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Development of test-system for detection of H5 and H7 avian influenza virus RNA by multiplex real-time RT-PCR assay using internal control

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ABSTRACT

Introduction. High pathogenicity avian influenza is a dangerous highly contagious viral infection of domestic and wild birds that recently has become widespread in Europe, Asia, Africa and Americas. The causative agent of the disease is type A influenza virus of subtypes H5 and H7. Real-time RT-PCR is one of the most rapid and effective techniques for avian influenza virus identification and typing, so development of the test system based on this technique with internal control to be used for control of the reaction main stages is of current importance. At the same time, the multiplex format of RT-PCR allows for simultaneous identification of several targets that reduces the consumption of reagents and the reaction time.

Objective. Development of test-system for detection of H5 and H7 avian influenza virus RNA with multiplex real-time RT-PCR in biological samples and its characterization.

Materials and methods. H5, H7, H3, H4, H10, H16 avian influenza virus, Newcastle disease virus, infectious bursal disease virus, infectious bronchitis virus, Marek's disease virus, avian adenovirus isolates were used. MS2 bacteriophage was used as internal control.

Results. Optimal primer-probe combinations were selected, test-system characteristics were determined: specificity for homologous and heterologous avian disease viruses, analytical sensitivity, reaction amplification efficiency, repeatability and reproducibility.

Conclusion. Determination of the developed test system validation parameters has shown that it is specific only for H5 and H7 avian influenza virus, its analytical sensitivity for each subtype was 1.5 lg EID_{50}/cm^3, and the amplification efficiency was 92 and 97%, respectively. The test system was validated through its use for testing biological samples submitted to the laboratory, the test results were consistent with the results of tests with standard diagnostic methods used in the Reference Laboratory for Avian Viral Diseases of the Federal Centre for Animal Health.

Keywords: high pathogenicity avian influenza, H5 avian influenza virus, H7 avian influenza virus, real-time RT-PCR, test-system

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Разработка тест-системы для выявления РНК вируса гриппа птиц подтипов H5 и H7 методом мультиплексной ОТ-ПЦР в режиме реального времени с использованием внутреннего контрольного образца

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РЕЗЮМЕ

Введение. Высокопатогенный грипп птиц является особо опасной высококонтагиозной вирусной инфекцией домашних и диких птиц, в последние годы получившей широкое распространение на территории стран Европы, Азии, Африки и Америки. Возбудитель заболевания – вирус гриппа типа А подтипов H5 и H7. Одним из наиболее быстрых и эффективных способов идентификации и типирования вируса гриппа птиц является ОТ-ПЦР в режиме реального

времени, в связи с чем представляется актуальной разработка тест-системы на основе данного метода с использованием внутреннего контрольного образца для возможности контроля основных этапов проведения реакции. При этом постановка реакции в мультиплексном формате позволяет одновременно идентифицировать несколько целевых мишеней, что уменьшает расход реагентов и время постановки реакции.

Цель исследования. Разработка тест-системы для выявления в пробах биологического материала РНК вируса гриппа птиц подтипов H5 и H7 методом мультиплексной ОТ-ПЦР в режиме реального времени и определение ее основных характеристик.

Материалы и методы. Использовали изоляты вируса гриппа птиц подтипов H5, H7, H3, H4, H10, H16, вирусы ньюкаслской болезни, инфекционной бурсальной болезни, инфекционного бронхита кур, болезни Марека и аденовирус птиц. В качестве внутреннего контрольного образца служил бактериофаг MS2.

Результаты. Подобраны оптимальные сочетания систем праймеров и зондов, определены характеристики тест-системы: специфичность в отношении гомологичных и гетерологичных вирусов болезней птиц, аналитическая чувствительность, эффективность реакции амплификации, повторяемость и воспроизводимость.

Заключение. При определении валидационных характеристик разработанной тест-системы установлена ее специфичность в отношении только вируса гриппа птиц подтипов H5 и H7, аналитическая чувствительность для каждого подтипа составила 1,5 lg $ЭИД_{50}/см^3$, эффективность амплификации – 92 и 97% соответственно. Проведена апробация тест-системы при исследовании поступающих в лабораторию проб биологического материала, результаты соответствовали таковым для стандартных диагностических методов, используемых в референтной лаборатории вирусных болезней птиц ФГБУ «ВНИИЗЖ».

