic and are notifiable to the OIE. NA subtype should be identified for precise virus characterization.

Neuraminidase inhibition (NI) test is one of the methods used for NA typing [8], but it takes at least two days and requires a panel of high-quality monospecific antisera and control antigens. The test should be carried out under specified conditions since highly toxic reagents have to be used.

Therefore, new highly sensitive, specific and reliable techniques for NA subtype detection and identification are urgently required for prompt diagnosis when it is necessary to identify AIV neuraminidase within a short period of time (within one working day).

Currently, N2 subtype AI virus is quite widespread and actively circulates in domestic and wild bird populations. In 2018, H9N2 AI virus was detected in three commercial poultry establishments in the Primorsky Krai, Russian Federation, as well as on one poultry farm in the Republic of Tajikistan. In 2017–2018, highly pathogenic H5N2 AI outbreaks were reported on one poultry farm in the Kostroma Oblast. In 2019, H9N2 AI virus was detected in the Chelyabinsk Oblast and Zabaikalsky Krai [9, 10].

Hence, development of high sensitive and specific real-time RT-PCR-based assay for N2 subtype AIV genome detection that enables faster results and rapid diagnosis for improvement of preventive measure effectiveness is urgent.

The works were aimed at development of the real-time RT-PCR-based assay with original primer and probe system and optimized parameters for N2 avian influenza virus RNA detection.

MATERIALS AND METHODS

The following N2 AI virus isolates available in the working collection of the FGBI "ARRIAH" Reference

Table 1
Primers and probes used for real-time RT-PCR amplification of N2 AIV N gene fragments

Таблица 1
Праймеры и зонды для амплификации фрагментов гена N подтипа N2 в ОТ-ПЦР-РВ

No.	Primer designation	5'-3' sequence	Nucleotide base number
1	AIVN2-1316f	GARACYAGAGTRTGGTGGAC	20
2	AIVN2-1319f	ACYAGAGTRTGGTGGACYTC	20
3	AIVN2-1325f	GTRTGGTGGACYTCAAAYAG	20
4	AIVN2-1379-FAM	(FAM) GGAACAGGCTCATGGCCTGATGG (BHQ1)	22
5	AIVN2-1414r	TTTTCTAAAATTGCGAAAGC	20
6	AIVN2-1421r	GGAGTTTTTTTYTAAAATTG	20
7	AIVN2-1432r	AGTAGAAACAAGGAGTTTTT	20
8	AIVN2-1370-FAM	(FAM) GGTACHTATGGAACAGGCT CATGGCCTGATGGRGC (BHQ1)	34
9	AIVN2-1376-FAM	(FAM) TATGGAACAGGCTCATGGCCTGATGG (BHQ1)	26
10	**AIVN2-1367F**	AGTCTGGTGGACYTCAAAYAG	21
11	AIVN2-1488R	AATTGCGAAAGCTTATATAGVCAT	24
12	AIVN2-1444_FAM	(FAM) CCATCAGGCCATGAGCCT (RTQ1)	18
13	**AIVN2-1418r**	GCGAAAGCTTATATAGSCAT	20
14	AIVN2-1428r	TTTTCTAAAATTGCGARAGCTT	22
15	AIVN2-1430r	TTTTTTCTAAAATTGCGARAGC	22
16	AIVN2-1383FAM	(FAM)-CAGGCTCATGGCCTGATGG (RTQ1)	19
17	**AIVN2R-1383FAM**	(FAM) CCATCAGGCCATGAGCCTG (RTQ1)	19

Laboratory for Viral Avian Diseases were used for selection of primers and probes providing high real-time RT-PCR sensitivity and specificity: A/ty/Mass/65 H6N2, A/w.duck/Vladimir/446/09 H4N2, A/bird/Amursky/21/12 H9N2, A/chicken/Kostroma/3175/17 H5N2, A/chicken/Kostroma/2367/18 H5N2, A/chicken/Primorsk/419/18 H9N2, A/chicken/Tadjikistan/2379/18 H9N2, A/chicken/Primorsk/3124/18 H9N2, A/chicken/Chelyabinsk/30/19 H9N2, A/duck/Primorie/2621/2001 H5N2.