Ключевые слова: высокопатогенный грипп птиц, вирус гриппа птиц подтипа H5, вирус гриппа птиц подтипа H7, ОТ-ПЦР-РВ, тест-система

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INTRODUCTION

Avian influenza is one of the most dangerous viral diseases of poultry and wild birds, that affects primarily respiratory and digestive systems. The disease is caused by virus of genus *Alphainfluenzavirus*, *Orthomyxoviridae* family. The virus genome is a single-stranded (–)RNA composed of the 8 segments allowing high-rate virus evolution owing to reassortment [1]. Avian influenza virus (AIV) is classified into 16 subtypes by hemagglutinin (HA) and 9 subtypes by neuraminidase based on antigenic differences in surface proteins [2].

The natural reservoir of the AIV is wild waterfowl. The infected wild waterfowl are asymptomatic or demonstrate mild clinical signs. The virus naturally spread along the migration routes of wild migratory birds and at the same time is transmitted to poultry [3, 4]. High pathogenicity AIV is the most dangerous for poultry industry, since it can cause severe, rapidly developing disease with 100% mortality. High pathogenicity AIVs are believed to evolve under natural conditions from low-pathogenic H5 and H7 viruses through point mutations in HA gene causing accumulation of multiple basic amino acid at the HA cleavage site [4, 5, 6, 7, 8]. High pathogenicity avian influenza is to be notified to the World Organization for Animal Health (WOAH), regardless of the causative virus subtype.

Outbreaks of the disease caused by H5 high pathogenicity AIV were regularly reported in poultry and wild birds in the Russian Federation in 2021 – early 2024 (H5N1, H5N5 and H5N8 virus isolates were recovered) [9]. In 2024, high pathogenicity avian influenza outbreak caused by H7N3 AIV was reported in poultry kept at a poultry establishment in Australia [10]. Previously, disease cases, including cases in humans, caused by H7 AIV were reported in the North and South American, European, African and Asian countries [11, 12].

Current high pathogenicity avian influenza situation in the Russian Federation requires ongoing monitoring. Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) is the most rapid and accurate technique for detection of AIV RNA in biological materials from various poultry and wild bird species that enables simultaneous virus typing. This technique has high sensitivity, specificity, and is relatively rapid allowing high throughput.

Highly effective test system for detection of H5 and H7 AIV RNA by real-time RT-PCR is proposed in this paper. The developed test system contains exogenous internal control, that allows control of the main reaction stages (extraction of nucleic acids, reverse transcription and PCR) and elimination of false negative results.

The study was aimed at the development of real-time RT-PCR-based test system enabling simultaneous detection H5 and H7 virus RNAs in biological materials and control of the reaction procedure at all stages, starting with the nucleic acid extraction.

MATERIALS AND METHODS

Viruses. Isolates of AIV of various subtypes, Newcastle disease, infectious bursal disease, infectious bronchitis, Marek's disease viruses and avian adenovirus obtained from the working collection of the Reference Laboratory for Avian Viral Diseases of the Federal Centre for Animal Health were used (Table 1). MS2 bacteriophage with an infectious activity titre of 10^6 PFU/cm^3 served as internal control [13].

RNA extraction was performed using a "RIBO-prep" reagent kit for RNA/DNA extraction from clinical samples

(AmpliSens®, Russia) according to the manufacturer instructions. At the extraction stage, internal control was added to each sample (including negative extraction control), 0.01 mL of internal control per sample.

Primers and probes. Several sets of primers and probes for amplification of H5 and H7 AIV HA gene fragments were selected and tested based on the analysis of publications on development of test systems and methods for detection of H5/H7 AIV in biological samples with real-time RT-PCR [14, 15, 16, 17]. Since real-time RT-PCR was performed in a multiplex format, the dyes included in the TaqMan probes were selected in such a way as to generate a stable fluorescent signal and not inhibit the signals in other detection channels (Green/H5, Orange/H7, Crimson/internal control). The selected primers and probes were synthesized by the Syntol company (Russia), and the specific primers and probe for internal control [13] were synthesized by the Alkor Bio Company Ltd. (Russia).