Real-time RT-PCR primers were optimized using low virulent H9N2 AIV isolate, A/chicken/Primorsk/419/18, recovered in the Russian Federation (Primorsky Krai) in 2018. The real-time RT-PCR assay was tested for its analytical sensitivity using serial ten-fold dilutions of extracted total RNA of the following isolates in triplicate: A/ty/Mass/65 H6N2, A/bird/Amursky/21/12 H9N2, A/duck/Primorie/2621/2001 H5N2, A/chicken/Tadjikistan/2379/18 H9N2, A/chicken/Primorsk/3124/18 H9N2, A/chicken/Chelyabinsk/30/19 H9N2.

N gene nucleotide sequences of AIV/N2 isolates published in the electronic NCBI GenBank database from 1999 to 2008 (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/) were used for primer selection. Multiple nucleotide sequence alignment was performed with Clustal W tool and BioEdit 7.0 software programme was used for searching for the most conserved regions. The primers and probes were tested for their specificity using on-line Blast resource (NCBI) (http://blast.ncbi.nlm.nih.gov) and AIV isolates of other NA subtypes: (A/w_duck/Altai/1732/2013 H3N6, A/shoveler/Krasnoyarsk/1586/08 H4N6, A/chicken/Adygea/203/06 H5N1, A/mallard/Khabarovsk/12/2014 H10N7) as well as Winterfield 2512 strain of infectious bursal disease virus, H120 strain of infectious bronchitis virus and APMV/wild duck/Rus/Vladimir/44/15 isolate of Newcastle disease virus. Selected primers and probes were synthesized by SINTOL Company (Russia) (Table 1).

All primers and probes except for No. 10–12 were selected in the FGBI "ARRIAH" Reference Laboratory for Viral Avian Diseases. Reverse primer, AIVN2-1418r, and probe (FAM), CCATCAGGCCATGAGCCTG (RTQ1), were modified relevant oligonucleotides described by B. Hoffmann et al. in their publication. Probe and primers No. 10–12 were also recommended by B. Hoffmann et al. [11].

The RNA was extracted from allantoic fluid of SPF chicken embryonated eggs infected with N2 AI virus isolates with 'AmpliPrime RIBO-sorb kit' in accordance with the instruction of its use. Real-time RT-PCR was carried out using deoxynucleoside triphosphates (dNTPs) (Fermentas, cat. No. R0181), GoTaq® Flexi DNA Polymerase, thermostable Taq-DNA-polymerase (Promega, cat. No. M8295) and MMLV reverse transcriptase (SINTOL, cat. No. E-040) with Rotor-Gene Q cycler (Germany).

Table 2
Ct-values for AIV isolates obtained by M gene-targeted real-time RT-PCR

Таблица 2
Значения порогового цикла для изолятов ВГП в ОТ-ПЦР-РВ на ген М

No.	Isolate designation	Original materials (Ct)	Ct-value for dilution					
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	A/bird/Amursky/21/12 H9N2	11.27	14.01	17.08	20.49	23.93	26.86	–
2	A/chicken/Primorsk/419/18 H9N2	11.22	14.98	18.67	22.38	28.46	30.59	36.20
3	A/chicken/Kostroma/2367/18 H5N2	12.47	15.80	19.72	23.48	28.58	30.66	35.31
4	A/w.duck/Vladimir/446/09 H4N2	14.04	18.08	22.07	26.27	30.22	34.35	–
5	A/chicken/Tadjikistan/2379/18 H9N2	12.62	16.49	20.21	23.77	27.14	–	–
6	A/chicken/Chelyabinsk/30/19 H9N2	11.09	15.30	19.06	22.29	26.26	32.21	–
7	A/chicken/Primorsk/3124/18 H9N2	11.08	13.85	17.45	20.86	23.71	27.41	30.45
8	A/duck/Primorie/2621/2001 H5N2	11.27	15.02	18.00	21.50	24.63	28.51	–
9	A/ty/Mass/65 H6N2	14.36	17.37	20.67	24.25	27.28	30.32	–

«–» – negative result (отрицательный результат реакции).

RESULTS AND DISCUSSION

Development of the real-time RT-PCR assay for detection of N2 AI virus RNA included selection of the primers-probe system providing sufficiently high assay sensitivity and specificity. Also, optimal real-time RT-PCR conditions, reaction mix component concentrations and temperature-time mode had to be determined.

Selection of primers and probe. The full-length NA gene nucleotide sequences of N2 AI virus isolates recovered in Eurasian and African countries in 1999–2008 and belonging to different genetic groups that had been published in the electronic NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/) were analyzed to select the primer system for neuraminidase detection.