Real-time RT-PCR was performed in one step using amplification reagents manufactured by the Syntol company (Russia) in Rotor-Gene 6000 programmable amplifier (Corbett Research Pty Ltd, Australia). The reaction mix (20 µL per sample) contained: deionized (bidistilled) water – 5.35 µL; 10× PCR buffer – 2.5 µL; 25 mM $MgCl_2$ solution – 4 µL; 25 mM deoxynucleoside triphosphate (dNTP) solution – 0.4 µL; forward and reverse primer solutions for AIV/H5, 10 pmol/µL – 1 µL of each primer; fluorescent probe for AIV/H5, 10 pmol/µL – 0.75 µL; forward and reverse primer solutions for AIV/H7, 10 pmol/µL – 1 µL of each primer; fluorescent probe for AIV/H7, 10 pmol/µL – 0.75 µL; forward and reverse primer solutions, fluorescent probe solution for MS2, 10 pmol/µL – 0.5 µL of each primer; SynTaq DNA polymerase – 0.25 µL; MMLV-revertase – 0.5 µL. The reaction was carried out according to the following procedure: reverse transcription – 20 min at 40 °C; polymerase activation – 8 min at 95 °C; 40 PCR cycles – 10 s at 95 °C; 35 s at 55 °C; 15 s at 72 °C. The fluorescence signal was detected at the stage of primer annealing using the Green/H5, Orange/H7, and Crimson/internal control channels.

Table 1
Avian virus isolates used for the study

Virus	Strain/isolate
H5N2 avian influenza virus	A/duck/Italy/5952/2015
H5N2 avian influenza virus	A/avian/Italy/6558/2015
H5N2 avian influenza virus	A/duck/Italy/6926/2017
H5N1 avian influenza virus	A/duck/Altai/469/2014
H5N1 avian influenza virus	A/dalmatian pelican/Astrakhan/485-1/2022
H5N5 avian influenza virus	A/shelduck/Kalmykia/1814-1/2021
H5N8 avian influenza virus	A/duck/KChR/1590-20/2020
H7N2 avian influenza virus	A/chicken/Italy/1670/2015
H7N3 avian influenza virus	A/turkey/Italy/9289/02
H7N7 avian influenza virus	A/duck/Italy/4932/2018
H3N8 avian influenza virus	A/wild duck/Primorsky/1872-13/21
H4N6 avian influenza virus	A/wild duck/Primorsky/1872-11/21
H9N2 avian influenza virus	A/chicken/Udmurtya/2008-1/21
H9N2 avian influenza virus	A/gull/Tyva/767-113/21
H10N7 avian influenza virus	A/wild duck/Primorsky/1872-13/21
H16N3 avian influenza virus	A/mallard/Khabarovsk/12/14
Infectious bronchitis virus	H-120
Infectious bursal disease virus	Winterfield 2512
Newcastle disease virus	LaSota (genotype II)
Avian adenovirus	KR95 (type C)
Marek's disease virus	3004

The test system was examined for its *specificity* by performing real-time RT-PCR using RNAs extracted from homologous and heterologous viruses (Table 1).

The test system was assessed for its *analytical sensitivity* by performing real-time RT-PCR with extracted RNA of serial 10-fold dilutions (10^{-8}–10^{-3}) of a virus-containing suspension (AIV strains: A/duck/KChR/1590-20/2020 H5N8 and A/turkey/Italy/9289/02 H7N3, initial infectivity titre was 8.5 lg EID_{50}/cm^3) with internal control in triplicate for each dilution. The reaction sensitivity for each sample was estimated as the virus amount (measuring units – lg EID_{50}/cm^3) corresponding to the last dilution, at which at least 95% of positive results were obtained (in 20 repeats) [18].

For *repeatability* assessment, positive samples were tested 3 times in 5 repeats during three days. Mean threshold cycle (Ct), standard deviation, and coefficient of variation for the obtained results were determined within one real-time RT-PCR run and between runs.

To determine the *reaction efficiency (E)*, the results obtained during the reaction runs for analytical sensitivity testing were used. The reaction efficiency was estimated after plotting a linear regression graph (in the coordinates "virus dilution" / "threshold amplification cycle Ct") according to the following formula:

$$E = (10^{(-1/m)} - 1) \times 100\%,$$

where m is the slope coefficient of the straight line [19, 20, 21].