Initially, 2,822 N gene nucleotide sequences of AIV/N2 isolates were selected for comparative analysis. One hundred and sixty-nine sequences the most different from each other were finally selected for the analysis after step-by-step optimization and removal of the most genetically similar sequences from the selected ones. All sequences contained relatively conserved segments located at both gene ends. The 200-bp region at the N-gene end was considered optimal and selected for the primer incorporation since number of substitutions in the said region was the least in all selected sequences as compared to the other gene segments.

The data previously published by foreign authors were analyzed concurrently with the tests for in-house primer selection. The structure of the primers proposed by B. Hoffmann et al. [11] (No. 10–12, Table 1) was taken into account during in-house primer selection.

As a result, 11 primers and 6 probes were selected and synthesized for further real-time RT-PCR testing to identify the most specific and sensitive system.

One the most suitable primer system (indicated in bold in Table 1) was selected during experimental real-time RT-PCR runs using various primer-probe combinations with N2 AIV isolates and used for further assay optimization aimed at its sensitivity improvement. The reverse primer and fluorescent probe were designed in the FGBI "ARRIAH" Reference Laboratory for Viral Avian Diseases with minimum modifications of the nucleotide sequence proposed by B. Hoffmann et al. Forward primer sequence was identical to that one given in the above said publication [11].

Optimization of real-time RT-PCR conditions. It was required to optimize reaction mix component concentrations and temperature-time mode in order to increase sensitivity, specificity and rate of the real-time RT-PCR assay for N2 subtype avian influenza virus genome detection.

Prepared ten-fold dilutions (10^{-1}–10^{-6}) of extracted total RNAs of N2 AIV isolates were tested with the said real-time RT-PCR targeted for M gene using the primers-probe system recommended by the OIE immediately before tests for optimization of N-targeted real-time RT-PCR conditions [5–7]. The reaction was carried out in accordance with the Methodical Guidelines developed earlier in the FGBI "ARRIAH" [12]. The results of tests of N2 subtype AIV isolates with M-targeted real-time RT-PCR assay (Table 2) were compared to the results obtained by optimized N-targeted real-time RT-PCR assay.

The main task was to optimize reaction mix component concentrations in order to increase sensitivity and specificity of the real-time RT-PCR assay for N2 AIV RNA detection. To do this, N gene-targeted real-time RT-PCR assays using ten-fold dilutions of low virulent H9N2 AIV isolate recovered in the Russian Federation (Primorsky Krai) in 2018 were carried out. A temperature-time mode similar to that one of the M gene-targeted real-time RT-PCR assay was used. Tables 3–6 show cycle threshold (Ct) values for two AIV RNA dilutions (10^{-3} and 10^{-4}) tested in triplicate.

Selection of optimal magnesium chloride concentration for the real-time RT-PCR assay. Mg^{2+} ions as a required reaction mix component are crucial for proper DNA-polymerase functioning. They also have a significant impact on primer hybridization specificity. Optimal Mg^{2+} ion concentration can vary within a fairly wide range

Table 3
Ct-values obtained by real-time RT-PCR during optimal $MgCl_2$ concentration selection

Таблица 3
Значения порогового цикла при подборе оптимальной концентрации $MgCl_2$ в ОТ-ПЦР-РВ

Dilution	$MgCl_2$ amount, µl (concentration 25 mM)							
	1.0	2.0	3.0	4.0	4.5	5.0	5.5	6.0
10^{-3}	–	28.19	27.19	22.74	23.74	23.63	24.13	22.88
10^{-3}	–	27.06	29.33	22.49	23.63	22.58	23.81	23.34
10^{-3}	–	28.14	28.57	22.25	24.08	23.89	23.99	23.22
Mean	–	**27.8**	**28.36**	**22.49**	**23.82**	**23.37**	**23.98**	**23.15**
10^{-4}	–	32.97	31.16	27.03	28.96	27.85	27.55	27.07
10^{-4}	–	32.09	31.12	28.24	28.95	27.03	28.72	27.30
10^{-4}	–	33.16	33.53	28.12	29.76	27.59	29.45	27.34
Mean	–	**32.74**	**31.94**	**27.8**	**29.22**	**27.49**	**28.57**	**27.24**

«–» – negative result (отрицательный результат реакции).

depending on used primers and enzymes [13]. For best results, it is recommended to select Mg^{2+} concentration for the used system of primers and enzymes empirically. The test results are given in Table 3.

Data given in Table 3 show that change in magnesium salt concentration has a significant impact on the amplification process. Specific PCR-product yield was obtained when 2 µl of $MgCl_2$ were added to the reaction mix. Analysis of the obtained results showed that the amplification was effective when 4–6 µl of $MgCl_2$ solution were added. Therewith, Ct-value deviation was minimal, when 5 µl of magnesium chloride solution was used. This amount was considered optimal. It should be noted that increase in magnesium salt concentration could result in decrease in the assay specificity.

Selection of optimal primer concentration for the real-time RT-PCR assay. Optimal concentrations of AIVN2-1367f and AIVN2-1418r primers were selected experimentally. Reaction mixes were prepared using dilutions given in Table 4 and different amounts of the primers, stating from 0.5 µl and up to 2 µl, were added to each of them. Amounts of both primers, forward and reverse, are given in Table 4.

Performed tests show that increase in the primer concentration results in decrease in the assay sensitivity that is clearly demonstrated by data given in Table 4. The least Ct-values were obtained by the assays when 0.5 µl of each of the primers was added to the reaction mix.

Selection of optimal fluorescent probe concentration. Amount of the probe to be added was optimized to increase amplification effectiveness and fluorescence intensity of amplification curves. Test results are given in Table 5. Insignificant changes in real-time RT-PCR assay sensitivity were registered when 0.75 up to 2 µl of the

Table 4
Ct-values obtained by real-time RT-PCR during optimal primer concentration selection

Таблица 4
Значения порогового цикла при подборе оптимальной концентрации праймеров в ОТ-ПЦР-РВ

Dilution	Amount of primers, µl (concentration 10 pmol/µl)			
	0.5	1	1.5	2
10^{-3}	21.03	22.13	22.61	23.34
10^{-3}	21.04	21.98	22.80	23.31
10^{-3}	21.13	22.40	22.09	23.23
Mean	**21.07**	**22.17**	**22.50**	**23.29**
10^{-4}	25.08	26.96	27.44	30.16
10^{-4}	24.69	27.07	28.14	31.07
10^{-4}	24.82	26.58	28.41	28.95
Mean	**24.86**	**26.87**	**28.00**	**30.06**

Table 5
Ct-values obtained by real-time RT-PCR during optimal fluorescent probe concentration selection

Таблица 5
Значения порогового цикла при подборе оптимальной концентрации флуоресцентного зонда в ОТ-ПЦР-РВ

Dilution	Amount of probe, µl (concentration 10 pmol/µl)				
	0.5	0.75	1.0	1.5	2.0
10^{-3}	24.22	22.61	22.71	22.22	22.19
10^{-3}	24.55	23.47	22.65	21.97	22.56
10^{-3}	24.58	22.72	22.96	21.47	21.69
Mean	**24.45**	**22.93**	**22.77**	**21.89**	**22.15**
10^{-4}	29.26	26.20	26.63	25.70	26.88
10^{-4}	28.05	27.61	27.62	25.62	26.32
10^{-4}	27.47	25.91	27.40	25.84	25.98
Mean	**28.26**	**26.57**	**27.22**	**25.72**	**26.39**

probe were added to the reaction mix. Mean Ct-values differed by no more than 1.5 cycles. The most stable results were obtained when 1.5 μl of AIVN2R-1383FAM probe were used. Moreover, changes in the maximum levels of positive sample fluorescence intensity were observed in the reaction graph when the said component in the specified amount was added.

Concentrations of the reaction mix components (magnesium chloride, primers, fluorescent probe) were optimized to increase sensitivity and specificity of the real-time RT-PCR assay for N2 subtype AIV RNA detection. Other components were used in accordance with their manufacturer instructions.

Optimization of the real-time RT-PCR temperature-time parameters. The next step of our works was optimization of temperature-time mode of the PCR itself since temperature parameters of reverse transcription were defined by the enzyme (reverse transcriptase) used. In our case, optimal annealing temperature for the primer system containing selected oligonucleotide primers and fluorescein-labeled probe was determined. The next stages of the PCR – DNA denaturation and synthesis – were carried out in rather narrow temperature range. Denaturation is usually performed at 90–95 °C and DNA chain elongation – at 68–72 °C [13]. Results of primer annealing temperature selection for the real-time RT-PCR assay are shown in Table 6.