RESULTS AND DISCUSSION

Optimal combinations of primers and probes were determined based on the results of testing of primer and probe combinations for amplification of H5 and H7 AIV HA gene fragments. Nucleotide sequences are shown in Table 2.

Selected sets were tested in real-time RT-PCR with AIV strains of H5N2, H5N1, H5N5, H5N8, H7N2, H7N3 and H7N7 subtypes and internal control in mono- and multiplex reaction format. Threshold cycle value, above which the reaction results should be considered negative, was set at 36.00 for the Green/H5 and Orange/H7 channels. In all tests, H5 or H7 AIV RNAs were confirmed to be present only in the samples containing the relevant virus subtype.

Optimal internal control concentration was determined by performing real-time RT-PCR using several 10-fold dilutions of the MS2 virus-containing suspension (10^5–10^7 PFU/cm^3) and simultaneous identification of one or two specific targets of the test system (AIV/H5 and AIV/H7). Based on the test results, concentration of 10^6 PFU/cm^3 was selected that enabled a stable

fluorescent signal increase in detection channel for internal control without inhibiting the signal in other channels for AIV/H5 and AIV/H7 (Fig. 1) and did not exceed sensitivity of the primers-probe system for internal control while simultaneously identifying AIV/H5 and AIV/H7 at high concentrations. When the Ct value is > 35 for Crimson/internal control detection channel, the results of the entire study are considered unreliable, i.e. errors were made at the stage of nucleic acid extraction, reverse transcription, or PCR, or the test sample contains impurities capable of inhibiting the reaction.

Test of real-time RT-PCR test-system for its specificity using extracted RNAs of H3, H4, H9, H10, H16 AIV and other RNA- and DNA-containing viruses (Newcastle disease,

Table 2
Primers and probes used for amplification of H5 and H7 AIV HA gene fragments

Name	Oligonucleotide structure
H5LH1	ACATATGACTACCCACARTATTCAG
H5RH1	AGACCAGCTAYCATGATTGC
H5Zond	(FAM)TCWACAGTGGCGAGTTCCCTAGCA(RTQ1)
LH6H7	GGCCAGTATTAGAAACAACACCTATGA
RH4H7	GCCCCGAAGCTAAACCAAAGTAT
H7Zond	(ROX)CCGCTGCTTAGTTTGACTGGGTCAATCT(BHQ2)