According to the data given in Table 6 compatible Ct-values were observed when the primer annealing temperature was 55–60 °C. Generally, this temperature range is suitable for the majority of primer systems used for the real-time RT-PCR assays intended for molecular avian influenza diagnosis. So, it was reasonable to use the same annealing temperatures for N2 subtype AIV identification. Thus, it is possible to carry out real-time RT-PCR assay targeted to different genes (M, H, N) during one thermocycler run and thereby to get the results and to make the diagnosis more quickly. The following temperature and time parameters were used for the optimized real-time RT-PCR: 20 min at 40 °C (reverse transcription); 10 min at 95 °C (polymerase activation); and further 40 PCR cycles comprising DNA denaturation: 10 sec at 95 °C; primer annealing: 35 sec at 55 °C and cDNA elongation: 10 sec at 72 °C.

Comparative sensitivity and specificity of the real-time RT-PCR assay for N2 subtype AIV genome detection. Sensitivity is one of the crucial parameters of real-time RT-PCR assay. Usually analytical sensitivity, i.e. minimum amount of the agent that can be detected by the method in the particular clinical samples, is reported. In this paper comparative sensitivity is reported since two real-time RT-PCR assays: one – for M-gene detection (data are given in Table 2) and the other – for N gene detection (Table 7) have been compared by the said parameter. Serial ten-fold dilutions of total extracted RNAs of 6 AIV isolates were made to test the developed assay for its sensitivity. The real-time RT-PCR assay was carried out in triplicate to obtain more reliable data.

After optimization of component concentrations and temperature and time parameters of the N gene-targeted assay Ct-values for ten-fold AIV isolate dilutions obtained by the said assay were consistent to Ct-values obtained by the M gene-targeted assay. Number of the dilutions tested positive for each AIV isolate was similar for the compared real-time RT-PCR assays.

The assay was tested for its specificity using samples containing N2 and N1, N6, N7, N8 AIV genetic materials

Table 6
Ct-values obtained during optimal primer and probe annealing temperature selection

Таблица 6
Значения порогового цикла при подборе оптимальной температуры отжига праймеров и зонда

Dilution	Annealing temperature, °C		
	60	55	50
10^{-3}	22.04	22.22	21.91
10^{-3}	21.77	21.97	22.82
10^{-3}	21.79	21.47	22.47
Mean value	**21.87**	**21.89**	**22.4**
10^{-4}	25.12	25.70	27.20
10^{-4}	25.39	26.62	27.06
10^{-4}	25.86	25.84	26.58
Mean value	**25.46**	**26.05**	**26.95**

Table 7
Ct-values for AIV isolates obtained by N gene-targeted real-time RT-PCR

Таблица 7
Значения порогового цикла для изолятов ВГП в ОТ-ПЦР-РВ на ген N

Isolate designation	Original materials (Ct)	Ct-value for dilution					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
A/chicken/Tadjikistan/ 2379/18 H9N2	12.19	15.92	19.24	23.16	26.11	–	–
A/chicken/Chelyabinsk/30/19 H9N2	10.30	13.88	17.53	20.75	25.91	29.82	–
A/chicken/Primorsk/3124/18 H9N2	10.62	13.43	17.22	21.05	24.77	29.10	36.73
A/bird/Amursky/21/12 H9N2	10.00	13.57	17.22	21.11	25.54	30.04	–
A/duck/Primorie/2621/2001 H5N2	11.30	14.52	18.31	21.72	25.14	29.25	–
A/ty/Mass/65 H6N2	10.90	14.34	18.54	21.24	26.04	29.70	–

«–» – negative result (отрицательный результат реакции).

as well as samples containing Newcastle disease virus, infectious bronchitis virus and infections bursal disease virus RNAs. All samples containing non-specific pathogens were tested negative with the developed real-time RT-PCR assay that confirmed the said assay specificity.

CONCLUSION

Primer system for N2 subtype AI virus genome detection was selected and the real-time RT-PCR conditions, reaction mix component composition and temperature-time mode, were optimized based on the performed test results. It was demonstrated that the proposed assay was able to detect N2 AIV RNA in biological material samples. High specificity and sensitivity of the real-time RT-PCR assay were proved by successful identification of N2 AIV genetic materials in samples collected from birds and submitted for testing from several regions of the Russian Federation in 2019–2020.

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