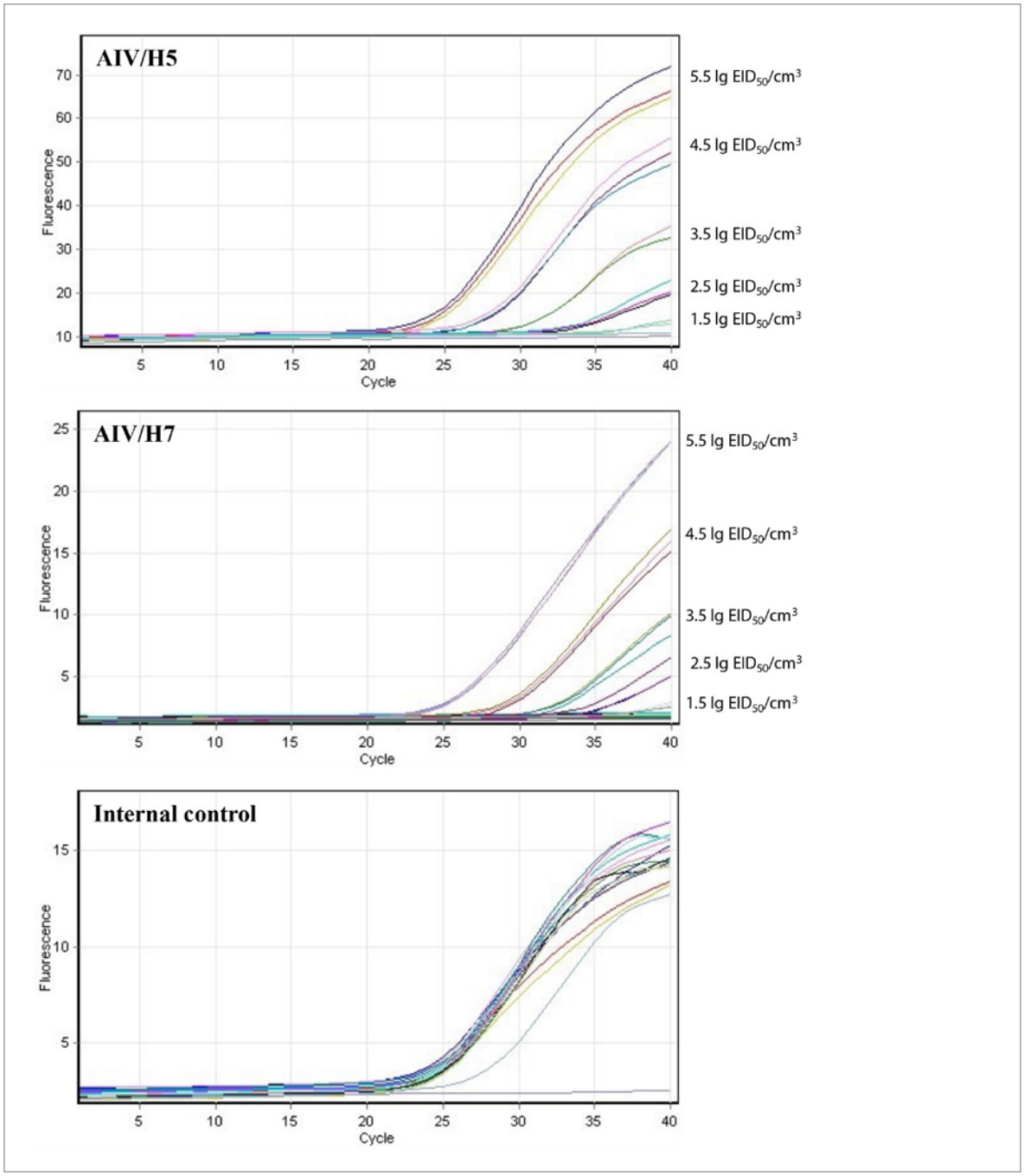

Fig. 1. Graphs of fluorescence intensity increase during real-time RT-PCR on Green (10-fold AIV/H5 dilutions), Orange (10-fold AIV/H7 dilutions) and Crimson (internal control) channels

Table 3
Real-time RT-PCR Ct values for 10-fold AIV/H5 and AIV/H7 dilutions

Detection channel / AIV subtype (virus titre in the initial suspension was 8.5 lg ED_{50}/cm^3)	The average Ct value for dilution, $n = 3$					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Green/H5	19.74 ± 0.13	23.13 ± 0.02	26.76 ± 0.53	30.37 ± 0.44	33.80 ± 0.07	–
Orange/H7	21.20 ± 0.11	25.17 ± 0.08	28.23 ± 0.03	31.39 ± 0.39	35.08 ± 0.52	–

"–" – negative result.

infectious bursal disease, infectious bronchitis, Marek's disease viruses, adenovirus) showed the absence of cross-reactions with the above-listed pathogens.

Analytical sensitivity of the test-system used for testing 10-fold dilutions of the virus with an infectious titre of 8.5 lg EID_{50}/cm^3 (Fig. 1, Table 3) for AIV/H5 the sensitivity limit corresponded to 10^{-7} dilution of the virus (in 20 repeats a positive result was obtained in 95% of cases) at the average Ct value of 34.16 ± 0.54 and a coefficient of variation of 1.59%; for AIV/H7 the sensitivity limit corresponded to a virus dilution of 10^{-7} (in 20 repeats, positive result was obtained in 100% of cases) with average Ct value of 35.17 ± 0.65 and a coefficient of variation of 1.84%.

Accordingly, the minimum virus amount that can be detected by the developed test system is 1.5 lg EID_{50}/cm^3 for AIV/H5 and AIV/H7.

Linear regression graphs were plotted for reactions with AIV/H5 and AIV/H7 to determine the efficiency parameters (Fig. 2). The following parameters should be taken into account for evaluation of the reaction efficiency:

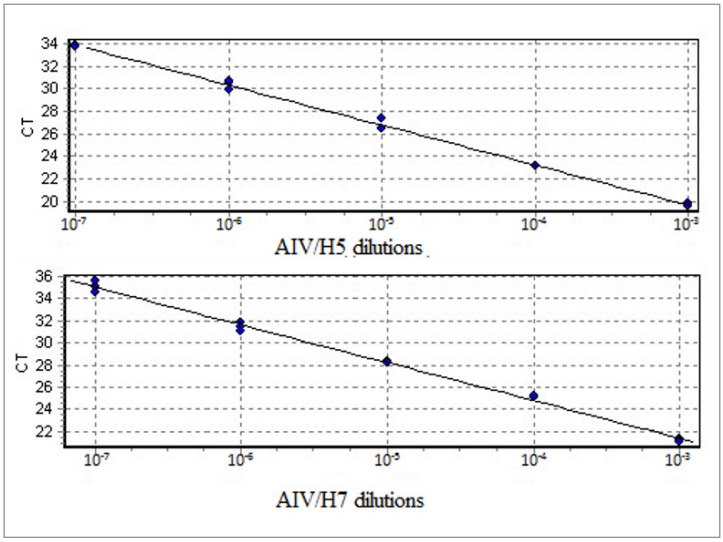

Fig. 2. Graphs of standard straight lines based on real-time RT-PCR results when 10-fold H5 and H7 AIV dilutions were used

Table 4
Reaction efficiency parameters for AIV/H5 and AIV/H7

Detection channel / AIV subtype	Correlation coefficient (R^2)	Straight line slope (m)	Reaction efficiency (E), %
Green/H5	0.997	–3.537	91.74
Orange/H7	0.996	–3.398	96.92

straight line slope (m) and correlation coefficient (R^2). Ideally (at 100% efficiency), m is –3.32, but values in the range from –3.2 to –3.5 are considered optimal. Values greater than 0.98 are optimal for R^2 [20, 22]. Determined reaction efficiency parameters for the developed test system are presented in Table 4.

The reaction efficiency for AIV/H5 (Green channel) was 91.74%, for AIV/H7 (Orange channel) was 96.92%. Parameters such as straight line slope coefficient and the coefficient of determination for AIV of both subtypes correspond to optimal values [20, 22].

The reproducibility of the test system was assessed based on standard deviation (SD) for each series of 10-fold dilutions (10^{-7}–10^{-3}, $n = 3$). For AIV/H5 standard deviations varied from 0.02 to 0.53; for AIV/H7 standard deviations varied from 0.03 to 0.52.

For repeatability assessment, the same viruses were used at a 10^{-4} dilution, each sample was tested in 5 repeats. For AIV/H5, the average Ct value within the runs varied from 22.89 to 23.36; SD was 0.22–0.33; the coefficient of variation was from 0.92 to 1.17%. For AIV/H7, the average Ct value ranged from 24.53 to 25.06; the SD – from 0.18 to 0.23, and the coefficient of variation – from 0.71 to 0.94%. Repeatability values between runs for AIV/H5 were as follows: average Ct – 23.09 ± 0.32, coefficient of variation – 1.41%; for AIV/H7: average Ct – 24.53 ± 0.31, coefficient of variation – 1.25%.

A total of 434 biological samples were tested for H5 and H7 AIV RNA with the developed test system, AIV/H5 RNA was detected in 268 samples. No AIV/H7 RNA was detected in tested samples. The results obtained using the developed test system correspond to those obtained during testing of the same samples by standard molecular diagnostic methods used by the Federal Centre for Animal Health Reference Laboratory for Avian Viral Diseases [23].

CONCLUSION

The test system for detection of H5 and H7 AIV RNA by real-time RT-PCR was developed. The proposed test system parameters were determined: the specificity was 100% (AIV/H5 and AIV/H7), analytical sensitivity limit was 1.5 lg EID_{50}/cm^3 (AIV/H5 and AIV/H7), the reaction efficiency was 92% (AIV/H5) and 97% (AIV/H7). The developed test system can be used for qualitative analysis of H5 and H7 AIV RNA in biological samples from birds and other animals.

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